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Indonesian Betel Quid and Oral Cancer

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A thesis submitted in total fulfilment of the requirements for the degree of

Doctor of Philosophy

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Declaration

This is to certify that:

1. This thesis comprises only my original work toward the degree of a PhD
2. Due acknowledgement has been made in the text to all other materials used
3. This thesis is less than 100,000 words in length, exclusive of tables, figures, bibliography and appendices as approved by the Research Higher Degrees Committee

Elizabeth Fitriana Sari

22th January 2020

Abstract

Purpose

Oral cancer (OC) is considered as a public health problem that carries significant morbidity and mortality. The disease represents a group of conditions with a range of sites and varied etiology. Indonesia has insufficient data of epidemiology studies in relation with oral pre-malignant disorders (OPMD) or OC prevalence and their risk factors. It is also important to understand the knowledge of GDPs in recognizing OC risk factors, early symptoms of OC, as well as in performing conventional oral examination. Without a comprehensive understanding of these factors, efforts to prevent, detect and manage this disease are likely to be ineffective in terms of outcomes and use of resources.

Hypothesis and Aim

The overall hypothesis was that Indonesia has a high prevalence of OPMD or OC that are strongly associated with the actual found risk factors. The overall aims of the epidemiology studies were to understand the prevalence and risk factors of OC and OPMD among Indonesians as well as to assess Indonesian GDP's knowledge in risk factors for OC and in performing COE. The aims of the laboratory studies were to identify antioxidants activities and phytochemical components of Indonesian BQ using HPLC, LCMS, and GC-MS, as well as to know the pathobiological response of Indonesian BQ components on oral keratinocyte (OKF-6) and oral fibroblast (MMF-1).

Materials and Methods

A cross-sectional study was undertaken using a pre and post-training questionnaire and training intervention with both theory and practical based. The study conducted in five different provinces of Indonesia; Aceh, Banda Aceh (BA); Bandung, West Java (WJ); special district

Jakarta, Jakarta (JKT); Pontianak, West Kalimantan (WK); and Sorong, West Papua (WP). The local Dental Association or Faculty of Dentistry invited the GDPs to attend a one-day standardized education program of oral malignant and OPMDs detection and 3 days hospital-based or community service-based practice.

A cross-sectional study was undertaken in multiple geographical areas within five Indonesian provinces, namely West Java (WJ), Jakarta (JKT), West Papua (WP), West Kalimantan (WK) and Banda Aceh (BA) regions. Individuals attending community health services, dental hospitals and selected villages were recruited. Volunteer respondents answered a previously validated extensive questionnaire that included socio-demographic factors, oral hygiene practices, diet, adverse oral habits and frequency of dental visits. An oral examination was undertaken using standardized WHO methodology. Data were analyzed using ANOVA, Chi-Square, and logistic regression to assess association between risk factors and mucosal disease.

The total phenolic content (TPC), antioxidant activity (by mean of ferric reducing antioxidant power, FRAP), radical scavenging activity (DPPH test), phenolics profile and arecoline content in different components of BQ which were AN, Leaf or SI, Husk, and blended BQ (BQ mixture). BQ mixture were a blending form contained AN, Leaf or SI and slaked lime. Samples were imported from 4 major regions of Indonesia, namely: Banda Aceh (BA), North Sumatra (NS), West Kalimantan (WK) and West Papua (WP). The highest TPC, FRAP, and DPPH values were detected in AN samples compared to other BQ components, while samples from WP region were of higher values compared to the other regions. High performance liquid chromatography - Mass Spectrometry (LC-MS) analysis was used to identified and quantified polyphenols and arecoline.

The gas chromatography mass spectrometry (GC-MS) platform was used to analyse the alkaloids present in betel quid originated from four different regions of Indonesia, Banda Aceh

(BA), North Sumatra (NS), West Kalimantan (WK), and West Papua (WP). Further, our study profiled the plant metabolites present in different components of betel quid, including areca nuts, betel leaf, stem inflorescence and betel quid as whole originated from each region.

Fluorescein diacetate assay (FDA) was used as a cell cytotoxicity test of BQ components on oral keratinocytes (OKF-6) and oral fibroblast (MMF-1) cells. The cytotoxicity effects of areca nut (AN), leaf of *piper betle* (Leaf) or stem inflorescence of *piper betle* (SI), areca husk (Husk) and whole BQ Mixture was tested on the growth of oral keratinocytes (OKF-6) and oral primary fibroblasts (MMF-1). On the basis of their chemical characteristics, BQ from Banda Aceh (BA) and West Papua (WP) regions were selected for *in vitro* testing.

Results

177 GDPs were registered in the one-day standardised training and 3 days practice. The highest number of registered GDPs was from WJ region (n=63), followed by GDPs from BA (n=44), JKT (n=27), WK (N=23), and WP (20) region respectively in rank. A total of 144 (81.37%) GDPs sent back their response to the post-training questionnaire 2-6 weeks after attending the training. Female respondents predominated with 157 (88.7%) returning the pre-training questionnaire and 126 (87.5%) returning the post-training questionnaire. Only 20 (11.3%) and 18 (12.5%) male GDPs answered the pre- and post-training questionnaire, respectively. Most of Indonesian GDPs demonstrated inadequate knowledge, awareness, and confidence in performing COE. The OC training could positively increase their knowledge and confidence of early screening.

A total of 973 respondents were enrolled (WJ 35.5%; JKT 13.3%, WP 18.3%, WK 9%; BA 23.9%). Smoking (14.8%), BQ chewing (12.6%) and alcohol drinking (4%) varied geographically. An OPMD was detected in 14.3% respondents and 0.2% had history of OC. Leukoplakia was the most common OPMD (8.6%), while the prevalence of

erythroplakia/erythroleukoplakia was 0.6%, and oral submucous fibrosis (OSMF) 1.9%. Leukoplakia was the most common type OPMD found in the survey most likely due to high number of smoking habit related. Overall, there was a strong correlation between the prevalence of OPMD and risk factors for OC. In particular, high use of BQ chewing in WK and WP, related to high prevalence of OPMD. The knowledge of OC risk factors, oral hygiene behaviour, ethnicities and low-income SES group were significantly associated with OPMD.

High performance liquid chromatography - Mass Spectrometry (LC-MS) analysis showed that Husk contains the widest range of polyphenols, including hydroxybenzoic acids, hydroxycinnamic acids, flavanols, flavonols and stilbenes. Catechin and epicatechin were the main polyphenols detected in BQ, and they were present at the highest concentrations in WP-AN sample. Arecoline was detected in all AN and BQ mix samples and was significantly correlated with catechin and epicatechin, and significantly negatively correlated with *p*-hydroxybenzoic acid. The current study is the first to extensively characterise the chemical composition of BQ and provides insight for the interactions of BQ alkaloids and phenolics in the development of oral submucous fibrosis and oral cancer.

Three types of alkaloids (arecoline, arecaidine, and guvacoline) were successfully identified using GC-MS. However, guvacine could not be analysed due to low volatility. This currently also profiled the metabolites in different BQ components and identified high concentrations of benzenoid and terpense, in stem inflorescence samples, especially the carcinogenic constitute, safrole. Interestingly, high concentrations of sesquiterpenes, such as gamma-cadinene, spatulenol, alpha-copaene, linalool, alpha-gurjunene, gamma-amorphene were also identified in stem inflorescence.

The study found that WP-AN exhibited cytotoxic effect on OKF-6 at a concentration of 100 μg/ml, as measured by a 50% reduction of cell viability (IC50), while BA-AN exhibited cytotoxic effect at a higher concentration (500 μg/ml) after 2 days of incubation. WP-SI also

showed cytotoxic effect at 500 μg/ml after 2 days incubation. In contrast, the extract of Leaf, BQ Mixture and Husk did not exhibit cytotoxic effect for up to 3 days of incubation. The exposure of BQ components to MMF-1 showed no cytotoxicity at any concentration assessed. Incubation of MMF-1 with BA-AN and WP-AN led to an increase of cell proliferation (1 day) followed by a decline of cell growth (2 days) in a dose and time dependent manner. Thus, the results of our study showed distinctive cytotoxic profiles in stromal and epithelial cells of the oral cavity induced by BQ components.

Conclusion

A previously underestimated high prevalence of OPMD in Indonesia strongly correlates with OC risk behaviour. There is an urgent need for primary prevention of OC programs in Indonesia. GDP knowledge of OC risk factors and COE is important to be addressed, as the lack of their knowledge and awareness can impact to delaying OC early screening and diagnosis. Therefore, improving the national curriculum in Indonesia on OC is more likely the best way for GDPs to have sufficient knowledge and confidence regarding OC before graduation.

The ripeness of AN is directly related to the amount of both polyphenols and arecoline. The unripe AN contained a higher concentration of polyphenols and arecoline compared to ripe AN, suggesting that consuming the ripe AN could lower the potential of developing OSMF. Further, our finding that safrole was detected in betel stem inflorescence (SI) as well as husk. This finding suggests that adding betel SI and husk into BQ compound could raise the risk of having OSMF and oral cancer even more as safrole is a possible potential human carcinogen

Preface

Publication

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Abstracts

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Poster presentations

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Abbreviations

µ	Micro
©	Copy right
mg	Milligram
ml	Millilitre
g	Gram
AN	Areca nuts
ANE	Areca nut extract
BA	Banda Aceh
BA-AN	Banda Aceh areca nut
BQ	Betel quid
BQ Mixture	Betel quid mixture
BA-Husk	Banda Aceh husk
BA-Leaf	Banda Aceh betel leaf
CI	Confident interval
COE	Conventional oral examination
CS	Community service
DPPH	1,1-diphenyl-2- picrylhydrazyl
ENT	East Nusa Tenggaranese
FDA	Fluorescein diacetate assay
FRAP	Ferric reducing antioxidant power
GC-MS	Gas chromatography mass spectrometry
GDP	General dental practitioner
HPLC	High pressure liquid chromatography
LC-MS	Liquid chromatography mass spectrometry

NS	North Sumatra
NS-AN	North Sumatra areca nut
NS-Leaf	North Sumatra betel leaf
OC	Oral cancer
OH	Oral Hygiene
OPMD	Oral premalignant disorders
OSMF	Oral submucous fibrosis
PLS	Plain language statement
SD	Standard deviation
SES	Socioeconomic status
SI	stem inflorescence
TPC	Total phenolic content
VI	Village
WJ	West Java
WK	West Kalimantan
WK-AN	West Kalimantan areca nut
WK-Husk	West Kalimantan Husk
WK-Leaf	West Kalimantan betel leaf
WP	West Papua
WP-AN	West Papua areca nut
WP-Husk	West Papua husk
WP-SI	West Papua betel stem inflorescence

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Chapter 1: Literature Review

1.1 Oral cancer

Oral cancer (OC) is ranked as the sixth most common cancer worldwide and accounts for 3% of cancer globally (1). The disease represents a group of conditions across a range of with varied etiology (1). The incidence of OC annually is estimated to be about 354,864 with 177,384 number of deaths in 2018 and the number is increasing (2-6). More than half of OC incidence globally occurs in Asia where an estimated 168,850 new OC cases arise in this region per year. Of these, approximately 11% were from South East Asia (SEA) countries such as Indonesia (7, 6).

The disease has wide geographic variation with two-third of the disease occurring in low-income and middle-income countries such as South and SEA, Latin America and Eastern Europe. However, the incidence of OC continues to increase in the West (1, 8). In SEA population, OC occurs predominantly in females, while globally it is more common in males (7, 9, 10). The incidence and mortality ratio of OC in SEA population is among the highest in Asia (11, 12), and it was recorded in 2012 that the mortality associated with OC in SEA was estimated 8505 cases (9).

Globally, the most common OC risk factors are the use of tobacco, alcohol drinking, betel quid (BQ) chewing, age, gender, and sunlight (1). However, the risk habit associated with OC in Asia and other developing countries were mostly tobacco use, both smoking and smokeless and BQ chewing. Whereas in the developed countries the OC associated risk habits were tobacco use and alcohol drinking. The demographic and health surveys on tobacco use reported that in all countries of SEA, more than 50% men aged 15–49 were tobacco user (13), and some of these countries, notably Indonesia and Timor-Leste, are among those with the

highest smoking rates among males in the world. Respectively, 72.3% and 96.5% men smoke in Indonesia and Timor-Leste (14, 9).

The real problem of OC is not simply related with its rising incidence, as the results of people continue to perform OC risk habits through smoking, excessive drinking and BQ chewing. However, a greater challenge is that OCs are not detected early enough for successful treatment (15). The most effective means of reducing mortality due to OC by preventing late-stage diagnosis. This could happen by increasing awareness among the population and thus encouraging earlier presentation, or secondary prevention by screening and early detection of OC and OPMD (16). Studies have showed that approximately 30% of patients postpone seeking help for more than 3 months following the self-discovery of OC symptoms (16) and 40% of patients present OC with advanced stage (stages III and IV) (17). The poorer prognosis of the disease has been shown to be associated with the delay in patient's presentation and diagnosis made by health care providers (18). Lack of awareness of the signs, symptoms, and risk factors for oral cancer (19), as well as the absence of prevention and early detection by health-care providers (20), are both believed to be responsible for the diagnostic delay.

OC is therefore a public health problem which carries significant morbidity and mortality (21). While to date Indonesia has insufficient data of epidemiology studies in relation with OC/OPMD prevalence and risk factors. It is also important that the knowledge of GDPs in recognizing early OC symptoms or OPMD is assessed. Without a comprehensive understanding of these factors, efforts to prevent, detect and manage this disease are likely to be ineffective in terms of outcomes and use of resources (22). Thus, this thesis aimed to discuss the broad evidence base on early screening to assess the prevalence and risk factors of OC and OPMD among Indonesians, as well as survey Indonesian GDP's knowledge of OC. Secondly, the laboratory studies focused on identifying antioxidants activities and phytochemical

components of BQ using HPLC, LCMS, and GC-MS, as well as determining the pathobiological response of Indonesian BQ components on the human cell lines.

1.2 OC and OPMD Screening

A visible early transformation changes in oral mucosal almost always precede an OC, and this visible changes namely as an oral potentially malignant disease (OPMD). The visible OPMD will allow the practitioners to detect and effectively manage OC at an early intraepithelial stages (23, 24).

OC screening has been defined as "the process by which a practitioner evaluates an asymptomatic patient to determine if he or she is likely or unlikely to have a potentially-malignant or malignant lesion" (25). The OC screening can occur as population-based screening, opportunistic screening, and targeted screening. The population-based screening is when a population being assessed specifically for the purpose of OC detection, 'the opportunistic screening', when patients who are attending a health care provider for another purpose are examined for signs of OC or PMD, or as 'targeted screening' when high risk individuals are selected for OC screening (1). In any of these contexts, along with a visual and tactile examination of the oral mucosa, the practitioner should ask the patient about their health history including tobacco and alcohol use. The risk of oral cancer is increased with age, alcohol and tobacco use and a history of upper aerodigestive tract cancer (22, 25)

The histological examination is always the gold standard for the diagnosis of oral dysplasia, and malignant lesions (26). Conventional oral examination (COE) is considered to be the best approach in OC screening, however, several aids for OC screening have been suggested to clearly discriminate the lesions, to increase the sensitivity, accuracy, and specificity of COE (26).

According to Mehrotra *et al,* there are two approaches for detection of OC and OPMD (27), the first is to undertake a program for screening OC that identify asymptomatic individual with suspicious oral lesions and second is to use specific diagnostic tools to identify OPMD and early OC in asymptomatic individual" (27).

1.2.1 Conventional oral examination (COE)

COE is the current accepted mucosal examination for OC and OPMD detection, this examination has been proven to have high discriminatory ability (28). A complete mucosal examination include visual and tactile examination, namely COE, should be part of a routine dental examination by all GDP (28). The effectiveness of COE could be influenced by several important factors such as practitioners' experience and confidence, as well as the acceptance of the patient of the procedures (22).

A standard method of COE for OC screening is using normal (incandescent) light. Several studies have reported COE to have a relatively high degree of sensitivity, specificity and positive predictive value (29). However in contrast, a number of studies suggested COE may have limited value as a method for detecting OC and OPMD (30-32).

A study done for oral cancer-screening programs involving over 2300 individuals who were examined for red or white lesions or ulcers of greater than 2 weeks duration (33, 34). A general dental practitioner examined each individual and recorded the presence of lesions. Independently, an oral medicine specialist also examined all individuals for a "true" clinical diagnosis. The biopsy was done to confirm the diagnosis in patients who screen negative by COE (clinically normal) has been deemed inappropriate and an ethically questionable practice (35). The sensitivity and specificity of the COE was 74% and 99% respectively, indicating that a visual oral examination can detect relevant lesions with a sensitivity and specificity similar to that found in other screening programs.

A randomize control trial (RCT) of COE involving 130,000 individuals was carried out in India by Kerala group (36, 37). The early results presented at 3 (37) and 6 (36) years. Surrogate markers of improved outcome including 3-year survival, stage of presentation and yield were all significantly greater in the intervention group compared to the control group. However, they could not provide the evidence of reduced mortality with regard to oral cancer (38). Kujan *et al*. (39), also concluded that there was no evidence to support the use of a visual examination as a method of screening for oral cancer. Subsequently, the Kerala group reported their results at 9 years (40). Although it showed no increase in survival for the overall population, however, a significant increase in survival was observed among males with high-risk habits, such as tobacco use. This result became the first clear evidence to support the efficacy of an oral cancer screening program, as measured by reduced mortality. Further, the broader use of oral cancer screening measures were prompted throughout the world (38).

Although COE does not identify all potentially premalignant lesions, nor does it accurately detect the small proportion of biologically relevant lesions that are likely to progress to cancer, however, COE may be useful in the discovery of some oral lesions for early prevention of oral cancer (29).

A COE consists of 8 steps of examination included intraoral and extraoral visual assesment and palpation using white light (41). The steps are as including :

1) Extraoral examination:

   Inspection of the region of head and neck for asymmetry, pigmentation, tenderness or swelling, numbness or pain. Palpation of the lymph nodes region in the submandibular, neck and supraclavicular region, paying particular attention to size, number, tenderness and mobility. Finally, an inspection and palpation of the lips and perioral tissues for any abnormalities (Figure 1.1.A and 1.1.B)

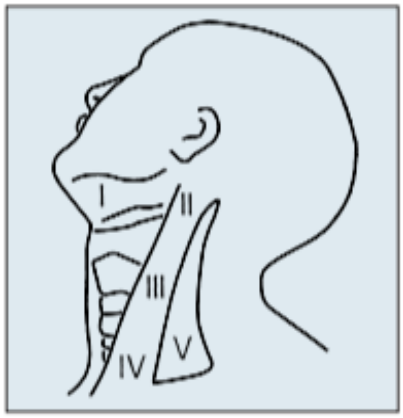
2) Intraoral examination:

A sound and systematically inspection and palpation all oral soft tissues, especially the high-risk sites for oral cancer development that include the lateral and ventral aspects of the tongue, floor of mouth and the soft palate complex (Figure 1.1C-G).

3) Lesion inspection:

Each lesion needs to be evaluated for specific characteristics paying particular attention to size, colour, texture and outline. Focusing attention on predominantly indication of white, red, red and white, ulcerated and/or indurated lesions.

A



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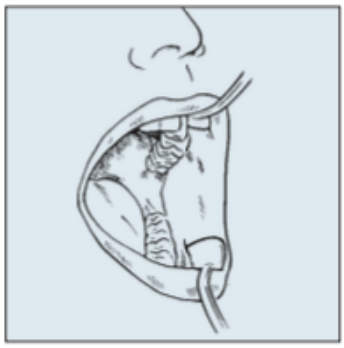

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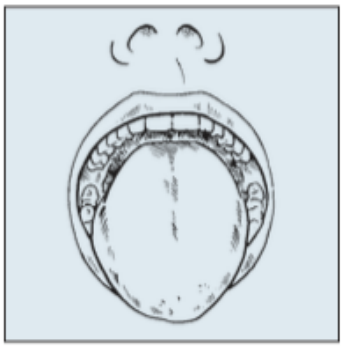

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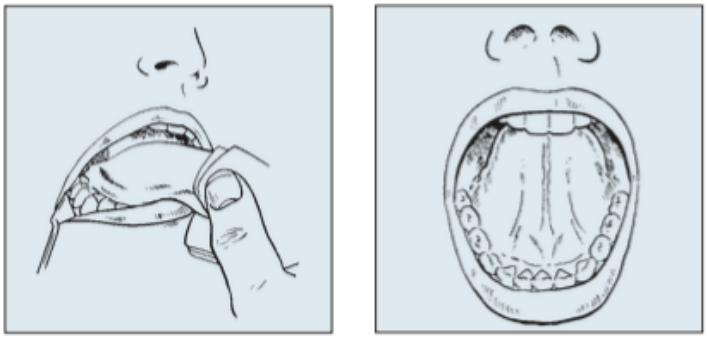


F G

Figure 1. 1 COE Steps (15)

1.2.2 Brush cytology

The Brush Biopsy (CDx Laboratories, Suffren, NY) was introduced for the first time in 1999 as a potential OC screening adjunct tool. It was designed for the low level of suspicion for carcinoma based on clinical features, the lesion would otherwise not be subjected to biopsy at the clinical examination (42, 43). When the brush cytology shows an abnormal result (*atypical* or *positive*), the practitioners must follow-up with an incision scalpel biopsy to provide a definitive diagnosis (29).

Brush biopsy is a technique of cytologically minimal invasive which obtains cells of all epithelial layers, a special circular bristled has been designed for this purpose (44). With this methods, even the small epithelial tissue fragments can be contained and further be embedded in paraffin to be examined as histological specimens, this method also called “cell block method” (45, 46). The advantages of using oral brush biopsy are that it is chair-side, easy to perform, and painless (47).

The initial sensitivity and specificity assessment of OralCDx was undertaken in a multi-centre US study with 945 patients, it was more than 96% of histologically evaluation confirmed oral pre-malignant and malignant lesions (48). Although the investigators reported that OralCDx had a sensitivity rate of 100%, the majority of the negative brush results were not followed by incisional scalpel biopsy for diagnostic confirmation (49). Since that initial

study, conflicting data has been presented in relation to the accuracy of OralCDx (49). In another study (50), 112 brush biopsy cases were performed and of interest the sensitivity was only 71.4% and the specificity was only 32%. The positive predictive value of an abnormal result was 44.1 per cent and the negative predictive value was 60 per cent. Remmerbach et al (51), reported a sensitivity evaluation of cytologic diagnosis in oral smears for the detection of OC was 100%, however, the specificity for the detection of non-neoplastic cells was 93%, the positive predictive value was 91%, and the negative predictive value was 100% (51). In 25 cases, the brush biopsies were performed on suspicious oral lesions and were diagnosed cytologically as non-malignant (tumor cell negative). Four cases were ‘‘atypical,’’ 5 cases were diagnosed as ‘‘suspicious for tumor cells,’’ and 13 specimens were diagnosed as ‘‘positive for tumor cells.’’ The medical reports indicated that, in all 18 specimens that were diagnosed as ‘‘suspicious’’ or ‘‘tumor cell positive’’ and in 2 of 4 specimens that were diagnosed as ‘‘atypical,’’ squamous cell carcinomas were verified by subsequent histopathologic diagnosis (correctly positive) (51).

Some studies have reported its sensitivity and specificity in dysplasia and OSCC detection (42, 48, 50, 51). However, in majority of cases, surgical biopsy still has to be performed after brush biopsy of both innocuously-looking lesions and highly clinically suspicious dysplasia (49, 52, 44).

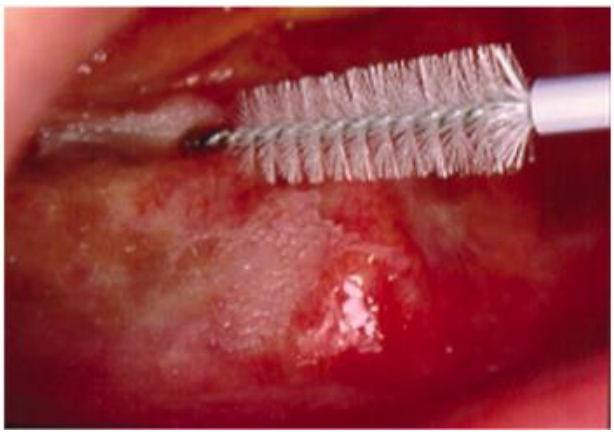


Figure 1. 2 Circular bristled for brush biopsy (53)

1.2.3 Toluidine blue staining (TBS)

TBS, which has a chemical name tolunium chloride, is a vital staining technique (29, 47)**.** It has been used for many decades as an adjunctive tool to identify mucosal abnormalities of the oral cavity as well as in the cervix (29).

Vital staining is staining of living cells or tissues. It is divided into two techniques, firstly, intra-vital staining within the living body or namely in vivo, and secondly is supra vital staining outside the body which usually involves detached cells on slides (In vitro) (47). TBS is an in vivo staining technique**;** it is a basic metachromatic dye that potentially stain malignant neoplasm leaving normal epithelium unstained. The increased amount of DNA and RNA in neoplastic cells and the wider intercellular canals facilitate the dye to enter are postulated to be responsible for TBS (54, 55). This toluidine blue dye in vivo staining is an acidophilic cationic metachromatic dye of thiazine group that selectively binds to free anionic acidic tissue components, such as sulfates, carboxylates and phosphate radicals belonging to DNA and RNA that increase in dysplasia and in situ carcinoma (54, 56).

TBS method for OC screening is considered to be non-invasive, practical, rapid, inexpensive, and effective diagnostic adjunct aids (57, 56). TBS also has been suggested as to assist biopsy site selection, for assessment of margins of OPMD or OC and in determining OPMD lesion at risk of progression to malignancy (57, 55, 56). While the FDA does not approve the use of toluidine blue for OC screening technique in the United States (58), some studies have shown evidence that TBS methods is a useful as an adjunct to COE practice for the identification of OPMD (29). To date, however, the use of it is limited in a secondary care environment in the hands of specialists (29).

When applying TBS, after COE is done, and if there is a suspicious lesion it should be followed by an oral rinse of 1% acetic acid solution for 20 seconds before expectorating. Toluidine blue (1% W/W) applied as an oral rinse for 20 seconds and then 1% acetic acid used

for to eliminate mechanically retained stain.

A complex study carried out on patients with a previous history of oral cancer and who had been seen regularly in follow up in a specialist clinic (59). Patients were joined into a screening program where they were re-examined and screened with TBS. Fifty out of 235 people were screened, had visible lesions that clinically detected, and a further 32 patients had areas that retained dye without a visible lesion. All 82 lesions were biopsied and 6 out of the 32 clinically invisible lesions, showed toluidine blue-positive, and lesions proved to be carcinomas. Of the 50 clinically visible lesions, 20 stained positively with TBS and 6 of these were carcinomas. One of the clinically visible carcinomas was dye negative. Therefore, 6 OC were identified out of 82 patients that would have been otherwise undetected. The overall sensitivity and specificity were 92% and 42% respectively. This study showed that TBS had quite good sensitivity but low of specificity (59).

A previous study undetaken by Warnakulasuriya and Johnson (60), showed 100% sensitivity of TBS for detection of oral carcinoma. Eighteen patients have been proven to have oral carcinoma and all of their respective malignant lesions were dye-positive with TBS. They stained the oral mucosa of 102 patients with clinically suspicious lesions (60). However, the overall specificity was low (62%), and the results of this study raise several issues (29), such as; the dye-positive carcinomas were all clinically detected and would have been aimed to biopsy in the absence of TBS; only 29 (74%) dye positive out of the 39 lesions that proved be dysplastic in biopsy confirmation; 18 cases (50%) of oral lichen planus showed positively stained. Thus, the overall sensitivity and specificity of the staining for the detection of dysplasia was only 74% and 66% respectively (29). Another study by Onofre et al (61) showed that all carcinomas were dye-positive, however, only 50% of dysplasias were dye-positive and 13 of 37 (35%) benign lesions also stained. The overall sensitivity and specificity were 77% and 67% respectively.

1.2.4 Light-based detection system

Light-based detection systems are based on the assumption that the changes in mucosal structure and metabolic during carcinogenesis gives rise to distinct profiles of absorption and refraction when exposed to different types of light or energy (62). There are two groups of light-based detection system, the group of Chemiluminescence (ViziLite Plus; Microlux/DL, Orascoptic-DK) and tissue fluorescence imaging (VELscope) (46).

1.2.4.1 Chemiluminescence (reflective tissue fluorescence)

The light emission of limited heat (luminescence) is called Chemiluminescence. It produced various colours such as blue, green, yellow-green, yellow, orange, and red. The luminol and peroxyoxalate based systems are the most common used systems of chemiluminescence (56). This oral examination aid was proposed to improve identification, evaluation and monitoring of OPMD (56). The field of obstetrics and gynaecology have been used chemiluminescence as its principal for early detection of cervical cancer and pre cancer (56). This tool, Vizilite plus consists of a 1% acetic acid solution, a capsule (which emits light), a retractor and the manufacturer's instructions (63, 56). The acetic acid removes all debris on the surface, as well as exclude the glycoprotein barrier on epithelium's surface, purportedly allowing better light penetration. Normal mucosal cells absorb the blue light produced, and the same colour will be reflected by cells with abnormal nuclei including dysplastic cells (64). The reflection of light back from neoplastic cells caused by shorter wavelength light that are more reflected via scattering by neoplastic cells. This is thought to occur as there is an increase replication of DNA, causing DNA to take up a greater volume of cytoplasm (56). The abnormal cells will also supposedly contain increased protein content and the light reflection commonly observed in this state (56).

In many cross sectional studies, Vizilite (Zila Pharmaceuticals, Phoenix, Az, USA) has

been suggested to have a high sensitivity (100%) but low of specificity (0-14,2%) and low of positive predictive value (65, 46, 63, 62). Therefore, the combination of Vizilite with toluidine blue has been subsequently suggested to reduce the number of false positive (62).

The use of Vizilite as COE aid has been proven to increase the sensitivity in recognizing OPMD by GDPs, although the disadvantage of it probably could have shown to increase the number of unnecessary referral due to false positive (66). Most of studies have shown that chemiluminescence increases oral white lesions' brightness and margins (65). Vizilite also can assist to identify undetected lesion by COE (65). However, some have shown conflicting results (65). Ram *et al.* found that ViziLite had 100 % sensitivity with a low (12.5%) specificity for detecting of OPMD and OSCC (67). Rajmohan *et al* examined oral mucosa in normal, precancer and cancer patients using ViziLite and found to have 77.8 % sensitive for detecting dysplasia and 90 % sensitive for detecting OSCC (68).

In contrast, Mehrotra *et al.* found that Vizilite was not sensitive (0%) in detecting dysplasia and OSCC and does not give benefit in detecting OSCC and OPMD (14). Ujaoney *et al.* compared the use of chemiluminescence and toulidine blue staining (TBS) for detecting high risk oral pre-cancerous lesions and TBS showed a better diagnostic test than chemiluminesecence (69).

Another previous study on sensitivity and specificity of ViziLite showed 95.45% and 84.6% respectively (56). ViziLite could discover early epithelial dysplastic changes in one high risk patient with clinically normal appearing oral mucosa. Sensitivity and specificity of toluidine blue were also calculated to be 86.36% and 76.9% respectively. 60 patients were enrolled to this study, and 25 patients had oral leukoplakia, 10 patients had diagnosed with OSCC clinically and 25 high risk patients had invisible lesion clinically. All patients were assessed using ViziLite and toluidine blue staining, and further followed by incisional biopsy (56).

Vizilite has various limitations as visualization adjunct such as; it needs a dark environment to examine oral mucosa, high cost, unable to record the picture unless it is photographed, low specificity for detecting dysplasia, it could contribute to high referral rate and over-treatment, unable to detect some red lesions, the use of acetic acid as pre-rinse could increases salivary flow that interferes with mucosal surface reflectance, inability to objectively measure the visualization results. Thus, Vizilite can give one-dimension information for the horizontal extent of the lesions. However, it cannot assess the depth of the lesion which is more important in predicting the malignant (70).

1.2.4.2 VELscope (narrow-emission tissue fluorescence)

Approximately 30 years ago, it was observed that the autofluorescence of tissues (tissue fluorescence) could potentially be used for cancer detection. In early 2006, a direct fluorescence visualization device for examining oral cavity tissues was released and tested in a pilot study (71). This device is named a Visually Enhanced Lesion Scope (VELScope). It is a handheld device that facilitates the direct visualization of oral cavity fluorescence for the detection of high-risk, potentially malignant and early malignant lesions. A blue excitation light, between 400 to 460 nm, is employed to excite green-red fluorescence from fluorophores in the oral tissues. Tissue fluorescence is viewed directly along an optical axis collinear with the axis of excitation to reduce inter- and intra-operator variability (72, 62). The VELScope is used to shine a light onto the suspicious lesion in oral cavity that is observed through an attached eyes piece for colour changes. Normal oral tissue is said to omit a pale green fluorescence while potentially early tumour or dysplastic cells appear dark green to black (73).

It has been proposed that abnormal tissue is associated with a loss of auto-fluorescence (LAF) and appears dark in contrast to the surrounding tissue. LAF occur in dysplasia due to alterations in the stromal architecture. Lesions that completely blanch on pressure and return

to a normal fluorescence pattern should be considered negative for LAF. This phenomenon is termed diascopic fluorescence (74, 73).

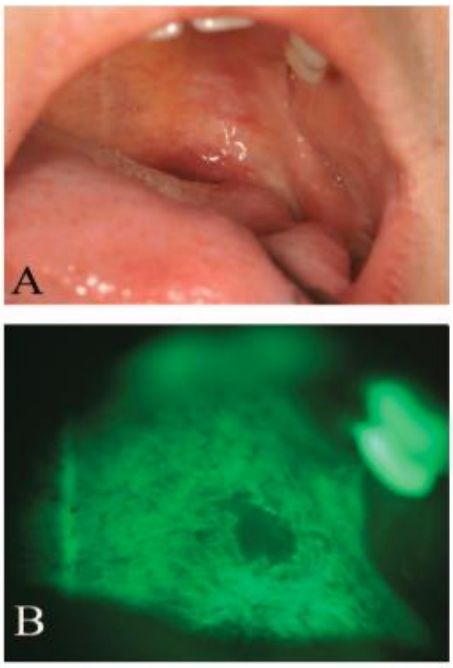


Figure 1. 3 COE of the oral cavity showing an ill-defined leukoplakia in the left soft palate (A), and same lesion demonstrated with VELScope fluorescence imaging showing loss of auto- fluorescence (B)(75)

Farah et al (75) assessed the efficacy of VELScope in detecting oral mucosal lesions (75). A total of 112 patients were referred with OPMD and were examined under routine incandescent light, and then assessed with VELScope, loss of autofluorescence and presence of blanching were noted. Further, incisional biopsies were performed to provide definitive histopathological diagnoses. The results showed that VELScope enhanced the visibility of 41 lesions and discover 5 clinically undetected lesions. Assessment with VELScope alone showed 30% sensitivity and 63% specificity. Its accuracy at identifying dysplasia was 55%.

The authors concluded that VELScope cannot provide a definitive diagnosis for the presence of epithelial dysplasia. The loss of autofluorescence is not even useful in diagnosing epithelial dysplasia in its own right without relevant clinical interpretation (75).

McNamara et al evaluated the benefit of direct visual fluorescence examination (DVFE) using VELSCope as adjunct tool to conventional oral examination (COE) for screening OPMD in a general population of patients who present for dental care (76). A total of 130 patients were examines by COE followed by VELScope. The surgical biopsy was performed to the clinically suspicious areas examined by COE or with positive visual fluorescence loss (VFL). The results showed 42 subjects had one or more areas of VFL, however, histologic evidence of premalignancy/malignancy was only identified in a single individual. One lesion negative by VELScope was found to have epithelial dysplasia. The authors suggest that COE is more valid compared to VELScope at discriminating benign mucosal changes from premalignancy and do not support use of DVFE as an oral cancer screening adjunct.

Awan *et al* (77) evaluated the accuracy of autofluorescence (VELScope) against conventional oral examination and surgical biopsy (77). A total of 126 patients who presented to the Oral Medicine Clinics at King's and Guy's Hospitals, London with oral white and red patches suspicious of OPMD were enrolled. Following a complete COE and autofluorescence examination (VELScope), all underwent an incisional biopsy for histopathological assessment. Seventy patients had oral leukoplakia/erythroplakia, Of the 126 lesions, 105 (83%) showed loss of fluorescence. Following incisional biopsy, 44 had oral epithelial dysplasia (29 mild, 8 moderate and 7 severe). The VELScope had a sensitivity of 84.1% and specificity of 15.3%. The authors concluded that VELscope was useful in confirming the presence of oral leukoplakia and erythroplakia and other oral mucosal disorders, however, the device was inability to discriminate high-risk from low-risk lesions (77).

A study reported the strengths and limitations of using VELscope (78). The strengths included non-invasive aids and simple to use, does not require consumable reagents, no recurrent cost, provides real time results, a wide range of operator can simply operate it after short training, variability of operator is limited, sensitivity for any oral mucosal disorder is

considerably high (78). The limitations of using Velscope that it requires a dark environment, high initial setting-up cost, no permanent record unless photographed, specificity for dysplasia is low leading to high referral rate and over-treatment (78).

1.2.5 Salivary detection

Saliva is composed of multifarious components which not only preserve the oral tissue integrity, but also allow numbers of clues of local and systemic condition to be manifested in it. That is why saliva has been considered as a mirror of bodily health (46). Exploration of the saliva components as general health markers has been continually done over the years (79). The multiplex biomarker detection systems have been created through remarkable progress in the development of lab-on-a-chip and point-of-care technologies (80). Several saliva markers include oncogenes (e.g., C-myc, c-Fos, C-Jun), anti-oncogenes (e.g., p53, p16), cytokines (e.g., transforming growth factor-β1, interleukin (IL)-8, and -1β), growth factors (e.g., vascular endothelial growth factor, epidermal growth factor, and insulin- like growth factor), extracellular matrix-degrading proteinases (MMP1, MMP2, MMP9), hypoxia markers (hypoxia-inducible factor-α, carbonic anhydrase-9), epithelial-mesenchymal transition markers (e.g., E- cadherin, N-cadherin, and β-catenin), epithelial tumor factors (Cyfra 21–1), cytokeratin (CK13, 14 and 16), microRNA molecules, and hypermethylation of cancer-related genes (p16 and death-associated protein kinase) (46, 81).

Body fluids such as serum, urine, cerebrospinal fluid, and saliva are circulating body fluids where tumour markers may be present as intracellular substances. The recent advance technology in diagnosis techniques allows saliva to become a tool for diagnosis that has grown exponentially. Saliva-based oral cancer diagnosis is a non-invasive alternative to serum testing and has an 85% overall accuracy rate. The saliva based is an effective modality for diagnosis, determining prognosis of oral cancer and monitoring post-therapy status (82, 83).

Salivary mRNA is speculated to contain apoptotic bodies (84, 85) or actively released in exosomes or micro vesicles (86, 87). The clinical accuracy of saliva was compared to blood RNA biomarkers for oral cancer detection and it was found four RNA biomarkers that have a sensitivity and specificity of 91% and 71%, respectively, and a collective receiver operator characteristic value of 0.95 (88, 89). Speight and Morgan found seven mRNA molecules significantly more frequently occur in OSCC patients rather than in healthy controls (90).

1.3 General dental practitioner's role in OC and OPMD screening

General dental practitioner (GDP) and other dental health care providers have an important frontline role in the early screening of OC (91, 92, 5). However, some studies indicated that most of the general dental practitioners (GDP) have an insufficient knowledge of oral cancer sign and symptoms, and inadequate skill to perform a complete examination as part of dental routine examination (93, 94). GDPs' lack of knowledge and experience in oral cancer examination become one of obstacles to early diagnose and treatment of oral cancer (93, 94). In addition, inadequate GDPs' skill, knowledge, and confidence to perform OC and OPMD detection may also impact on an inadequate education provided to their patients (22).

A hands-on training courses and continuing education seem to be an essential strategy to increase and maintain knowledge of healthcare provider specifically GDP and dental health providers (91, 95). However, the study which evaluated training for continuing education of oral cancer early screening to the GDPs and other dental health providers is very limited to none.

A previous study aimed to implement the national project created within the framework of the state task of the Ministry of Education and Science of the Russian Federation, namely Development of the program of screening of cancer and precancerous diseases of the oral mucosa and red border of the lips. A national research groups have been created at the bases of the Department of Therapeutic Dentistry of the Moscow State Medical and Dental University, the Tula regional dental clinic, City dental clinic in Noyabrsk City (Yamal-Nenets autonomous district). A total 217,806 people aged 18-92 years old were enrolled to epidemiology study of oral cancer and precancer within a period from 2015 to the first half of 2018 studied the epidemiology of cancer and precancer. Over that time, the effectiveness of the research team increased by 8.45 times. The study also found that dentist had a low

awareness level of oral cancer. A training for oral cancer and precancer screening was set and developed and 574 dentists were enrolled within continuous medical education programs (96).

Maybury et al reported the reduction of oral cancer incidences and mortalities in Maryland from 1995 to 2009, which significantly improved the state's ranking in the nation (97). It suggested that the Comprehensive Cancer Control Plan developed in the state has contributed to the success and that the Maryland approach may serve as a model for other states. A state-wide oral cancer prevention program with emphasis on problem identification (survey), training, and coordination is unique and effective.

Most of the studies documented the relation between the importance of educating dentists and other health providers of oral cancer with the reduction of oral cancer incidence and mortalities over period of time, however, they did not clearly explain what kind of training given to the dentists and how the training impact the dentists' and other dental health workers' awareness of oral cancer.

1.4 Oral potentially malignant disorders (OPMD)

Asymptomatic clinical lesions mostly preceded OC in most cases and collectively referred to as oral potentially malignant disorders (OPMDs) (98, 99). According to the new classification of the World Health Organization, OPMDs are defined as "clinical presentations that carry a risk of cancer development in the oral cavity, whether in a clinically definable precursor lesion or in clinically normal oral mucosa" (100).

OPMD has been defined as oral lesions with a predisposition to malignant transformation (101). The OPMD definition, terminology and classifications have been discussed and recommended in a World Health Organization (WHO) Workshop, held in 2005 (101). The classification of OPMD by WHO was separated into two subgroups as follows: "a) premalignant lesion, a benign lesion with morphologically altered tissue, which has a greater than normal risk of transforming into malignancy; b) premalignant condition, a disease or patients' habit that does not necessarily alter the clinical appearance of local tissues but is associated with a greater than normal risk of precancerous lesion or cancer development in that tissue" (102, 101) (Figure.1.4).

Premalignant lesions	Premalignant conditions
Leukoplakia	Lichen planus
Erythroplakia	Discoid lupus erythematosus
Proliferative verrucous leukoplakia(PVL)	Epidermolysis bullosa
Viadent leukoplakia	Verruciform xanthoma
Candida leukoplakia	Graft-versus-host-disease
Reverse smokings' palate	Cheilitis glandularis
Verrucous hyperplasia	Xeroderma pigmentosum
Oral verrucous carcinoma	Syphilis (third stage)
Dyskeratosis congenita	Plummer-Vinson syndrome
Actinic cheilosis	Malnutrition
Keratoacanthoma	Vitamin A, B, C deficiency
Oral submucous fibrosis	Immunosuppressive diseases [AIDS]

Figure 1. 4 Oral potentially malignant disorders (OPMD) (101)

The information in regards of prevalence of OPMD in the general population is still limited, however a commonly accepted prevalence of OPMD was reported between 1–5% (103). OPMD mostly affected patients in average 50-69 years of age, which is 5 years before oral cancer presentation. Unfortunately, the recent studied found 5% OPMD occurred in person under 30 years of age. The common site of OPMD is on the buccal mucosa, followed by gingivae, tongue and floor of the mouth (104, 105, 102). The most common OPMD that have a high malignant transformation rate are leukoplakia, oral submucous fibrosis and erythroplakia (103).

Beside a COE that is required for detection of OPMD, the following criteria should be taken into consideration in terms of the importance of early diagnosis: (1) symptomatic and/or non-symptomatic non-healing lesions of oral mucosa, (2) history of smoking, chewing tobacco,

alcohol consumption, oral HPV infection, drug use, long-term exposure to sunlight, (3) advanced age, (4) the presence of immunodeficiency, (5) the presence of genetic disease, and (6) poor oral hygiene (103).

1.4.1 Oral leukoplakia

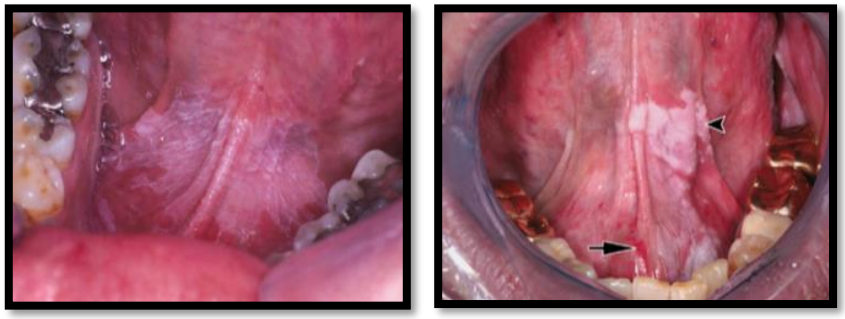
Leukoplakia is defined as "a predominantly white lesion of the oral mucosa that cannot be characterised as any other definable lesion" (106, 101). A report by Warnakulasuriya proposed that: "OL should be used to recognise white plaques of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer" (107, 101). The prevalence of leukoplakia in the recent studies showed varies between 1.1% and 11.7%, with a mean value of 2.9% (108). Leukoplakia mostly occurs in individual under the age of 40, however, there is possibility to occur at any age (109). Petti showed that there was no prevalence differences between individuals aged 50 years or older and those aged less than 50 years (110). Oral leukoplakia occurs at least three times more often in males than females (110, 111).

In the developing countries, tobacco use and areca nut chewing habit, either alone or in combination are strongly associated with the occurrence of oral leukoplakia, whereas in the developed countries, majority of oral leukoplakia are associated with just the use of tobacco (112, 113).

Clinically, leukoplakia can be divided into two subtypes including homogeneous and non-homogeneous types (107). Homogenous lesions (**Figure 1.5**) are characterized by uniformly flat, thin, white in colour and shows shallow cracks of the surface keratin (109, 107). Non-homogenous type have been defined as mix of a white and red lesion, also known as *erythroleukoplakia*, either irregularly flat (speckled) or nodular (103), as can be seen in **Figure 1.5**.

Verrucous leukoplakia is yet another type of non-homogenous leukoplakia (114) . Proliferative verrucous leukoplakia (PVL), was first described by Hansen *et al* (114) in 1985, is a form of verrucous leukoplakia and characterized by multifocal presentation. Malignant transformation rate of OPL is high and resistance to treatment (107). PVL seems more prevalent among elderly women. There may or may not be a history of tobacco use (107).

There are few studies about aetiology of PVL that tried to identify the presence of *Candida albicans and Epstein Barr Virus (EBV)* (115). Silverman et al reported 19 of 38 specimens with *C. albicans* positivity, however, they found no correlation either between the fungal infection and PVL occurrence or transformation to carcinoma, it might be a secondary infection (116). Similarly, Hansen et al found that 12 of 30 patients were positive for *C. albicans* (117). Differently, Bagan et al was the only one to detect EBV in a PVL group, they found 4 of 6 patients with EBV (60%) (118). Therefore, the exact role of microbiological agents in PVL pathogenesis have not been established yet in those studies (115).



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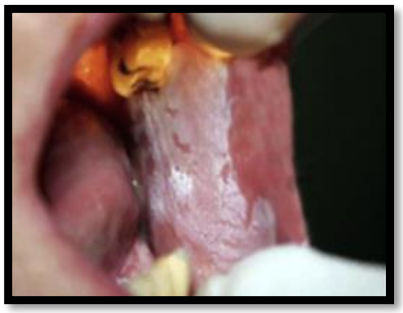

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Figure 1. 5 Leukoplakia (115, 119).

A. Homogenous type (119), B. Non-homogenous leukoplakia (erythroleukoplakia) (119), C. Proliverative verrucous leukoplakia (115) (PVL).

Histopathologically, leukoplakia may be seen as two distinct appearances as dysplastic or non-dysplastic (111). Oral leukoplakia characterized by hyperkeratosis of the stratified squamous epithelium, with or without acanthosis and/or epithelial dysplasia (111). There are miscellaneous benign and/or OPMD that may be seen similarly white or predominantly white appearance, this condition needs to be distinguished. Differential diagnosis of those disease that can be considered as leukoplakia, including aspirin burn, chemical injury, oral pseudomembranous and hyperplastic candidiasis, frictional lesions, oral hairy leukoplakia, leukoedema, linea alba, lupus erythematosus, morsicatio buccarum, papilloma and allied lesions, mucous patches in secondary syphilis, tobacco-induced lesions, smoker's palate

(nicotinic stomatitis), snuff-induced lesion, white sponge nevus, oral lichen planus, and lichenoid reaction (109, 107).

The malignant transformation rate of leukoplakia is reported in some studies associated with several risk factors such as female gender, long duration, leukoplakia in non-smokers (idiopathic), location on the tongue and/or floor of the mouth, size more than 200 mm, non-homogenous type, presence of *C. albicans* and epithelial dysplasia (120, 121, 107), as can be seen in **Figure 1.6**.

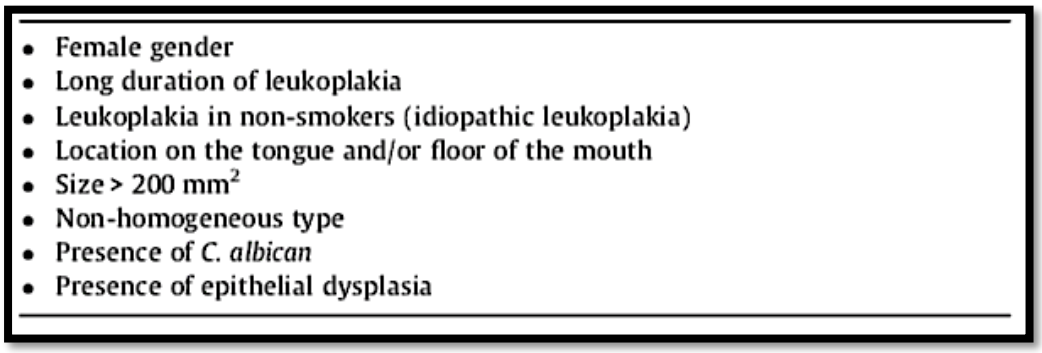


Figure 1. 6 Reported risk factors associated with malignant transformation of leukoplakia (107)

In 2005, WHO Collaborating Centre for Oral Cancer and Precancer coordinated a workshop for international working group comprising specialists in the fields of epidemiology, oral medicine and pathology and molecular biology with a special interest in oral cancer (101). In this meeting, they also discussed a schema in recognition of leukoplakia to avoid confusion. The schematic flowchart (Figure 1.7) shows that after a white patch is eliminated from any other oral mucosal diseases, leukoplakia is the provisional clinical diagnosis, and should be followed by biopsy, and subsequently if biopsy revealed confirmation of no other disorders, the lesion is further characterized as leukoplakia with or without dysplasia (101).

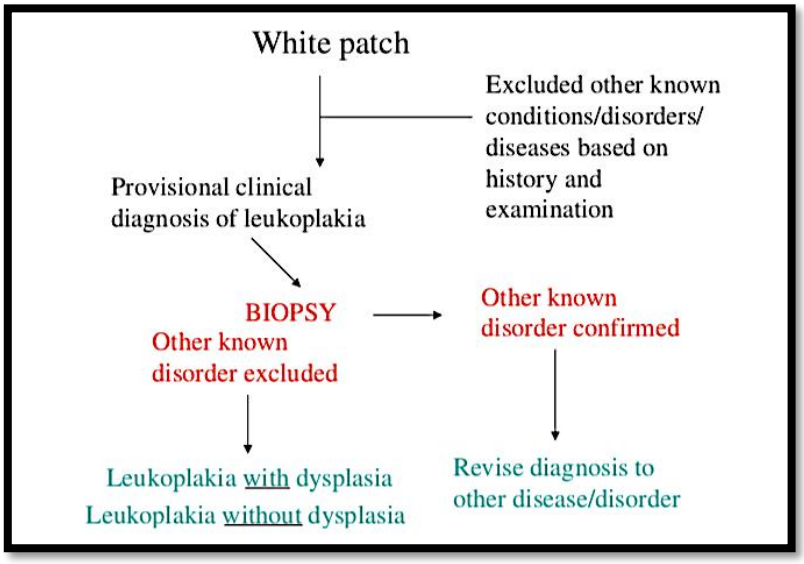


Figure 1. 7 Schematic representation of the steps in diagnosis of oral leukoplakia (101).

If the possible associated risk factors are present, including tobacco, alcohol, and betel quid use (112, 102, 101, 103), the patient needs to cease all possible risk factors, and an observation period of not more than 2–4 weeks seems acceptable to observe a possible regression after elimination of such factors. At this stage, histopathologic examination is the gold standard for diagnostic aims, some adjunctive tools may help to assist biopsy area (107).

The treatment of leukoplakia aims to diminish the possible symptoms and as an attempt to prevent malignant transformation, for that reason, an active treatment of leukoplakia would appear to be a more safe practice, whether there is epithelial dysplasia or not (122). There are various treatment modalities for leukoplakia such as surgical excision, cryosurgery, laser surgery that includes evaporation (123, 124), administration of retinoids, either topically or systemically (125, 126), mouthwash therapy containing an attenuated adenovirus (127, 128) and photodynamic therapy (129). Surgical excision and laser therapy are the most commonly used treatment for leukoplakia (107)

1.4.2 Oral Erythroplakia

Erythroplakia is defined as “A fiery red patch that cannot be characterized clinically or pathologically as any other definable disease” (130). This condition has been considered to have the greatest potential malignant transformation in oral mucosal. The definition has not changed over a period, and the definition by WHO (1978) is still widely used (130).

Clinical appearance is often appears as a flat red either with smooth or granular surface (Figure 1.5) (131). Erythroplakias seem to be relatively uncommon to appears on their own and often as mixed red-and-white lesions that refer as “*erythroleukoplakia*” (131). Prevalence of erythroplakia varies between 0.02% and 0.83%. It mainly presence in the middle aged and the elderly. Male gender is predominantly affected. Mostly, oral erythroplakia occurs at any part of oral mucosa as a solitary lesion. Studies indicated that the most commonly affected areas were soft palate, the floor of the mouth, and the buccal mucosa (132, 107). Erythroplakia (**Figure 1.8**) is usually asymptomatic, but some patients may complain of a burning sensation (133, 134). According to Shafer and Waldron (135), 51% of erythroplakias transformed into OSCC, 40% were carcinoma in situ and 9% showed mild to moderate dysplasia.

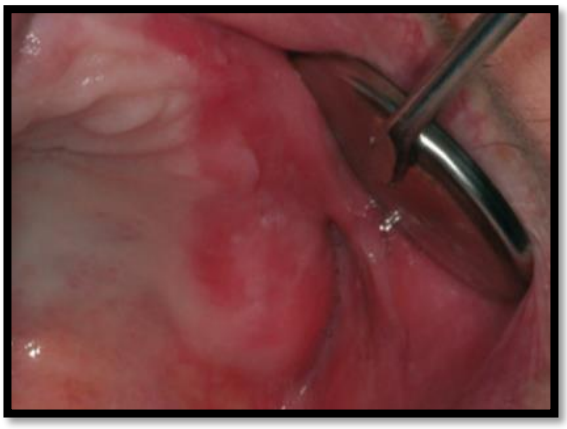


Figure 1. 8 Erythroplakia (112)

The exact etiopathogenesis of oral erythroplakia is unknown, however the possible etiology of chewing tobacco and alcohol use have been reported. Furthermore, investigation by Hashibe *et al* reported that in Indian population, chewing tobacco and alcohol drinking are strong risk factors associated with erythroplakia (107).

A common finding in the histopathological examination is moderate or severe dysplasia in lesions with erythroplakia. Malignant transformation rates is high and vary between 14-50%, and as such the treatment of oral erythroplakia is mandatory and should be done swiftly after initial finding (136, 131, 107). The recommended therapy for oral erythroplakia is surgery, either by cold knife or by laser (107). In lesions with severe epithelial dysplasia or carcinoma *in situ*, surgical excision may necessary to be undertaken (131).

Oral erythroplakia should be distinguished from other oral diseases which clinically appears as red patch. Clinician may be confuse with some oral diseases that is similar to oral erythroplakia, such as oral candidiasis, oral histoplasmosis, oral tuberculosis, atrophic OLP, lupus erythematosus, pemphigus, pemphigoids, amelanotic melanoma, haemangioma, telangiectasia, lingual varies, Kaposi's sarcoma, local irritation, mucositis, drug reaction, median rhomboid glossitis, and oral purpura may be confused with oral erythroplakia (131).

1.4.3 Oral Submucous Fibrosis (OSMF)

OSMF is a chronic and potentially malignant disorder characterized by juxtaepitelial fibrosis of the oral cavity (137). The disease was first described by Schwartz in 1952. Fibroelastic change of the lamina propria and epithelial atrophy occur in consequence of juxta epithelial inflammatory reaction, and eventually develops stiffness of oral mucosa, trismus and an inability to eat (137). A previous study in prevalence of oral submucous fibrosis which examined 5 groups, approximately 10,000 people were enrolled in each of Indian villagers in 4 states of India. The prevalence rate varied between 0 to 4%. Clinical data are given on the 63

cases found in the survey. Atrophy on tongue papillae were seen in 60% cases. This finding support OSMF as premalignant condition (4).

Mostly, OSMF cases are seen in Asian population. OSMF predominantly occurs in the second and third decade and may affected both gender (137). Rarely seen in pediatric group of age (138-140). The malignant transformation rate has been reported between 7%-30% (137).

According to Guo et al (141), OSSC originating from OSMF occurs at a younger age (45.8 vs. 55.9 years). OSMF was seen more common in males compared to females (male-to-female ratio 32.1:1 vs. 2.3:1). Management of OSMF is with surgical excision, furthermore, intralesional injections of interferon-γ has been also recommended (133).

In a study from Allahabad, India, 239 OSMF patients were enrolled; 46% were in their 3rd decade of life. Buccal mucosa was found to be the most common affected site (20.8%), followed by palate (17.7%). Patients had trismus (37.2%), burning sensations (29.5%), excessive salivation (22.5%), and 14.2% suffered from recurrent oral ulcerations (142).

The aetiology of OSMF is not well established and thought to be multifactorial (137). Several epidemiology and clinical studies reported the strongest risk factor associated with OSMF is BQ chewing containing areca nut. Genetic and immunologic factors may also contribute to OSMF development as OSMF was reported in non-BQ chewer families (143, 4, 142).

Clinical presentation of OSMF depends on the stage of the diseases (144). Initial symptoms that mostly occur is the present of a burning sensation or intolerance to spicy food, and vesicles may appear particularly on the palate. In later stages, ulceration and mouth dryness is later followed by fibrosis of the oral mucosa, which leads to rigidity of the lips, tongue, and palate, and trismus (Figure 1.9) (144). Petechiae without blood dyscrasias or systemic disorders, are found in about 22% of OSMF patients and mostly occur on the tongue, followed by the labial and buccal mucosa (145, 146).

The diagnosis of OSMF is based on clinical and histological finding. Clinically, one or more of the following symptoms should be present; A). blanching of oral mucosa defined as a persistent, white, marble-like appearance of the oral mucosa, which may be localized, diffuse or reticular; B) tough, leathery texture of the mucosa; C) palpable, whitish, fibrous bands. Those clinical characterisations should be accompanied by histopathological investigations (147).

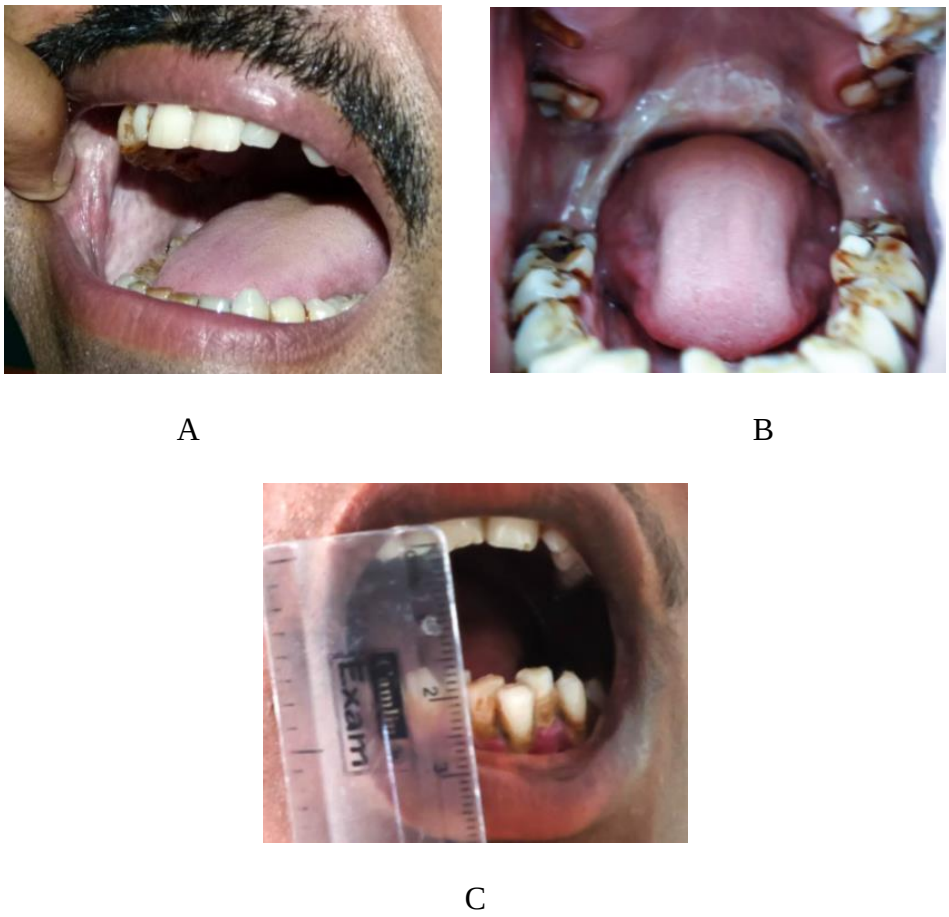


Figure 1. 9 Clinical appearance of OSMF (147).
A. Blanching of buccal mucosa, B. Blanching of soft palate, C. Decrease of mouth opening.

Histologically, OSMF is characterized by epithelial atrophy with loss of rete ridges and hyalinization of the lamina propria and the underlying muscle (148). The initial stage of OSMF

is characterized by mixed inflammation, oedema, and large fibroblasts. Later, there can be seen collagen bundles with early hyalinization, and the lymphocyte and plasma cell infiltrate, and occasionally resembling lichenoid mucositis (48). In more-advanced stages, the thick collagen bands and hyalinization extending into the submucosal tissues and decreased vascularity.

Currently, there are three treatment modalities for OSMF including surgical, physical, and medical are available. Surgical treatments may be used to improve mouth opening and movements. Physical treatment is used to help correction for mouth opening including physical exercise regimen, splints or other mouth opening devices, and microwave diathermy. Some studies have reported numerous medical therapy alternatives can be used for OSMF management such as steroids, interferon gamma, placental extracts, immunized milk, pentoxifylline, buflomedil hydrochloride, nylidrin, isoxsuprine, β-carotene, lycopene, vitamins, micronutriens, collagenase, hyaluronidase, chymotrypsin, and aloe vera (149, 137, 150, 151, 103).

1.4.3.1 OSMF and betel quid

The strong association between the presentation of OSMF with areca nut/BQ chewing or pan masala has been directly related to increased OSMF, whereas chewing or smoking tobacco does not increase the risk for OSMF (152). A case–control study from Kerala, India, reported that BQ chewing alone increased the odds ratio for OSMF to 56.2 (153).

The ingredients of BQ differ in different countries, but the major components being used are nut of *Areca catechu L*. **(Figure 1.10A)**, leaf of *Piper betle* **(Figure 1.10B)** or stem inflorescence (SI) of *Piper betle* **(Figure 1.10C)**, and slaked lime **(Figure 1.11)**. Tobacco **(Figure 1.12)** and other spices are optional to be included into chewing practices (154-156).

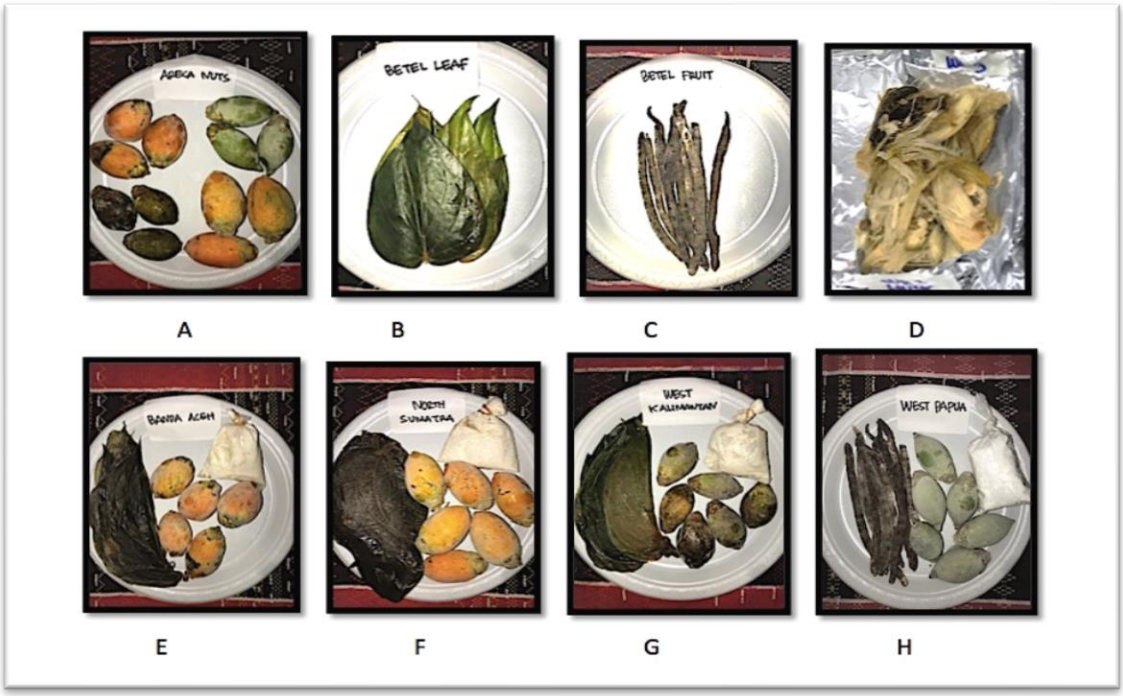


Figure 1. 10 Betel quid (BQ) From four regions of Indonesia.

A. Areca nuts, B. Betel leaf, C. Betel fruit, D. Husk, E. BQ Mixture from Banda Aceh, F. BQ mixture from North Sumatra, G. BQ mixture from West Kalimantan, H. BQ mixture from West Papua. (Personal documents)

A **B**

Figure 1. 11 Slaked lime, A. Slaked lime in powder form, B. Slaked lime in paste form (personal documents)

Figure 1. 12 Tobacco (personal documents)

Areca nut **(Figure 1.10A**) is the seed or endosperm of the palm Areca catechu (157, 155, 158). The synonymous term of 'betel quid' is 'pan' or 'paan', generally a mixture contains of areca nut, betel leaf (**Figure 1.10B**) or betel inflorescence stem (**Figure 1.10C**), and slaked lime **(Figure 1.11)**, some may contain tobacco. Other spices or substances are added according to local preference, including cardamom, saffron, cloves, aniseed, turmeric, mustard or sweeteners. The unripe areca nut has green in color, while the ripe areca nut looks yellow. In some countries such as Taiwan and China, the unripe areca nut is mostly consumed (155),

The *Piper betle leaf* (**Figure 1.10B**) is commonly used to wrap the whole package of areca nut, and the term betel nut has been used to describe this combination, incorrectly. To avoid the confusing term 'betel nut', it was recommended by a consensus workshop held in 1996 that the term 'quid' should be defined as 'a substance, or mixture of substances, placed in the mouth (**Figure 1.10. E, F, G, H**). Beside a fresh betel leaf, some people in Taiwan use the inflorescence stem (**Figure 1.10.C**) of *Piper betle* (155).

The husk (**Figure 1.10.D**) is the fibre pericarp outer layer of areca nut which appears green or yellow in colour, and some people in Papua new guinea include the husk when chewing areca nut (159).

Another most common component of betel quid is slaked lime. This is a white powder or paste which commonly placed in small plastic bags (**Figure 1.11**). This slaked lime is added to strengthen the taste of the BQ mixture. The powder or paste slaked lime could come from coral, shell fish, and quarried lime stone (155).

In Indonesia, the BQ chewing habit has been known since the 4th century on the island of Java, and 7th century on the island of Sumatera, whereas in Sulawesi it has been present since 14th century at the time of King Buton IV. However, there is no information when exactly the BQ habits spread across Indonesia (160). According to Budhy et al in 2001 (161), BQ chewing habit may be decreasing in Indonesia, however, presently many people remain

actively participating in the habit with some starting as young as 3- 5 years in certain regions such as Papua and North Sumatra.

1.4.3.2 OSMF and the addition of tobacco in BQ chewing

A number of studies have reported the increased risk of OSMF when tobacco is added into BQ mixture. A relative risk for OSMF of 489 has been reported in BQ combined tobacco chewer compared to non-users (162). The user of BQ-tobacco mixed products are often younger individual (163, 164). The development of OSMF in the mouth is faster with a mixed BQ-tobacco chewer reported to develop after 2.7 years of consumption, while BQ chewer without tobacco develop OSMF after 8.6 years. Malignant transformation also appeared at an early age (165).

Nitrosamines, reactive oxygen species, and depletion of endogenous anti-oxidant capacity seems to be the dominant contributors for OSMF development in BQ-tobacco mixed (166, 167). Subepithelial fibrosis of oesophagus is seen more frequently in patients who had consumed areca nut/BQ-tobacco mixed for longer than 5 years (168).

Hosein *et al* (157) studied areca nuts and its derivates in a cross-sectional multi-centre study that was conducted over 8 years. OSMF was diagnosed clinically (n = 765) from both public and private tertiary care centres. Consumption of areca nut in different forms, frequency of daily usage, years of chewing, degree of mouth opening, and duration of the condition were recorded. They found that the addition of tobacco could contribute to the severity of OSMF (moderate OSMF cases, n = 176) and betel quid without tobacco (severe OSMF cases, n = 167).

1.4.3.3 The pathogenesis of OSMF

OSMF is essentially a collagen metabolism disease (169). There is strong evidence that the AN has a main role in the OSMF development **(Figure 1.13)**, but it has not been elucidated (144, 170). However, the extracellular matrix changes seems are likely to have a key role (170). Some studies reported an increased synthesis, or reduced degradation, of collagen, as possible mechanisms in the OSMF development(170); there are changes in the normal collagen metabolism at different stages.

The homeostasis of the extracellular matrix is interfered by such as alkaloids, flavonoids, and copper which are present in AN. Four major alkaloids; arecoline (the most potent), arecaidine, guvacine, and guvacoline have been known to stimulate fibroblasts to produce collagen (170). Flavonoids such as tannins and catechins can inhibit collagenase, stabilise the collagen fibrils, and render them resistant to degradation by collagenase (144, 170).

AN causes a localised mucosal inflammation results in the recruitment of activated T-cells and macrophages that lead to an increase of cytokines and tumour growth factor beta (TGF-β) production(169). The latter considerably increases the production of collagen by activating procollagen genes, and upregulating procollagen proteinase enzymes and lysyl oxidase activity (169, 171). TGF-β inhibits collagen degradation simultaneously by activating the tissue inhibitor of matrix metalloproteinase (TIMP) genes and plasminogen activator inhibitor (PAI) (169). The high concentration of copper in AN has been shown to stimulate lysyl oxidase activity, an enzyme essential to the final cross-linking of collagen fibres (172, 169). Increased copper concentration has been seen in mucosa affected by OSMF, which supports its role in fibro genesis by enhancing lysyl oxidase activity (144, 173, 174).

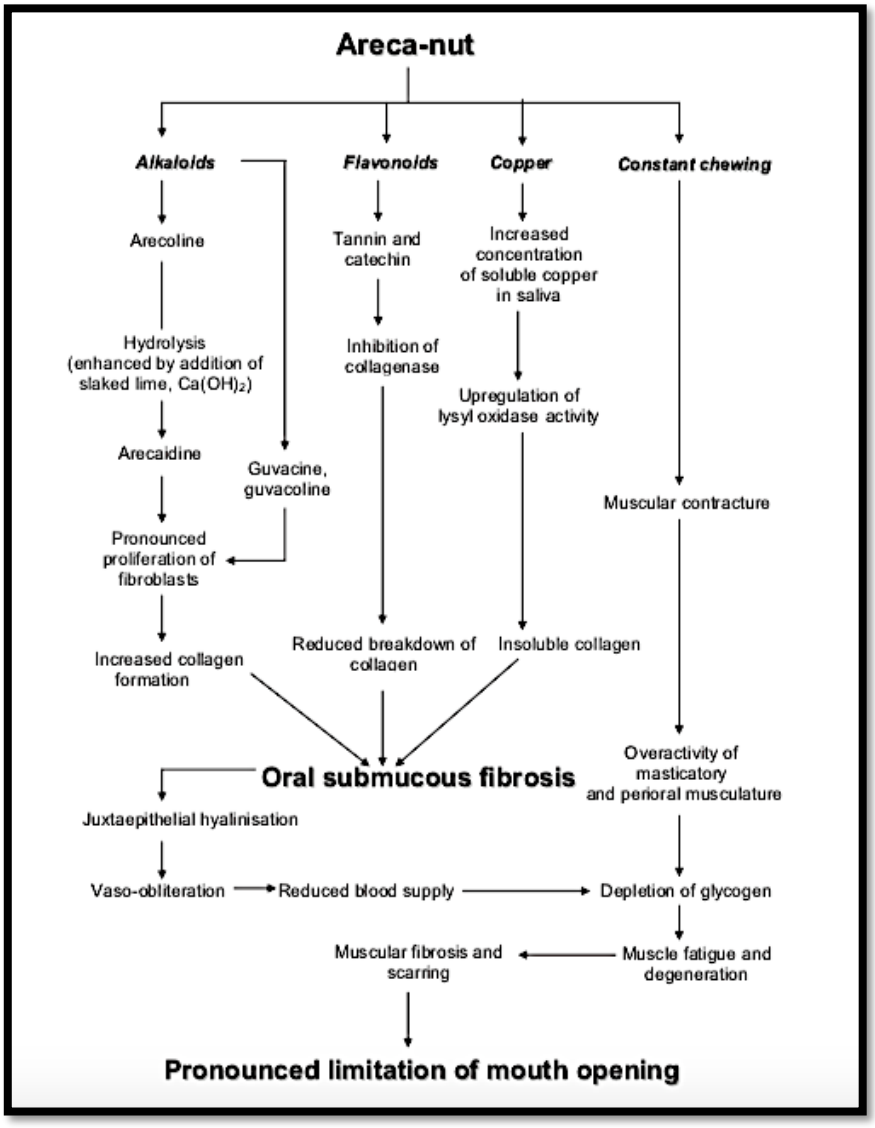


Figure 1. 13 The role of areca nut in the pathogenesis of OSMF(137)

Consuming AN continuously leads to an increased activity of the masticatory muscles, glycogen, depletion and muscle fatigue. The decreased blood supply following fibrosis further triggers muscle fatigue and causes extensive degeneration and fibrosis in the muscles(175).

Sarode, S. C., Mahuli, A., Sarode, G. S. and Mahuli, S. (176) hypothesized that areca nut alone cannot trigger OSMF but must include other necessary required factors such as slaked lime and inflammation. These factors possibly make conversion into phenotypically altered fibroblasts, which leads to increased fibrosis in the oral mucosa causing OSMF. The study done by Thomas in 1992 (177), showed that powdered slaked lime applied to the chewed areca nut

and betel inflorescence at the corner of the mouth in Papua New Guinea, causes the PH change to 10, at which reactive oxygen species (ROS) are produced from betel quid in vitro. ROS together with lime-induced cell proliferation suggested as a possible mechanism of oral carcinogenesis (177).

Another two possible predisposition of OSMF are autoimmune factors and genetic factors (144). The presence of circulating immune complexes, immunoglobulins, and autoantibodies in some patients with OSMF are an evidence, as well as altered cellular and humoral responses (144, 137, 178, 170). Genetic susceptibility is supported by increased production of HLA-A10, -B7, and DR3 in OSMF patients compared with normal controls (179).

1.4.4 Actinic cheilitis

Actinic cheilitis is one of OPMD that occurs on the lip caused by exposure to solar or ultraviolet radiation. The lower lip is mostly affected due to the anatomic proximity. The development of actinic cheilitis may also be triggered by tobacco use, lip irritation, poor oral hygiene, and ill-fitting dentures. Man is predominance rather than woman for this disease (180). Study by Martins-Filho *et al* (181),reported that there was a 16.7% prevalence of actinic cheilitis in farmers in a semi-arid area of Brazil. The estimation of malignant transformation rate ranging from 1.4% to 36% at 1 to 30 years interval (182).

At early stages, actinic cheilitis shows erythema and oedema, while in the late stage the disease may show diffuse scaling, thickened epithelium with small greyish-white plaques (known as *leukoplakia*), inflammatory areas (known as *erythroleukoplakia*), and linear fissures (180). Biopsy should be performed to confirm diagnosis to evaluate the degree of dysplasia.

Histopathological characteristic of actinic cheilitis shows hyperplasia, acanthosis or atrophy of the epithelium, thickening of the keratin layer, and/or dysplasia, which may range

from mild to severe. In connective tissue, basophilic degeneration of collagen fibers known as solar elastosis is usually detected, this solar elastosis occurs in addition to epithelial changes(183).

The available treatment modalities for actinic cheilitis such as 5-fluorouracil, scalpel vermilionectomy, chemical peel, electrosurgery, cryosurgery, CO2 laser, imiquimod, photodynamic treatment, and diclofenac 0.3% gel (180, 182, 103).

1.4.5 Oral lichen planus (OLP)

OLP is a chronic inflammatory disorder demonstrating some immune pathology. It is a cell-mediated immune condition. The exact aetiology is still unknown. In OLP T lymphocytes production accumulate beneath the epithelium of the oral mucosa and differentiation of stratified squamous epithelium is increase, resulting in hyperkeratosis and erythema with or without ulceration (184).

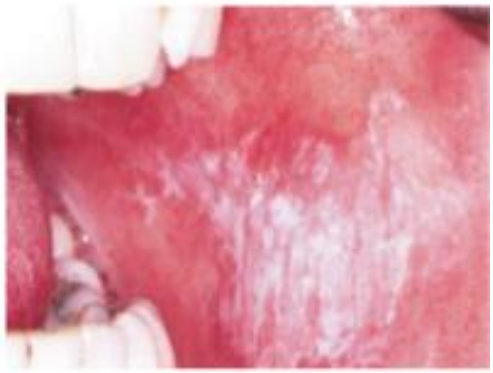


Figure 1. 14 OLP (15)

The annual malignant transformation rate is usually well below 1%. Apparently, such event may occur in all clinical types of OLP (185, 107). There is considerable debate as to the potentially malignant nature of this condition (186), while some authors state that OLP carries an unequivocal malignant potential and an unspecified risk (187). OLP and lichenoid lesions have characteristic, but not pathognomic, clinical and histological appearances, usually

allowing distinction from oral leukoplakia: the plaque type of lichen planus may, however, resemble leukoplakia (**Figure 1.14**), emphasizing the importance of biopsy in diagnosis. There are difficulties in distinguishing lichen planus from lichenoid lesions or lichenoid contact lesions (188).

1.4.7 Immunodeficiency

In patients with prolonged use of immunosuppressive drugs after solid organ transplants, human immunodeficiency virus-patients, and chronic graft versus host disease after stem cell transplantation are the patients in risk group for oral cancer development (181).

1.5 Betel quid carcinogenicity

Several studies have reported that a wide spectrum of therapeutic properties is possessed by betel leaf and areca nut (189). The betel leaf has prebiously been shown to have anti-proliferative effect towards KB and HeLa cells (190), antiplaque (191), anti-diabetic and anti-inflammatory properties (192, 193). While Areca nut has an analgesic and anti-inflammatory action (194), wound healing (195), antidepressant (196) and anti-HIV activities (197). In general, the BQ components have significant therapeutic properties and it is rich in phenolics and high antioxidant properties. This correlation has been confirmed with the antioxidant activity being detected in the extract of betel leaf (198) and areca nut (199).

Free radicals in our body can be neutralized by consumption of antioxidant-rich foods, thus preventing or delaying the oxidative damage of lipids, proteins and nucleic acids (200). It has been reported that the antioxidants could reduce the mortality rate associated with cardiovascular disease (201, 202), and protect against cancer and other chronic diseases (203). In addition, many studies have reported that the antioxidant is highly associated with the cytoprotective activity of plant (204, 205). However, the BQ antioxidant compounds that are responsible for reducing the risk of chronic disease have not been identified or limited (206, 189).

While various methods are available to test antioxidant activities, however, the commonly used methods for in vitro determination of antioxidant activity of BQ constituents are total phenolic content (TPC), the ferric reducing/antioxidant power (FRAP) assay, and DPPH (1,1-diphenyl-2- picrylhydrazyl) radical scavenging assay (189, 207, 208).

Sazwi et al (189), examined five samples, which were betel leaf, areca nut, gambir, betel quid and betel quid containing calcium hydroxide. Samples were extracted in deionized distilled water for 12 hours at 37°C. Antioxidant activities were evaluated for radical scavenging activity using DPPH assay, ferric reducing activity using FRAP assay and lipid

peroxidation inhibition activity using FTC assay. Total phenolic content (TPC) was determined using Folin-Ciocalteu procedure. Among the ingredients of betel quid, gambir demonstrated the highest antioxidant (DPPH - IC_{50} = 6.4 ± 0.8 μg/mL, FRAP - 5717.8 ± 537.6 μmol Fe(II)/mg), total phenolic content (TPC - 1142.5 ± 106.8 μg TAE/mg) and cytoprotective (100.1 ± 4.6%) activities. Betel quid, when compared with betel quid containing calcium hydroxide has high antioxidant (DPPH - IC_{50} =59.4 ± 4.4 μg/mL, FRAP - 1022.2 ± 235.7 μmol Fe(II)/mg), total phenolic content (TPC - 140.0 ± 22.3 μg TAE/mg), and cytoprotective (113.5 ± 15.9%) activities. However, for all a positive correlation was observed between TPC and radical scavenging (r = 0.972), reducing power (r = 0.981) and cytoprotective activity (r = 0.682). The authors concluded that betel quid has higher TPC, and antioxidant and cytoprotective activities than betel quid with slaked lime (calcium hydroxide), and samples showed good lipid peroxidation inhibition compared to vitamin E (189).

1.5.1 Total phenolic content assay

The TPC assay is used to estimate the total phenolic content (TPC), it was initially developed by Folin and Denis for free and conjugated phenolic compounds in urine (209) and modified by Singleton and Rossi (210). Finally, to increase the specificity of the technique, some modifications on the initial method were proposed (211). In TPC technique, a phosphotungstic-phosphomolybdic reagent with added lithium sulfate and bromine, the so-called 'Folin-Ciocalteu phenol's reagent' is used together with a saturated sodium carbonate solution to basify the reactional medium (pH 10-12) generating the phenolate anion. Thereafter, a redox reaction takes place between the phenolate anion and Folin-Ciocalteu reagent (yellow) in which the molybdenum undergoes reduction and the colour of the solution changes from yellow to blue after a period of approximately 60 minutes at room temperature and in the dark room. This colour shift from yellow is due to the development of a blue metallic complex and

is associated with the TPC of the test sample (Figure 1.17). The maximum absorption of the blue complex lies between 620 and 765 nm (211).

1.5.2 The ferric reducing/antioxidant power (FRAP) assay

The ferric reducing/antioxidant power (FRAP) assay is a direct test of "total antioxidant power." Antioxidant is used as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant present in stoichiometric excess. The FRAP assay does not use a lag phase type of measurement, which make the assay distinguishable from the many indirect radical scavenging tests (212).

In the concept of FRAP Assay, a biological antioxidant has been defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate." However, unless an antioxidant prevents the generation of an oxidizing species, for example, by metal chelation or enzyme-catalyzed removal of a potential oxidant, a redox reaction still generally occurs, even in the presence of an antioxidant. The difference is that the oxidizing species reacts with the antioxidant instead of the "substrate," i.e., the antioxidant reduces the oxidant. In simple terms, electron-donating antioxidants can be described as reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reactive species is reduced while another is oxidized. In this context, therefore, "total antioxidant power" may be referred to analogously as total reducing power (212).

The principle of FRAP Assay, is at low pH, reduction of a ferric tripyridyl triazine (Fe^{3+}-TPTZ) complex to the ferrous form, which has an intense blue color, can be monitored by measuring the change in absorption at 593 nm. The reaction is nonspecific, in that any half-reaction that has a lower redox potential, under reaction conditions, than that of the ferric/ferrous half-reaction will drive the ferric (Fe^{3+}) to ferrous (Fe^{2+}) reaction. The change in

absorbance, therefore, is directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture (212).

1.5.3 DPPH (1,1-diphenyl-2- picrylhydrazyl) radical scavenging assay

The DPPH assay is a common method employed to measure antioxidant activity, its benefit is simple and efficient technique (213). Initially, it was used to determine the antioxidant potential of phenolic compounds (214). Moreover, the other advantage of this method is easy, economic and rapid for the evaluation of the radical scavenging activity of non-enzymatic antioxidants (215, 216). The radical scavenging capacity of antioxidant is measured by donating hydrogen to the DPPH radical (214).

1.6 Identification and quantification of BQ constituents

Several studies have identified polyphenols and alkaloid in BQ components, especially in areca nut and betel leaf (217-221, 207). However, the information about BQ components' constituent in more detail part as in areca nut, betel leaf/betel stem inflorescence, husk, and BQ mixture with slaked lime is still very limited. The most common identification technique including high performance liquid chromatography, or also known as high pressure liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LCMS) (218-221, 207). The analysis of BQ components using gas chromatography mass spectrometry (GC-MS) is very limited to none.

1.6.1 High pressure liquid chromatography (HPLC)

This method is the most commonly used analytical methods in the pharmaceutical industry. HPLC is mainly used for the separation, identification, qualitative and quantitative analysis of the components in a sample mixture. Semi-volatile or non-volatile organic compounds identification is often using HPLC (222, 223).

HPLC is a column chromatography type, however HPLC is highly sensitive and extremely automated compared to the column chromatography. In column chromatography, the solvent allowed under gravity through a column, while in the HPLC technique it utilizes high pressure up to 400 atm (224, 225). Due to the high pressure and greater area of the packing material in HPLC, it allows faster and better separation than the column chromatography which is due to the smaller particle size accommodated. This greater surface area facilitates better interactions between the packing material and the molecules to be separated which is the stationary phase and also the molecules flow through the stationary phase (226, 227).

The practical technique of HPLC is complex. It involves a sampler, pump and a detector. HPLC uses the concept of affinity between the mobile phase which is the solvent and

the stationary phase (228, 229). A pump is used to inject a continuous flow of solvent. Into this solvent a dissolved sample is injected. Once the sample is in the solvent, it passes through the column (230). The components in the sample are separated based on their affinity to the molecules in the column. After the compounds in the sample are separated, they pass the detectors to be identified. The software is utilized in the HPLC technique for the data analysis (231).

There are two forms of HPLC technique, first is normal phase HPLC, this is the basic column chromatography. It is less commonly used, the column is filled with silica molecules, and non-polar solvent. Polar components in the mixture being passed through the column will adhere longer to the polar silica particles than non-polar compounds (232). The non-polar compounds will therefore pass faster through the column (232). Second, is reversed phase HPLC, this technique is most commonly used nowadays. In reverse phase HPLC, the silica is made non-polar by attaching long hydrocarbon chains to its surface. A polar mobile phase is used (233). Therefore, there will be a strong adherence between the polar solvent and polar molecules in the mixture being passed than between the hydrocarbon chains attached to the silica and the polar molecules in the solution (234). Non-polar compounds will attract with the hydrocarbon groups because of van der Waals dispersion forces. They are less soluble in the solvent. Therefore, this will slow them down on their way through the column. This resulting, the polar molecules will pass through the column quicker (234). The figure is reverse HPLC technique form.

A study by Chavan and Singhal have developed an effective method for maximum extraction of polyphenols with minimum arecoline, in areca nut. Several factors such as nature of the solvent, pH (2–10), substrate concentration (6–14 %) and extraction time (30–150 min) under shaking conditions were evaluated. Identification of polyphenols and arecoline were done using high-performance liquid chromatography (HPLC) (218). The results showed

maximum extraction of polyphenols (407.47 mg GAE g^{-1}), total tannin and its antioxidant activity with minimum arecoline (1.73 mg g^{-1} of sample) was achieved by using 80% acetone at pH 4 for 90 min with 10% w/v substrate under shaking conditions. Solvent extraction under optimized parameters gave maximum polyphenols with minimum extraction of arecoline, and highest ratio of polyphenols to arecoline. The presence of catechin and epicatechin in the extract was confirmed using HPLC and liquid chromatography–mass spectrometry results confirmed (218).

Another study done by Yang *et al* investigating chemical constituents of Areca catechu using HPLC. The results identified thirteen compounds from the 95% ethanol extract of the Areca catechu (235). The identified chemical constituents were cathecin, ferulic acid, vanillic acid, isorhamnetin，quercetin，liquiritigeni, β-sitosterol，cycloartenol de-O-methyllasiodiplodin, 5，7，4'-trihydroxy-3'，5'-dimethoxyflavanon, 5，8 - epidioxiergosta-6，2 2 -dien-3 β -ol，stigmasta-4-en -3-one.

1.6.2 Liquid chromatography mass spectrometry (LC-MS)

Over the past 15 years, liquid chromatography mass spectrometry (LC–MS) has evolved from a scientific curiosity into a routinely applied technique and increasingly more used in routine field laboratories. This has become possible largely due to the advent of the atmospheric pressure ionization (API) methods (236). The API sources are able to produce gas-phase ions with little or no spontaneous decomposition from delicate and high molecular weight analytes. This, combined with the intrinsic sensitivity of mass spectrometers, has revolutionized large areas of chemical analysis where traces of organic analytes are determined in complex matrices. Among the ionization methods electrospray ionization (ESI) has proven especially versatile (236, 237).

LC–MS offers additional selectivity and confirmation of identity compared to "traditional" chromatography (i.e., chromatography with unidimensional detector, such as UV absorbance) by determining the mass/charge ratio of the ion(s) or recording MS data (in the broadest sense of this word) for the whole chromatogram, often resulting in three-dimensional datasets. This has proven immensely useful in the wide range of fields where LC–MS is applied (238, 236, 239).

A study using LC-MS was undertaken by Sazwi (189) to identify five components of betel quid such as betel leaf, areca nut, gambir, betel quid and betel quid containing calcium hydroxide. Samples were extracted in deionized distilled water for 12 hours at 37°C. Phenolic composition was analyzed using LC-MS/MS. LC-MS/MS analysis revealed the presence of quinic acid as the major compound of gambir and betel quid. They concluded that quinic acid in betel quid may play an important role in the oral health protection.

1.6.3 Gas chromatography mass spectrometry (GC-MS)

GC–MS platform has a vigorous and excellent separation capability and high sensitivity as well as reproducibility (240-242). GC-MS is generally considered as a very compotent analytical method (243). Electron ionization (EI) and chemical ionization (CI) are the two main forms of ionization used in GC–MS (244). However, to date, most GC–MS methods in profiling metabolomics use EI. GC-MS is considered as an easy to use device, especially for analysis time and operating costs. Further, its capability to quantify and identify compound and availability of several mass spectral databases/libraries corresponding to EI-based GC–MS makes it the method of choice for many analysts studies (244).

Even though a variety of ionization sources are available for different analytical instruments used in metabolomics (244). However, electron ionization (EI) mass spectrometry is the most common form of GC-MS detector used, this is due to EI can lead to the reproducible fragmentation of molecules into well characterized mass spectral fingerprints, which is also considered as a '*hard*' ionization method (244).

GC-MS will generate the raw data files comprised of a complex three-dimensional data format that consists of retention time, *m*/*z* values and the intensity or abundance in each of the axes (for GC × GC derived data, there is an additional dimension resulting from the second column set) (244). This comprehensive data requires to be processed before using any statistical techniques (244). The raw data will be translated into a two-dimensional data matrix suitable for statistical analyses (244). This can be undertaken using software that is packaged with the GC–MS instrument that was used to collect the data or, alternatively, raw data can also be processed into an open standard form such as mzML (245). The conversion also can use an open source programs such as msConvert that is part of ProteoWizard (246).

Until recently, the study in detecting betel quid's components using GC-MS is none to very limited. One study using GC-MS analysis documented that betel leaf (*Piper betle L.*) has

been revealed thirty-three and thirty volatile compounds, representing 98.41% and 97.34% of the optimized fresh and cured leaf essential oils. Eugenol, estragole, linalool, α-copaene, an-ethole, chavicol, and caryophyllene were found to be highly abundant in both essential oil with different percentages (247). Therefore, the details identification and quantification of betel quid components for areca nut, husk, betel leaf/SI, and betel quid mixture using GC-MS platform has not yet been undertaken by researchers.

1.7 Cytotoxicity effect of BQ

Cytotoxicity assay is the simplest in vitro technique that was earliest designed to evaluate biocompatibility of materials (248). Several biological endpoints used in cytotoxicity examination include: A) morphological assessment using histology or ultrastructural analysis of the cells using the SEM or TEM; B) cell viability and proliferation assays such as the Alamar blue assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, neutral red uptake, propidium iodide assay, lactate dehydrogenase assay, bromodeoxyuridine incorporation assay, 3H-thymidine incorporation assay, and DNA or protein content measurement and C) cell function assays such as the measurement of the release of inflammatory markers, glutathione determination, heat-shock protein assay and apoptosis assay (248).

Areca nut (AN) as the main component of BQ is considered to be the major etiologic factor of oral cancer (249, 250). AN extract (ANE) contains mainly tannin and areca alkaloids (such as arecoline, arecaidine, guvacine, and guvacoline) are a potential carcinogen. Carcinogenic substances are released from BQ components during the period of chewing BQ, continue to irritate the oral mucosa, and lead normal oral mucosal into malignant transformation (251). BQ components could alter oral mucosal by causing endothelial cell damage and dysfunction. Cytotoxicity assay is the method to see pathobiological effect of BQ on oral mucosal, both stromal and endothelial. The data on BQ toxicity are diverse and numerous due to differences in methods of BQ extractions as well as cytotoxicity methods used (252, 250).

Yah-Yeh *et al* (251) studied C3H10T1/2 cells that were exposed to AN extract (ANE) for 24 hours. Cytotoxicity was evaluated by colony forming efficiency. The results showed ANE exhibited cytotoxicity to C3H10T/12 cells at concentrations higher than 320 mg/ mL as shown by a decrease in colony numbers. Another study of the effects of arecoline, a component

of BQ, on the growth and migration of endothelial cells (EAhy 926), and their adherence by U937 cells were investigated. EAhy926 endothelial cells were cultured and exposed to various concentrations of arecoline for 24 hours. Morphological changes were observed using phase-contrast microscopy. Cytotoxic effects were analyzed using a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. A wound closure assay was used to evaluate the cellular migration of EAhy926 cells. The addition of arecoline at concentrations >0.4 mM significantly decreased cellular viability. EAhy endothelial cells showed marked morphological changes, and cellular migration (253).

Van Wyk *et al* studied the effect of raw, baked and boiled areca nuts were tested on cultured buccal mucosa fibroblasts. Cells were exposed to extract concentrations of 50,100, 150, 300 and 500 μg/ml. Arecoline and arecaidine were determine using HPLC. They observed that areca nut extract is toxic to cultured fibroblasts and inhibits their proliferation in a concentration-dependent manner (254).

Numerous studies have reported the cytotoxicity effect of ANE, arecoline and BQ extracts on cultured oral keratinocytes and fibroblast separately (255-257, 253, 254, 251). However, no one has ever undertaken cytotoxicity test of BQ components in detail being as in AN, betel leaf/SI, husk and mixture BQ form on epithelial and stromal cell lines. Moreover, the information of Indonesian BQ in association with oral cancer is very limited.

1.8 Hypothesis

1. That Indonesian GDPs have inadequate knowledge of the risk of oral cancer and are inadequate in performing COE.
2. That there will be a significant improvement in Indonesian GDPs' knowledge of the risk factors for oral cancer as well as in performing COE after being given one day training for oral cancer.
3. That Indonesia has a high prevalence of OPMD or OC that are strongly associated with the actual found risk factors.
4. That each Indonesian BQ component from the four different regions has different carcinogenic and antimutagenic constituents identifiable using HPLC-MS or LC-MS.
5. That GC-MS can be used as an alternative method to identify carcinogenic and antimutagenic constituents in Indonesian BQ components.
6. That BQ components from WP and BA have different cytotoxic effects on oral keratinocytes and oral fibroblasts.

1.9 Aims

1. To determine Indonesian GDPs' knowledge, awareness, and confidence in oral cancer screening.
2. To assess the impact of a one-day training on OC on Indonesian GDPs' knowledge of the risk factors of oral cancer as well as in performing COE.
3. To assess the prevalence type, site, and regional variation of OPMD in Indonesian.
4. To assess the most significant risk factors for OPMD or OC in Indonesia.
5. To identify and quantify carcinogenic and antimutagenic constituents in Indonesian BQ components from the four different regions using HPLC-MS or LC-MS.
6. To determine the total phenolic content and antioxidant activities using the FRAP and DPPH assays in Indonesian BQ components.
7. To assess if GC-MS can be used as an alternative method to identify and quantify carcinogenic and antimutagenic constituents in Indonesian BQ components.
8. To identify and quantify other alkaloids and polyphenols in Indonesian BQ components that previously could not be identified using HPLC-MS or LC-MS.
9. To determine whether BQ components from WP have different cytotoxic effects on oral keratinocyte and oral fibroblast compared to BQ components from BA.

Chapter 2: Materials and Methods

2.1 Outline study of oral cancer (OC) and oral pre-malignant disorders (OPMD) detection among Indonesian and local GDP survey

The conceptual framework for this study shown in Figure 2.1 and outlines the two separate but linked aspect to this study. The first is Indonesian GDPs' knowledge in risk factors of oral cancers and in performing conventional oral examination for early screening, and the second is OC and OPMD detection among Indonesian; the prevalence and risk factors.

2.1.1 Ethical approval

Ethical approval was obtained from Faculty of Medicine University of Padjadjaran, Health Research Ethic Committee, Indonesia (reference number 701/UN6.C1.3/KEPK/PN/2016), and from The University of Melbourne (Ethics reference ID: 1748812).

2.1.2 Formal approval and arrangement

The formal approval for conducting a large-scale national wide research was granted by Indonesian Ministry of Home Affairs, Directorate General of Politic and National Affairs with reference number 440.02/4097/Polpum followed by approval from each provincial government, and local city government accordingly. All of these approvals were strictly required prior to the study being conducted. Furthermore, formal arrangement was made with Indonesian local dental association (*Persatuan dokter gigi Indonesia*) and the Faculty of dentistry as to;

1. Conduct a one-day standardize education training of oral cancer for GDP, as the Dental Association and/or Faculty or University are the only organization or

institution who have legal permission to conduct seminars or training in Indonesia.

2. Sought agreement for individual health service and Dental Hospital. This agreement was also sought by Local Dental Association or Faculty member together with researcher for data collection both in primary health care setting and Dental Hospital.

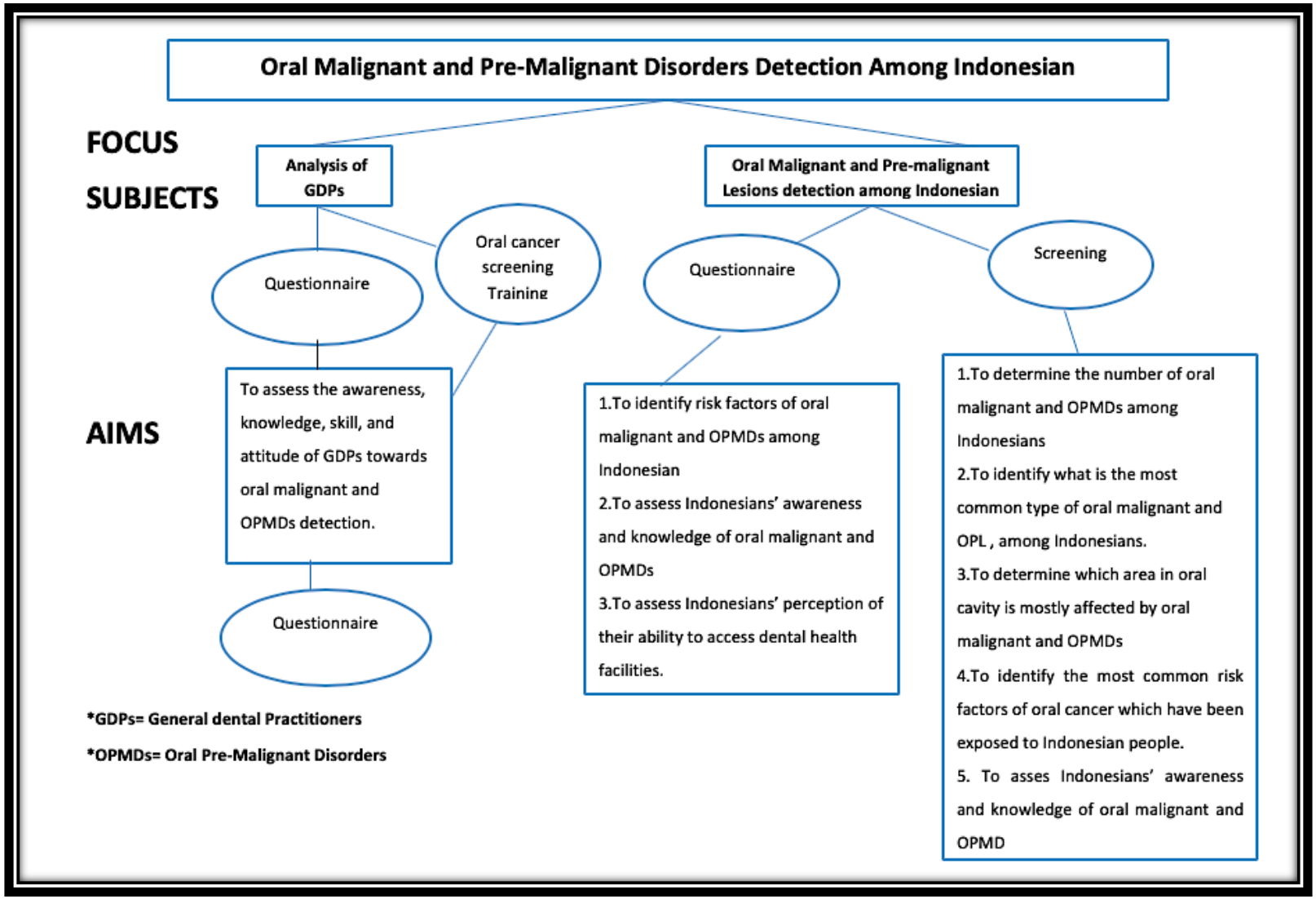


Figure 2. 1 Conceptual framework outlining the study of OC and OPMDs detection among Indonesian and local GDP survey

2.2 Indonesian GDPs' knowledge in risk factors of oral cancers and in performing conventional oral examination for early screening

This was a descriptive quantitative and qualitative cross-sectional study using a pre-training and post-training questionnaire and training intervention with both theory and practice. Consecutive sampling was used as a technique in recruiting respondents, and involved selecting all individual who agreed to participate, provided they meet pre-established criteria, until the desired number of subjects was recruited.

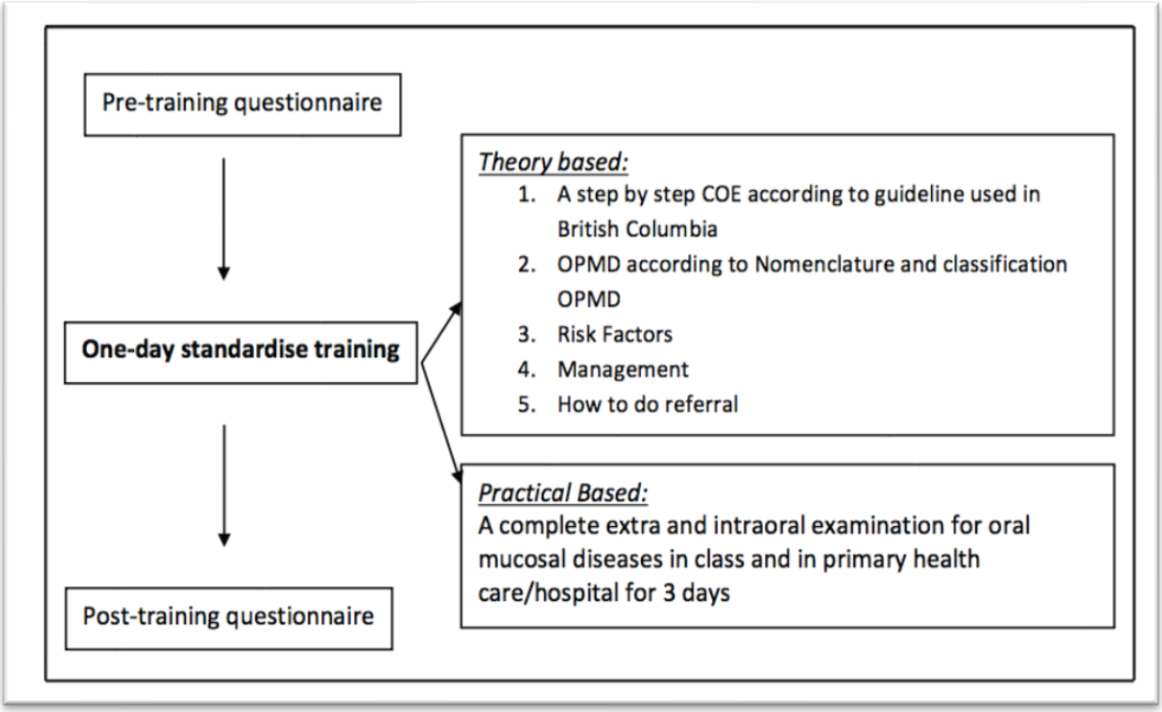


Figure 2. 2 Outlining the study design of a one-day standardize training program in oral cancer for GDPs in Indonesia that include both pre-training and post-training questionnaire

2.2.1 Participant recruitment

The local Dental Association or Faculty of Dentistry invited the GDPs to attend one-day a standardized education program of oral malignant and OPMDs detection. The GDPs who present in the meeting obtained clear explanation about the study and given a copy of plain

language statement (PLS) (**Appendix 1**). The GDPs who consented were enrolled as participant after signing the informed consent form (**Appendix 2**).

2.2.2 Pre-training questionnaire

The pre-training questionnaire consisted of 44 questions developed from existing literature and modified to suit the local situation (49, 258, 97, 95, 94). It underwent a validation process by a panel of senior researchers based in Indonesia to critically evaluate suitability, clarity, simplicity, understanding and sequencing of the questions (**Appendix 3**). Appropriate modifications to the questionnaire were made before being utilized in the main survey. The questionnaire contains multiple choice, open and closed question, Likert scale including GDP's knowledge, awareness, attitude, subjective assessment of their clinical competence for screening of oral cancer and level of education. A pre-training questionnaire was given to GDPs on the same day of the training. A pilot study has been conducted that consisted of 10 GDPs in Indonesia prior the survey modified.

2.2.3 One-day standardized education training program on OC and OPMDs detection

After completing a questionnaire, the GDPs undertook one-day standardise training on oral cancer. The GDPs were given intervention by theory-based and practical-based, a training to enhance confidence, expertise, knowledge, and ease of examination. The training was divided into two sessions. The first session focused on theory, explaining management of oral cancer and OPMD and how to undertake a referral, how to do a step by step conventional oral examination(41), the update classification of oral premalignant disorders and oral malignant (102, 101) with demonstrating more than 20 images of OPMDs and oral cancer, oral cancer risk factors (15) (**Figure.2.2**). The second session was practical-based intervention, participants were provided with intra oral mirror, hand gloves, white head lamp, and gauze,

divided into a small group and practiced on each other to undertake a proper COE. After adequate education for oral cancer detection, the GDPs subsequently were part of study as research team member and were involved in data collection in community services and selected villages under supervision of the principal researcher.

2.2.4 Post-training questionnaire

A-post training questionnaire was undertaken 6 to 12 weeks after the one-day standardized education program. This was essentially the same as the pre-training questionnaire with several additional questions directly related to training program (**Appendix 4**).

2.2.5 Location of one-day simple standardise education program for OC and OPMDs detection

Training was done in 5 different regions located the same as the OPMDs screening region (**Figure 2.3**). There were in Banda Aceh, West Java, Jakarta, West Kalimantan, and West Papua. The venues where the training was conducted are as follows:

1. Dental Hospital of Faculty of GDPry, University of Syiah kuala, Banda Aceh.

   Address: Teuku nyak arief, Kopelma Darussalam, Syiah Kuala, Kota Banda Aceh, Aceh 23111, Indonesia. A collaboration with Faculty of GDPry, University of Syiahwala.

2. Naripan Hotel, Bandung, West Java.

   Address: Naripan No.31-35, Braga, Sumur Bandung, Kota Bandung, Jawa Barat 40111, Indonesia. A collaboration with Indonesian Dental Association Bandung Branch and Forum Silaturahmi (Fosil), Indonesian department of Dental Health

3. Suku dinas kesehatan kota, Jakarta.

   Address: Percetakan Negara No. 82, Jakarta.

A collaboration with Indonesian Dental Association Jakarta branch

4. University of Tanjungpura (UNTAN)

   Address: Prof. Dr. H. Hadari Nawawi, Pontianak, West Kalimantan

   A collaboration with Indonesian association branch Pontianak, West Kalimantan

5. Indonesian General Local Hospital (RSUD) Sorong,

   Address: Kesehatan no. 36 Sorong, West Papua.

   A collaboration with Indonesian Dental Association branch Sorong, West Papua

2.2.6 Data analysis

Completed questionnaire data were stored in SurveyMonkey application. Data were exported to Microsoft Excel and were re-identified and coded to allow statistical analysis using Minitab Express© version 1.5.1. 2017 and R commander. The difference between means was determined using multiway analysis of variance (ANOVA) followed by Chi-Square and analysing Likert scale with logistic regression. Differences were considered significant where $P < 0.05$.

2.3 OC and OPMD detection among Indonesian; The prevalence and risk factors

This was a descriptive and qualitative cross-sectional study using questionnaire and a physical assessment. The physical assessment was referred to conventional oral examination (COE) using a step-by step guideline of British Columbia oral cancer prevention program (BCCA) in 2008 (41) and using a review of nomenclature and classification of potentially malignant disorders of the oral mucosa (259).

2.3.1 Questionnaire for respondents (patients)

A questionnaire consisting of 23 questions was developed from existing literature and modified to suit the local situation (**Appendix 5)** (1, 112, 18) .The questionnaire contained multiple choice, open and closed questions including socio-economic status, demographic information, oral health behaviour, diet, knowledge of risk factors, sun exposure, consumption of various type of cigarettes, alcohol, and the ingredient of betel quid used together with the frequency and duration of consumption. This questionnaire underwent a validation process by a panel of senior researchers based in Indonesia to critically evaluate suitability, clarity, simplicity, understanding and sequencing of the questions. Appropriate modifications to the questionnaire were made before being utilized in the main survey.

We conducted the pilot group that consist of 10 patients in Indonesia using test and re-test method modified. The questionnaire took 15 minutes to complete a questionnaire and 3-5 minutes to undertake COE or physical examination. Therefore, a total of 20 minutes for each respondent was required.

2.3.2 Conventional oral examination

Whilst the gold standard for the definitive diagnosis of OPMD is histological examination, conventional oral examination (COE) is considered to be the best approach in oral cancer screening (26). A COE consists of 8 steps examination included intraoral and extraoral visual assesment and palpation using white light (41). The steps are as follows :

4) Extraoral examination:

   Inspection of the region of head and neck for asymmetry, pigmentation, tenderness or swelling, numbness or pain. Palpation of the lymph nodes region in the submandibular, neck and supraclavicular region, paying particular attention to size, number, tenderness

and mobility. Finally, an inspection and palpation at the lips and perioral tissues for any abnormalities.

5) Intraoral examination:

   A sound and systematically inspection and palpation all oral soft tissues, especially to the high-risk sites for oral cancer development that include the lateral and ventral aspects of the tongue, floor of mouth and the soft palate complex.

6) Lesion inspection:

   Each lesion needs to be evaluated for specific characteristics paying particular attention to size, colour, texture and outline. Focusing attention to predominantly indication of white, red, red and white, ulcerated and/or indurated lesions.

7) Documentation:

   An image of any suspected lesion is strongly recommended to be obtained at the time of initial assessment and at each re-evaluation appointment and should examination form be completed (**Appendix 6**).

After the oral cancer screening procedure is completed, patient with a suspected OPMD should be asked to refrain from all suspected oral cancer risk factors and followed up for two weeks. If these lesion persistent or patient is having the oral malignant and OPMD lesion at the first appointment, then they are referred to selected referral places for further management. This occured at different location in each of the study region as follow;

- Aceh, Banda Aceh

  Patients referred to Oral Medicine Clinic of Dental Hospital, Faculty of Dentistry of University of Syiah Kuala. Banda Aceh.

- Bandung, West Java

Patients refered to Oral Medicine and Oral Surgery Department of Hasan Sadikin Hospital. Bandung.

- Pontianak, West Kalimantan

Patients referred to Dental Clinic, General Hospital (RSUD) Bethesda. Sorong, West Kalimantan

- Sorong, West Papua

Patients reffered to Government general hospital (RSUD)Sorong.

- Jakarta, Special District of Indonesia

Patients referred to Cipto Mangunkusumo Hospital (RSCM).

2.3.3 Participant recruitment

Consecutive sampling was used to recruit respondents. Respondents who attend to community services, hospital, and or selected village were given a clear explanation about the research, the time required to fill in the questionnaire and to be examined, the benefits and risks of becoming participant. The participants received a copy of plain language statement form (PLS) (**Appendix 7**). Those who freely consented to participate in this study were enrolled after signing the informed consent form (**Appendix 8**).

2.3.4 Selected location for oral cancer screening

The Indonesian Basic Health Research undertaken in both 2010 and 2013 indicated areas that have high level of tobacco use and betel quid chewing habit (260). According to that data, we focused on screening patients in five of the top 10 regions outlined (261). These are: Banda Aceh, Bandung (West Java), Jakarta (special city district of Indonesia), Pontianak (West Kalimantan), and Sorong (West Papua) **(Figure 2.3).**

Figure 2. 3 Selected location for OPMD and oral cancer screening in Indonesia. Banda Aceh, Jakarta, Bandung West Java, Pontianak West Kalimantan, Sorong West Papua (Mark with yellow pentagon)

2.3.5 Data analysis

Completed questionnaire were stored in SurveyMonkey application. Data were easily exported into Microsoft Excel. Re-identified clinical data was coded manually into Microsoft Excel to allow statistical analysis using Minitab Express© version 1.5.1. 2017 and R commander. The difference between means was determined using multiway analysis of variance (ANOVA), Chi-Square, T-test, and binary logistic regression analysis to assess association between risk factors and mucosal diseases. Differences were considered significant where $P \leq 0.05$.

2.4 Characterization of phenolics and arecoline constituent profile in betel quid from four regions of Indonesia

2.4.1 Chemicals and reagents

The reagents and solvents used for sample preparation and chemical assay were analytical reagent (AR) grades. AR grade methanol, sodium carbonate anhydrous, sodium hydroxide pellets and iron (II) sulfate heptahydrate were supplied by Chem-Supply Pty. Ltd. (Gillman SA, Australia). Hydrochloric acid (HCl) 36% grade AR, and iron (III) chloride anhydrous were purchased from Thermo Fisher Scientific (Scoresby VIC, Australia). Gallic acid, 2.0N Folin-Ciocalteu's phenol reagent (FCR), 2,4,6,-Tris(2-pyridyl)-s-triazine (TPTZ), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid 97% (Trolox) were obtained from Sigma-Aldrich (Castle Hill NSW, Australia). Acetic acid glacial was supplied by VWR International Pty. Ltd. (Tingalpa QLD, Australia). The chemicals and standard compounds applied in HPLC analysis were in HPLC grades. Acetic acid 50% solution and standard compounds [gallic acid, 5-(hydroxymethyl)furfural, protocatechuic acid, caftaric acid, p-hydroxybenzoic acid, caffeic acid, syringic acid, p-coumaric acid, trans-sinapic acid, ferulic acid, procyanidin B1, procyanidin B2, catechin, epicatechin, epicatechin gallate, quercetin, quercetin 3-*O*-galactoside, quercetin 3-*O*-glucuronide, quercetin 3-*O*-rhamnoside, kaempferol, kaempferol 3-*O*-glucoside, resveratrol, polydatin were purchased from Sigma-Aldrich (Castle Hill NSW, Australia). Acetonitrile was obtained from Merck Pty. Ltd. (Bayswater, VIC, Australia). All water used was prepared by Milli-Q Gradient water purification system (Millipore Australia Pty. Ltd., North Ryde, NSW, Australia). The alkaloids analytical standards were arecoline hydrobromide 250mg (cat #31593) and guvacine hydrochloride 250mg (cat # 6027914) from Merck Pty.Ltd. (Castle Hill, NSW, Australia). Guvacoline hydrobromide 10 mg (cat # 17190) and arecidine hydrobromide 10 mg (cat #A767000) from Shappire bioscience Pty Ltd (Roshill St, Redfern, NSW).

2.4.2 Experimental Design and sample preparations

Raw BQ components were collected from four regions of Indonesia; Banda Aceh (BA), North Sumatra (NS), West Kalimantan (WK), and West Papua (WP). The four regions were selected to represent the west, middle, and east part of Indonesia whose people perform a BQ quid chewing habit. Fresh BQs were bought from local traditional market from each region and transported to Australia. BQs were kept in the laboratory freezer (-20 ^{o}C) for 3 days to further freeze dried. A range of variation of BQ components within the selected region is highly possible, however we focused to study the most common BQ components as being AN of *Areca catechu*, Leaf/SI of *Piper betle*, Husk of *Areca catechu*, and BQ mixture. For BA, NS and WK regions, dried AN, Leaf, Husk, and BQ mix samples were analyzed, and the BQ mix contains dried AN, Leaf and lime at the ratio of 80.5:12.5:7 by weight (262). For WP region, dried AN, SI, Husk, and BQ mix samples were analyzed, and different from other regions, the BQ from WP region contains, betel inflorescence stem (SI) that commonly named as flower, instead of betel leaf (155). The outer cap colour differences of AN is a manifestation of the ripeness of AN, where the unripe AN looks green (shown by An from WP and WK regions), while the ripe AN looks yellow-orange colour (155) shown by AN from BA and NSBetel Quid samples were collected from four regions of Indonesia; Banda Aceh (BA), North Sumatra (NS), West Kalimantan (WK), and West Papua (WP). The four regions selected represented Aceh (Banda Aceh), Medan (North Sumatra), Pontianak (West Kalimantan) and Sorong (West Papua). For BA, NS and WK regions, dried areca nut, areca husk, betel leaf and mixed BQ samples were analyzed, and the mixed BQ contains dried areca nut, betel leaf and lime at the ratio of 80.5:12.5:7 by weight (262) (**Figure 1.14**). For WP region, dried areca nut, areca husk, betel inflorescence stem, and mixed BQ samples were analyzed, and different from other regions, the BQ from WP region contains, betel inflorescence stem commonly named as

flower, instead of betel leaf (155). All BQ mixture contains slaked lime, the white powder or paste that placed in the small plastic bag as can be seen in Figure 2.4. E-H.

2.4.3 Extraction of Betel quid

Extraction of dried areca hut, betel leaf, betel stem, and areca husk were performed following published method with modifications (263). Seeds and husks of areca nut were manually separated. All samples were freeze-dried for 72 hours using FD3 Freeze Drier (Dynavac Engineering, Australia), and finely grinded using electronic grinder (Multigrinder II, model EM0405, Sunbeam, Auckland, New Zealand). 5g of each sample powder was weighted into a 50mL centrifuge tube prefilled with nitrogen gas (99.99% Grade 4.0, Coregas, Yennora NSW, Australia), added with 50mL methanol as extraction solution. Extractions were carried out at 20ºC for 48hr under shaking condition at 150 rpm (Incubator shaker ZWYR-240, LABWIT scientific, Australia). After the extraction, the samples were centrifuged at 6500rpm for 10 minutes at 20ºC. The supernatants were passed to a clean tube and evaporated at 40ºC to remove the solvent entirely using Hei-VAO Value rotary evaporator (Heidolp Instruments GmbH & Co.KG, Schwabach, Germany), then redissolved in 10mL of LC grade methanol. The final extract was flushed with nitrogen gas and sealed with parafilm to avoid oxidation and storage at 4ºC in darkness until analysis. The extract stock solution was filtered using 30mm x 0.45µm nylon syringe filter (Thermo Fisher Scientific, VIC, Australia) to gain a clear solution before further analyses.

2.4.4 Determination of total phenolic content (TPC)

The sample extract total phenolic content was examined using a modified Folin-Ciocalteu reagent (FCR) based on Singleton and Rossi (1965) method (264). TPC measurement based on the spectrophotometric detection of chromogens established by the reaction between the

phenols from the functional hydroxyl groups with the phosphomolybdic/phosphotungstic acid complexes reagents. A standard calibration curve was constructed using gallic acid at concentrations from 50 mg/L to 500 mg/L, with 50 mg/L increment. The total phenolic content of sample was expressed as gallic acid equivalents (GAE) [mg GAE/g sample dry mass (DM)]. The assay was conducted by adding 100µl of diluted samples into clean tube containing 2.5mL 0.2N FCR (10-fold diluted 2.0N FCR with Milli-Q water). Add 2mL of saturated sodium carbonate solution (75g/l) after 5 minutes. The blend solutions were vortexed and allowed to react in the dark room for 1 hour. The absorbance was further quantified using Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Oy, Vantaa, Finland) at 765nm. Technical duplicates were performed for each sample replicate.

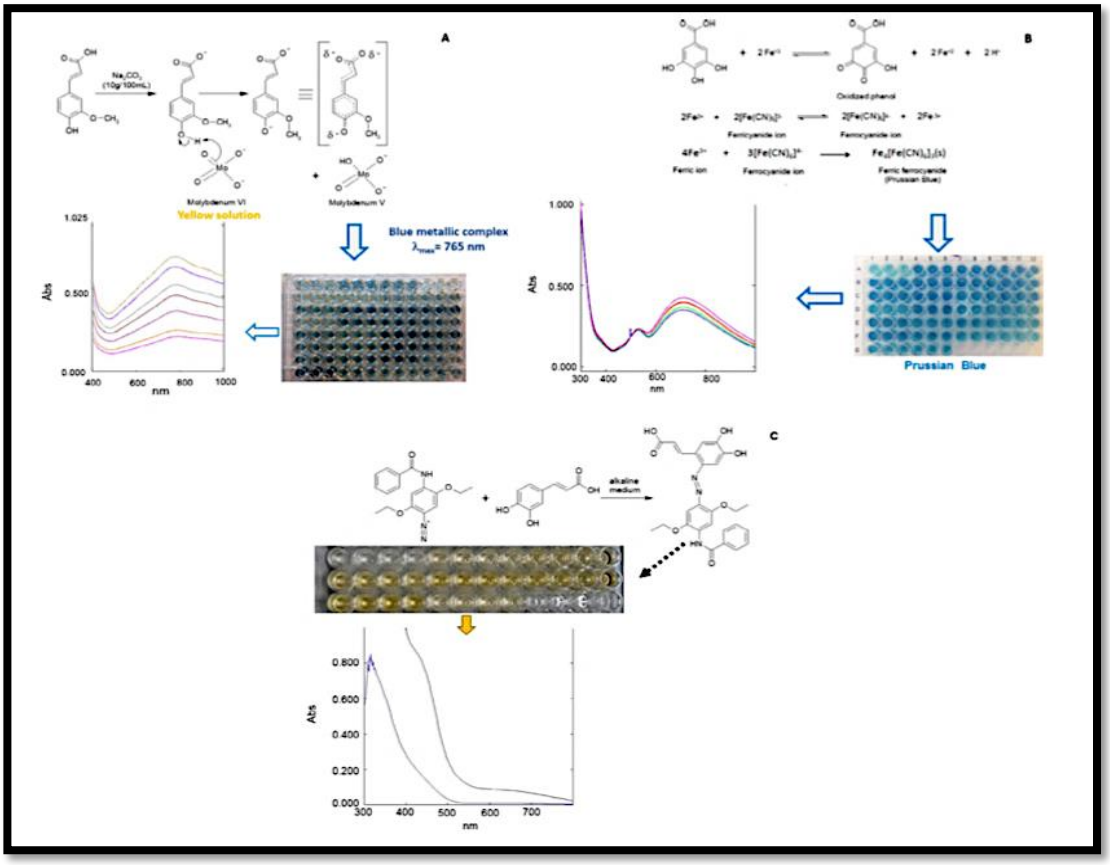


Figure 2. 4 Chemical reaction and UV/VIS spectra of total phenolic content of plant extracts at different concentrations measured by the Folin-Ciocalteu method (A), by the Prussian Blue assay (B): Modified from (265, 266), and by the Fast Blue BB hemi zinc salt assay (C): Adapted from (265, 267).

2.4.5 Determination of ferric reducing antioxidant power (FRAP)

The FRAP test of the sample extract was performed following the Benzie and Strain (1996) method (268). FRAP assay uses antioxidants iron (III) as reductants in a redox-linked colorimetric method, employing an easily reduced oxidant system presentin stoichiometric excess. Furthermore, Iron (II) sulfate heptahydrate was used as the standard composite to produce the calibration curve of Fe^{2+} standard solution with the following concentrations: 5µM to 45µM Fe^{2+} standard solution at 5 µM increment. Results were presented as the Fe^{2+} equivalents (mM Fe^{2+} eq./g sample DM). All reagents were prepared fresh on the day of examination. 5µL of diluted sample extract were added to 1.5mL centrifuge tube pre-filled with 495µL water and 1000µL FRAP reagent. The solution was mixed completely with vortex before incubation process at 37ºC for 5 min. The absorbance of the solution was quantified at 593nm using Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Oy, Vantaa, Finland). Technical duplicates were performed for each sample replicate.

2.4.6 Determination of DPPH radical scavenging capacity

The DPPH analysis was performed based on Gadow et al (1996) with modifications. The DPPH test measured the radical scavenging capacity of antioxidant by donating hydrogen to the DPPH radical. The standard compound using Trolox to construct the calibration curve. The Trolox concentration were presented as: 12.5mg/L, 25mg/L, 50mg/L, 62.5mg/L, 100mg/L, 125mg/L, and 250mg/L. The result were expressed as Trolox equivalents (mg Trolox eq,/g sample DM). 75µL of diluted sample extract was added into 1425µL pf DPPH working solution in 1.5mL centrifuge tube, vortexed and left in a darkness for 1 hour at room temperature to enable reactions. The absorbance then examined at 515nm using Multiskan™ GO Microplate Spectrophotometer (Thermo Fisher Scientific Oy, Vantaa, Finland). Technical duplicates were performed for each sample replicate.

2.4.7 Characterisation of phenolic by HPLC (High-Performance Liquid Chromatography) analysis

The HPLC phenolic characterization was performed by separation module Waters 2690 Alliance (Waters, Rydamere, NSW, Australia), paired with Waters 2998 Photodiode Array (PDA) detector following published protocols with modifications (218).The separation was executed using a Synergi Hydro-RP 80A, LC Column (250mm x 4.6 internal diameter, 4µm particle size) (Phenomenex Inc., Lane Cove Wes, NSW, Australia). The segregation of phenolics was operated at 23ºC for column temperature and acquisition range of 200-600nm with 1.25 scan/s (peak width=0.2 min) of spectral acquisition rate was placed for the photodiode array detector (269) (**Figure 2.5**)

2.4.7.1 System I (Phenolic acid)

The mobile phase involved solvent A [acetic acid in Milli-Q water (2:98, v/v)] and solvent B [0.5% (v/v)]. A gradient program was used as the following: solvent B increased from 10% to 15% in the first 10 min followed by isocratic of solvent B at 15% for 5 min. Solvent B was increased from 15% to 25% for 10 minutes, then further rose from 25% to 35% for 30 minutes. Afterwards, solvent B increased from 35% to 55% within 30 minutes, then increased 55% to 100% for 1 min and kept at 100% for 5 min. After that decrease the solvent B from 100% to 10% in 4 min. The total run time was 70 min. The flow rate of the elution was maintained constant at 1.0 mL/min. All samples were injected by volume 10µL. Synchronous detection wavelength was performed at 280nm for hydroxybenzoic acids and 320nm for hydroxycinnamic acids.

2.4.7.2 System II (Anthoxanthins and stilbenes)

System II used the similar composition of solvent A and solvent B as determined in System I (Phenolic acids), with a gradient program: solvent B rose from 10% to 24% in the initial 20 min, then increased to 30% in 20 min, and eventually improved from 30% to 55% for 30 min. Later, an increase of solvent B from 55% to 100% performed for 5 min, then kept at 100% for 5 min, finally decreased from 100% to 10% in 2 min. The total run time od elution was 82 min, and the flow rate was maintained constant at 1.0mL/min. All samples were injected by volume 10µL. Synchronous detection wavelength was performed at 280nm for flavanols, 320nm for stilbenes, and 370nm for flavonols.

2.4.7.3 System III (Alkaloids)

The isocratic mobile phase composed of 50% acetonitrile and 50% of 0.01M sodium hydrogenphosphate, adjusted at pH 7.8 by formic acid or triethylamine. The flow rate was 0.8ml/min passing through the Luna C18 column (250mm × 4.6mm internal diameter, 4µm particle size) (Phenomenex Inc., Lane Cove West, NSW, Australia). Total run time 10 minute. The column temperature was 30°C. The UV detector was set up at 216nm (219).

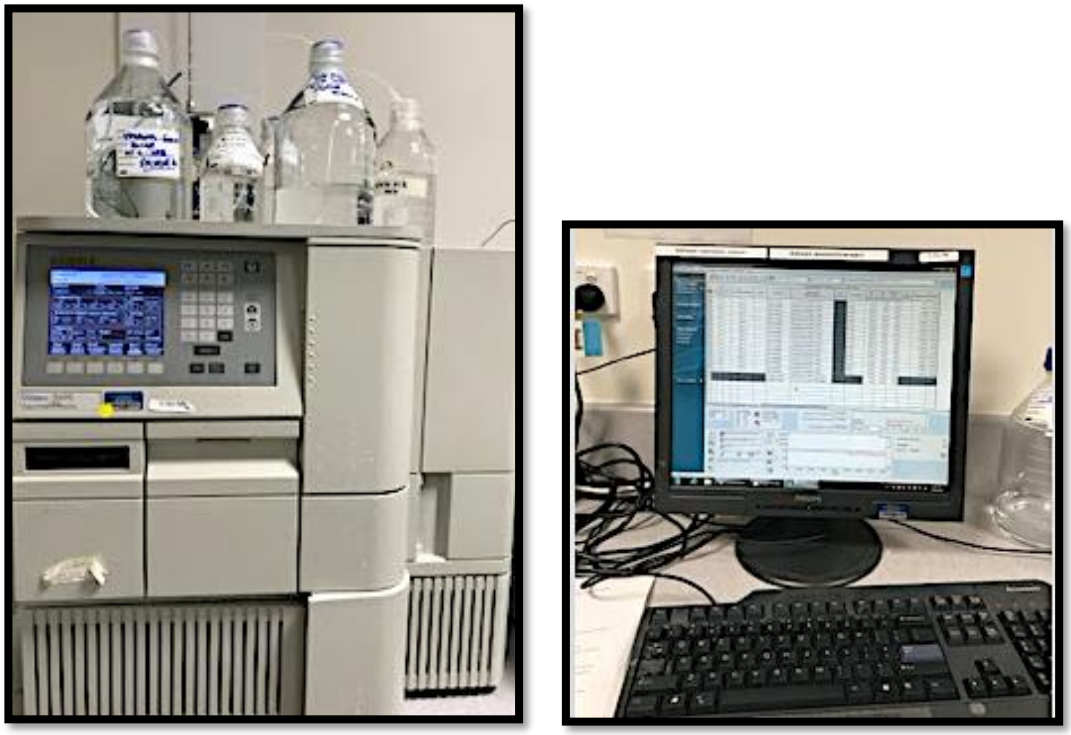


Figure 2. 5 Waters 2690 Alliance HPLC (Waters, Rydalmere, NSW, Australia). Waters 2998 Photodiode Array (PDA) detector

2.4.7.4 LC-DAD-ESIMS analysis

LC-MS analysis of polyphenols was performed with Agilent 1260 Infinity II LC system (Agilent Technology, Santa Clara, United States)**,** coupled to Agilent ESI MSD (G6125B, Agilent Technology). Data acquisition and machine control were achieved using the Openlab Chemstation software. Negative ion mass spectra of the column elute were analysed in simultaneous sim/scan mode for polyphenols based on a published method (263)**.**The scan covered the range m/z 90-600, while the target sim ions used for individual compounds are listed in **Table S1**. Nitrogen was used both as drying gas at the flow of 11.0 L/min and as nebulizing gas at pressure of 55 psi. The nebulizer temperature was set at 350 ^{o}C. The LC elusion program used was the same as the System I, with DAD synchronous detection wavelength set at 280 nm, 320 nm and 370 nm (**Figure 2.6**)

Figure 2. 6 Agilent 1260 Infinity II LC system (Agilent Technology, Santa Clara, United States)

2.4.8 Statistical analysis

Statistical analysis was conducted using MS-Excel, Minitab 17© 2016, MATLAB© by Mathwork, XLStat, and R software (v3.5.2 The R foundation). One-way ANOVA with Odd Ratio test were executed at $p < 0.05$. Group indicated with different letters showed statistically significant difference. The results were exhibited as the means $\pm$ standard deviation (SD) from four observations of individualistic replicates.

2.5 Metabolomic Analysis of BQ compounds by Gas Chromatography mass Spectrometry (GC-MS)

2.5.1 Chemicals and reagents

Arecoline hydrobromide and guvacine hydrocloryde was purchased from Sigma-Aldrich, (Castle Hill NSW, Australia), arecaidine hydrobromide and guvacoline hydrobromide were supplied by Shappire Bioscience, Pty Ltd (Redfern, NSW, Australia). Its purity and identity were checked by GC–MS and ^{1}H-NMR. Methanol (HPLC grade), chloroform, sulfuric acid and ammonia (analytical grade) were purchased from SDS (France). The hydrocarbon mixture (C7--C30, Restek, Cat no. 31614) was supplied by Teknokroma (Spain). 4-Octanol as internal standard (IS) and pyridine were purchased from Sigma–Aldrich (Castle Hill NSW, Australia)

2.5.2 Experimental design and sample preparation

The same as point 2.4.2 and 2.4.3

2.5.3 Standard solution

Alkaloids standards (arecoline hydrobromide, arecaidine hydrobromide, guvacoline hydrobromide and guvacine hydrochloride) were accurately weighted into a volumetric measuring flask of 10 mL. 40µl of 30% ammonia were added and then dissolved in 1.8 ml of each standard (with range of concentration 100mg/ml,50mg/ml, 25mg/ml, 12.5mg/ml, and 6.25 mg/ml).

2.5.4 Gas chromatography mass spectrometry analysis

GC-MS analysis was performed following published method on alkaloid (270). Briefly, an Agilent Technologies 6850 series II (GC; Agilent Technologies, Santa Clara, CA) connected to an Agilent Technologies 6973 mass selective detector (MSD) and coupled with an Agilent

PAL multipurpose autosampler was used for volatile analysis. The instruments were controlled using Agilent G1701EA MSC ChemStation software in conjunction with Agilent PAL autosampler Control software B.01.04 for ChemStation. The GC was fitted with a J&W DB-5ms column (30 m x 0.25 mm, 0.25 µm film df, with helium as carrier gas (ultrahigh purity, BOC Australia, North Ryde, NSW, Australia). The GC inlet was fitted with a borosilicate glass split inlet liner (volume 935 µl, Agilent Technology) and held at 250 ^{o}C. 1 µl of sample was injected to inlet in split mode at split ratio of 30:1, and a constant column flow rate of 1ml/min. The column temperature initated at 100 ^{o}C and increased to 180 ^{o}C at 15 ^{o}C/min, held at 180 ^{o}C for 1 min, then increased to 300 ^{o}C at 5 ^{o}C/min and held for 6 mins and post run at 100 ^{o}C for 2 mins. The MS source, quadruple and transfer line were held at 230 ^{o}C, 150 ^{o}C, and 280 ^{o}C, respectively. The MS was operated in scan/sim mode (35-350 m/z) with positive EI of 70 eV. The sim target ions for alkaloids quantification include: 53, 55, 69, 73, 82, 96, 126, 127, 140, 141, 155 m/z. The standard solution of arecoline, arecaidine, guvacoline and guvacine were analyzed using the same method as samples. All standard solutions were spiked with internal standard and analyzed as sample to generate standard curves (R^2>0.99). Alkaloids were identified by comparing the mass spectra and retention indices with the pure standard, while other metabolites in the sample were identified using NIST library in ChemStation and Webbook database and the standard solutions obtained. All compounds were quantified based on the standard curve curves using target ions. Blank and internal standard were checked regularly to check the sensitivity of GC-MSD system.

2.5.5 Statistical analysis

Statistical analysis was conducted using MS-Excel, Minitab 17© 2016, MATLAB© by Mathwork, XLStat (2019.3.2.61308) and R software (v3.5.2 The R foundation). One-way ANOVA with Odd Ratio test were executed at $p < 0.05$. Group indicated with different letters

showed statistically significant difference. The results were exhibited as the means ± standard deviation (SD) from four observations of individualistic replicates.

2.6. Cytotoxic effect of Indonesian betel quid on oral keratinocytes and fibroblast

2.6.1. Preparation of betel quid aqueous extracts

Areca nut, areca husk, betel leaf and stem were manually separated and chopped. All samples were freeze-dried for 72 hours using FD3 Freeze Drier (Dynavac Engineering, Australia), and finely grinded into powder using electronic grinder (Multigrinder II, model EM0405, Sunbeam, Auckland, New Zealand). The extraction was done following published method with some modification (255, 271). 5g of each sample powder (nut, leaf/stem, husk, BQ mixture). BQ mixture contains areca nut, leaf/stem, slaked lime with comparison 80.5:12.5:7, by weight (262). The samples were suspended with 50 mL milli-Q at 37ºC for 48hr under shaking condition at 150 rpm (Incubator shaker ZWYR-240, LABWIT scientific, Australia). After the extraction, the samples were centrifuged at 6500rpm for 10 minutes at 20ºC. The supernatants were passed to a clean tube and evaporated at 60ºC to remove the solvent entirely using Hei-VAO Value rotary evaporator (Heidolp Instruments GmbH & Co.KG, Schwabach, Germany), then redissolved in 10 mL of medium culture (Dulbecco's modified eagle's medium, From Merk, cat #D5796). The final extract was flushed with nitrogen gas and sealed with parafilm to avoid oxidation and stored at 4ºC in darkness until analysis. The extract stock solution was filtered using sintered glass vacuum and 0.22 μm Millipore nylon syringe filter (Thermo Fisher Scientific, VIC, Australia) to gain a clear solution before further analyses.

2.6.2. Primary fibroblast (MMF1)

Primary fibroblast (MMF1) cell line (**Figure 2.7**) was grown in the culture medium containing Dublecco's Modified Eagle's Medium (DMEM, From Merk, cat #D5796) with 2 mM L-glutamine, supplemented with 10% (v/v) FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5% CO2 in a humidified atmosphere.

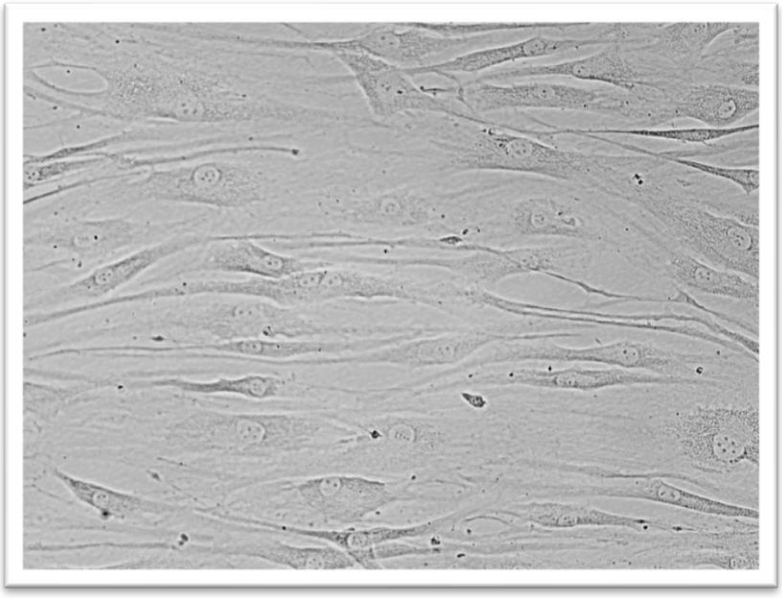


Figure 2. 7 Primary fibroblast (MMF1) after seeded for 24 hours. Picture taken with inverted microscope (Floid cell imaging station, Life Technologies)

2.6.3. Oral keratinocytes (OKF-6)

Immortalized normal human oral keratinocytes (OKF-6) cell culture (**Figure 2.8**) was used in this study. OKF-6 cells were cultured in standard cell suspending medium (CSM), containing GIBCO Keratinocytes Serum-Free Medium (GIBCO KSFM, #17005-042) supplemented with 25 µg/mL pituitary bovine extract (PBE), 0.2 ng/mL epidermal growth factor (EGF), 100 IU/ml penicillin and 100 µg/ml streptomycin, and containing 10% (v/v) new calf serum (NCS). Keratinocytes were maintained in a humidified incubator with 5% CO_2 at 37 ° C.

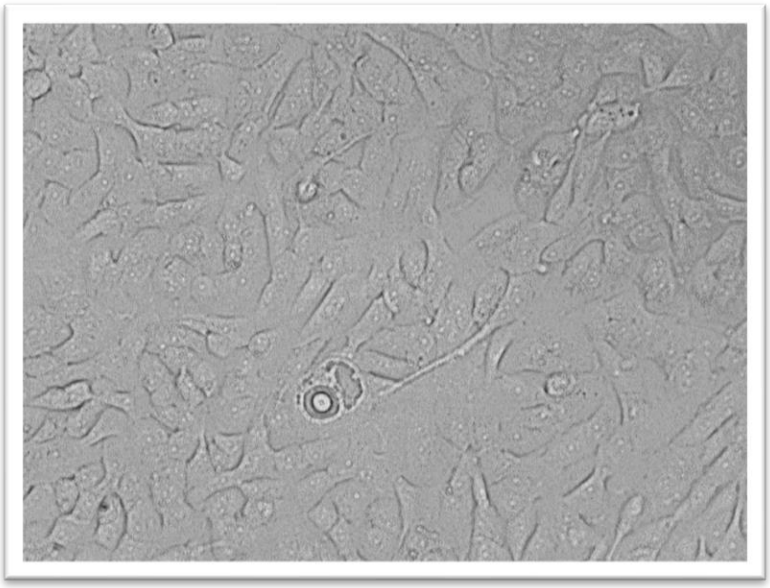


Figure 2. 8 Oral human keratinocytes (OKF-6) after seeded for 24 hours. Picture taken with inverted microscope (Floid cell imaging station, Life Technologies)

2.6.4. Preparation of betel quid solution

Sample solutions for treating cells were areca nut, leaf, husk, and BQ mixture from West Papua and Banda Aceh. Those BQs from two region were respectively selected based on HPLC results as the highest and lowest containing polyphenols and alkaloids. Samples were prepared in the cell culture media containing 1% Fetal Bovine Serum (FBS) for primary fibroblast (MMF1) and 1% containing New Calf Serum (NCS) for OKF-6. The extracts concentrations for the cytotoxicity assay were varies, for areca nut and leaf/stem were made at 100μg/ml, 250 μg/ml, and 500μg/ml, for BQ mixture was made at 1mg/ml, 22.5 mg/ml, and 45 mg/ml, for areca husk was made at 1mg/ml, 15 mg/ml and 30 mg/ml. Those concentrations were determined from an initial range previous finding study and preliminary study done by researcher.

2.6.5. Preparation of Fluorescein diacetate (FDA) solution:

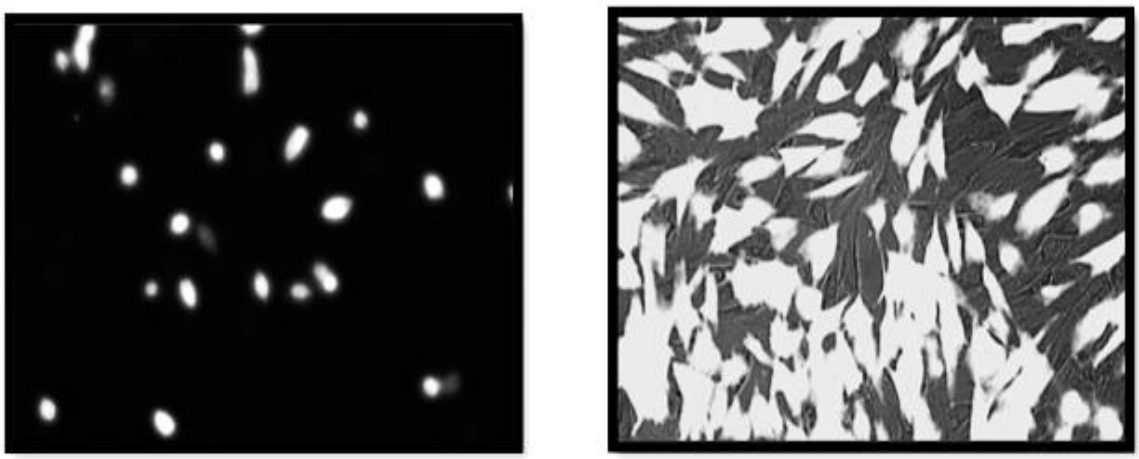
Fluorescein diacetate (FDA) stock solution was prepared by dissolving 5 mg of FDA (Sigma-Aldrich Co, F7378) in 1 ml of acetone. FDA solution was prepared by adding 1 µl FDA-stock per 1 ml fresh medium without FBS and supplements (12 µM) and vortex for 1 minute with vortex mixer (Ratek, Australia). This should be done immediately before addition to the plates to avoid precipitation (272, 273).

2.6.6. Cell cytotoxicity with FDA assay:

The assay was carried out according to the procedure reported by Gonzalez-Reyes et al with some modifications (272, 273). 96-well Costar® microplates (Sigma-Aldrich, Australia #CLS3603-48EA) were used and plated with 100 µl of $2x10^4$ cells/ml of OKF-6 or $1.35x10^4$ cells/ml of MMF1 cell suspension. Cell viability of seeded cells was at least 90% when determined with trypan blue stain. A humidified incubator (Thermo Scientific, Forma direct heat, CO_2 incubator) was used to incubate cells and this was performed at 37 °C in 5% CO2. After 24 hours of seeding, plates were removed from incubator and washed with 100 µl/well PBS and the cells treated with 100 µl/well BQ concentrations as previously prepared. Three wells/plate were used as controls. Control wells contained cells that were incubated with 100 µl culture medium containing Dublecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, supplemented with 10% (v/v) FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Blank wells were placed in empty wells (without cells), with 100 µl culture medium only. After treating cells with various BQ concentrations, the cells were incubated in a humidified at 37 °C/5% CO2 incubator and incubated for 1 day. After 1 day, control media or test compounds were removed, the cells were rinsed with 100 µl PBS and subsequently 100 µl of FDA solution prepared in serum free medium was added to each well. The plates were incubated at 37 °C/5% CO2 for 40 minutes, then FDA fluorescence was measured by

spectrometry at wavelength of 485/20 nm and 528/20 nm, respective excitation and emission, in Synergy HTX Multi-Mode Reader (Bio-Tek, USA). The FDA stained cells were viewed by an inverted microscope (Floid cell imaging station, Life Technologies) with fluorescence/green or white light as required to see whether the cells were correctly stained. The steps were repeated after treated with BQ for 2 days and 3 days of incubation. To validate the result of spectrometry readings in Synergy HTX Multi-Mode Reader, one of BQ cytotoxic concentration (IC50) was selected with its time point (1 time point over 3 days incubation). Cells were seeded in a 6-well plate in triplicates for 24-hour incubation to attach and after 24-hour, cells were treated with BQ at cytotoxic concentration (IC50) according to previous spectrometry finding. After exposing cell to BQ components at found IC50 and incubated for IC50's time point of incubation (whether the IC50 happen on day 1, day 2, or day 3 of incubation), cells were counted with automated cells counter (Bio-Rad, Australia) and stained previously with trypan blue to count viable cells. And if the results of IC50 consistently similar to spectrometry reading, then the IC50 read by spectrometry reading is correct.

The viable cells after exposed to arecoline uptake were via FDA stained and exhibited green or white light, while non-viable cells will remain dark as can be seen in **Figure 2.7**.



A B

Figure 2. 9 OKF-1 (A) and MMF-1 (B) stained by FDA assay.

The cells were exposed to 100 μg/ml arecoline after 3 days of incubation. A. Oral Keratinocytes (OKF-6), B. Oral Primary Fibroblast (MMF-1). Pictures were taken with inverted microscope with 100x magnification (Floid cell imaging station, Life Technologies)

2.6.7. Statistical analysis

Statistical analysis was conducted using MS-Excel, Minitab Express© version 1.5.1. 2017 and R commander. The significance different was tested by one-way ANOVA, with expected P-Value <0.05 and 95% CI. Tukey's HSD post hoc test was used to see significant differences between internal group. The cell cytotoxicity (IC_{50}) was measured by a 50% inhibition of cell viability.

Chapter 3: Indonesian Dentists' Knowledge in Risk Factors of Oral Cancer and in Performing Conventional Oral Examination

Abstract

The problem in managing oral cancer (OC) is not only related to the rising of its incidence. The greater challenge is that OC are not detected early enough for successful treatment. The most effective means of reducing mortality of OC by preventing late-stage OC, this could happen by increasing awareness among the population and health-care providers, specifically general dental practitioners (GDP). Therefore, our study aimed to assess GDPs' knowledge of OC risk factors and how they perform conventional oral examination (COE) as a complete examination that should be inseparable to routine dental examination.

Methods: This was a descriptive quantitative and qualitative cross-sectional study using a pre and post-training questionnaire and training intervention with both theory and practical based. The study conducted in five different provinces of Indonesia; Aceh, Banda Aceh (BA); Bandung, West Java (WJ); special district Jakarta, Jakarta (JKT); Pontianak, West Kalimantan (WK); and Sorong, West Papua (WP). The local Dental Association or Faculty of Dentistry invited the GDPs to attend a one-day standardized education program of oral malignant and OPMDs detection and 3 days hospital-based or community service-based practice.

Result: 177 GDPs were registered in the one-day standardised training and 3 days practice. The highest number of registered GDPs was from WJ region (n=63), followed by GDPs from BA (n=44), JKT (n=27), WP (N=23), and WP (20) region respectively in rank. A total of 144 (81.37%) GDPs sent back their response to the post-training questionnaire 2-6 weeks after attending the training. Female respondents predominated with 157 (88.7%)

returning the pre-training questionnaire and 126 (87.5%) returning the post-training questionnaire. Only 20 (11.3%) and 18 (12.5%) male GDPs answered the pre- and post-training questionnaire, respectively. Most of Indonesian GDPs demonstrated inadequate knowledge, awareness, and confidence in performing COE. The OC training could positively increase their knowledge and confidence of early screening.

Conclusion: GDP knowledge of OC risk factors and COE is important to be addressed, as the lack of their knowledge and awareness can impact to delaying OC early screening and diagnosis. Therefore, improving the national curriculum in Indonesia on OC is more likely the best way for GDPs to have sufficient knowledge and confidence regarding OC before graduation.

Key words: GDPs, OC risk factors, COE, GDPs knowledge, OPMD, OC

3.1 Introduction

The percentage of OC that arises from precursor lesions, namely oral potentially malignant diseases (OPMDs) varies between 16 and 62% (274). Furthermore, late stage OC is associated with significant morbidity and mortality rates. In contrast, early stage OC has a favourable prognosis, with a five-year survival rate of 66-85% of individuals without regional lymph node involvement (275). Disappointingly, almost half of the patients with OC experience diagnostic delay and over 50% present with advanced stage of disease (5). Thus, early diagnosis of OSCC is key and detection of OPMD is the single most important preventive measure to reduce the mortality for OC (259). The estimated global prevalence of potentially malignant disorders of the oral cavity is approximately 1-5% (276).

Several studies reported that developed countries have low rate of OC, while conversely developing countries show a higher rates both for females and males (277, 278). It has been shown that improved health system infrastructure has the potential to decline the worldwide OC mortality (279, 280). The increasing number of OC cases and unimproved 5-year survival rate, therefore, could be due to several factors, such as people's and practitioner's scarce awareness of OC.

General dental practitioners (GDPs) play a vital role as a frontline in detecting oral malignant and OPMDs. However, many GDPs fail to assess or diagnose OC and have poor recognition of early precursor lesions. The obstacles to undertake a routine OC examination by GDP has been recognised in some studies associated with their lack of knowledge and experiences (93, 94). In addition, inadequate GDPs' skill, knowledge, and confidence to perform oral malignant and OPMD detection may also as a result of inadequate preventive education provided to their patients (22). GDPs' knowledge or awareness in recognising early precursor lesion of OC, educating patients, their ability to perform a complete examination and appropriate referral has been studied separately or together to improve diagnosis delay and

decreased case fatality (281-284).

In the present study, we conducted a one-day standardized training intervention on oral malignant and OPMD in diverse regions of Indonesia, namely Aceh, Banda Aceh (BA); Sorong, West Papua (WP); Bandung, West Java (WJ); and Pontianak, West Kalimantan (WK); and Jakarta, Central District Jakarta (JKT). The GDP were given pre and post-training questionnaires with key points of question including their performance in OC screening examination, knowledge on recognising risk factors and early signs of OC or OPMD.

3.2 Methods

This was a descriptive quantitative and qualitative cross-sectional study using pre-training and post-training questionnaire and training intervention that included both theory and practice. Consecutive sampling was used as a technique in recruiting respondents, and involved selecting all individuals who agreed to participate, provided they meet pre-established criteria, until the desired number of subjects was recruited. Detailed methods are reported in the section 2.2.2.

3.3 Results

3.3.1 Demographics of GDPs who participated in the study.

The number of GDPs who enrolled in the one-day standardised training and filled in the pre-training questionnaire was 177 (**Table 3.1**). The highest number of registered GDPs was from WJ region (n=63), followed by BA (n=44), JKT (n=27), WP (N=23), and WP (20) (**Table 3.1**). A total of 144 (81.37%) GDPs returned responses to the post-training questionnaire 2-6 weeks after attending the training. Female respondents were predominant, although male respondents' rate percentage (90%) of returning post-training questionnaires was slightly higher than female respondents (80.25%).

Table 3. 1 The region distribution and gender of GDPs who return the pre and post training questionnaires

No	Variable		Before N	After N	Rate %
1	**Location**	BA	44	40	90.90
		WP	20	18	90
		JKT	27	20	74.07
		WK	23	11	47.83
		WJ	63	55	87.30
2	**Gender**	Female	157	126	80.25
		Male	20	18	90
	Total (N)		177	144	81.36
BA = Banda Aceh (Aceh), WP = West Papua (Sorong), JKT = Jakarta, WK = West Kalimantan (Pontianak), WJ = West Java (Bandung).					

3.3.2 GDPs' workplace

More than half (50.28%) of the GDPs who enrolled in this study worked in community service. The community service is the front-line health facility provided by the Indonesian government where most of the low and middle-income Indonesians seek for primary care as government subsidises the payment. Slightly over a quarter, 27% (N=48) of respondents work in private dental practice, 9.6% (N=17) worked in the dental teaching hospital, and the remainder worked in more than one location (**Table 3.2**).

GDPs' workplace varied considerably depending on the region examined. In the WJ region 93.85% GDPs who enrolled in this study worked in community service, whereas only 47.83% and 40% GDPs from WK and WP worked in community service, respectively. Conversely, most GDPs from JKT region (62.96%) and BA (40.91%) worked in private dental practice. (**Table 3.3**).

Table 3. 2 Distribution of GDPs' workplace

GDPs workplace	Before Training		After Training	
	N	%	N	%
Community service	89	50.28	55	38.19
Dental practice	48	27.12	24	16.67
Dental teaching hospital	17	9.60	25	17.36
More than one institution	23	12.99	40	27.78
Total (N)	177	100	144	100

Table 3. 3 Distribution of GDPs' workplace in each region studied

Workplace	WK		WP		WJ		JKT		BA		All Regions	
	N	%	N	%	N	%	N	%	N	%	N	%
Community Service	11	47.8	8	40	59	93.7	3	11.1	8	18.2	89	50.3
Dental practice	3	13	8	40	2	3.2	17	63	18	40.9	48	27.1
Dental hospital	4	17.4	3	15	0	0	6	22.2	4	9.1	17	9.6
More than 1 places	5	21.7	1	5	2	3.2	1	3.7	14	31.8	23	13
ALL	23	100	20	100	63	100	27	100	44	100	177	100

3.3.3 GDPs' awareness of OC varies in different Indonesian regions

GDPs' response towards questions related to their awareness of OC and OC training received at dental school from each region varied considerably (Table 3.4). A large proportion 92.66% (164 out of 177) of GDPs claimed to receive insufficient training on OC. One hundred percent GDPs from WP (N=20) responded to have insufficient training. Statistically significantly more, 18.18% (8 out of 44) GDPs from BA than any other region felt to have received sufficient OC training at dental school (**Table 3.4**).

An overwhelming majority, 81.36% of GDPs admitted that they failed to educate their patients on OC risk factors. 18 out of 20 (90%) GDPs from WP region confessed to neglect educating patients while 90.48% GDPs from WJ did not educate patients either.

Over the whole 5 regions only 51.41% of GDPs indicated that they did not perform a screening on high risk patients. In each region there was some variation with 75% (15/20), 61.9% (39/63), GDPs from WP and WJ reported skipping OC screening to high risk patients. Conversely, 65.91%, 52.17%, and 59.26% GDPs from BA, WK, and JKT regions, respectively, did undertake OC screening in high risk patients. As already mentioned in **Table 3.2**, more than half of GDPs work in the front-line at community service, however, the number of GDPs who undertook screening was not confidently sufficient to do OC prevention.

Similar to OC screening, the percentage of GDPs who performed complete mouth examination was 61.58% (109 out of 177). Of these, 75% (N=15 out of 20) WP GDPs admitted skipping a complete mouth examination and rather focused on dental aspects only. Interestingly, 70.45% GDPs from BA region did not commit a complete mouth examination, even though 65.91% of them did screening to high risk patients. This contradictory response makes the screening results in high risk patients questionable as they skipped the complete examination.

As many as 22.6% (N=40) of total GDPs choose not to refer to specialist when they found suspicious mucosal lesions. 13% (3 out of 23) and 30% GDPs (6 out of 20) from WK and WP, respectively, confessed not to refer the patient with mucosal lesions. Furthermore, there were also few GDPs from urbanised regions such as WJ, BA and JKT who also elected not to refer patients with oral mucosal lesions despite the presence.

Table 3. 4 GDPs' response regarding OC in selected study regions

Question	Response	WK		WP		WJ		JKT		BA		Total		P-Value
		N	%	N	%	N	%	N	%	N	%	N	%	
Training OC at Dental School	**Insufficient**	22	95.7	20	100	61	96.8	25	92.6	36	81.8	164	92.7	<0.05
	Sufficient	1	4.4	0	0	2	3.2	2	7.4	8	18.2	13	7.3	
Educated patient of OC Risk factor	**No**	14	60.9	18	*90	57	*90.5	23	85.2	32	72.7	144	81.4	<0.05
	Yes	9	39.1	2	10	6	9.52	4	14.8	12	27.3	33	18.6	
Perform Screening in high risk patients	**No**	11	47.8	15	*75	39	*61.9	11	40.7	15	34.1	91	51.41	<0.05
	Yes	12	52.2	5	25	24	38.1	16	59.3	29	65.9	86	48.7	
Perform complete examination	**No**	8	34.78	15	*75	40	63.5	15	55.6	31	70.5	109	61.7	<0.05
	Yes	15	65.2	5	25	23	36.5	12	44.4	13	29.6	68	38.4	
Refer if OPMD or OC observed?	**No**	3	13.04	6	30	7	11.1	10	37.0	14	31.8	40	22.6	<0.05
	Yes	20	86.9	14	70	56	88.8	17	62.9	30	68.2	137	77.4	
Total		23	100	20	100	63	100	27	100	44	100	177	100	

Significantly different

3.3.4 Referral patterns

Upon questioning regarding referral patterns after the recognition of a suspected oral mucosal lesions more than half of the GDPs (54.8%) indicated that they would refer to oral medicine specialists if they suspected their patients had either an OC or OPMD in their oral cavity (Table 3.5). It was disappointing to find out that 29.94% (N=54) of them do not know where best to refer. 13.56% decided to send the patient to oral surgery and 1.13% to oral pathologist. As high as 75% (16 out of 20) of GDPs from WP responded that they do not know where to refer. 59.26% (16 out of 27) GDPs from JKT also responded they were confused as to whom they have to send the patients for further treatment. Similarly, although not as high, 25% of GDPs from WJ and 26.1% from WP did not know where to refer a patient with OC or OPMD. The response from GDPs in BA was exceptional, as all enrolled GDPs seemed to know where to refer the patients, and most of them (90.91%) will send patients suspected with OC or OPMD to an oral medicine specialist.

Table 3. 5 Referral patterns

Referral patterns	WK		WP		WJ		JKT		BA		All Regions		P-Val
	N	%	N	%	N	%	N	%	N	%	N	%	
I don't know	6	26.1	15	75	16	25.4	16	59.3	0	0	53	29.9	<0.05
Oral medicine	15	65.2	1	5	32	50.8	9	33.3	40	90.9	97	54.8	
Oral surgery	2	8.7	4	20	14	22.2	1	3.7	3	6.8	24	13.6	
Oral pathologist	0	0	0	0	1	1.6	1	3.7	0	0	2	1.1	
Oncologist	0	0	0	0	0	0	0	0	1	2.3	1	0.6	
All	23	100	20	100	63	100	27	100	44	100	177	100	

Significantly different

3.3.5 OC risk factors before and after OC training

The number of GDPs who had sufficient knowledge of risk factors increased after OC training (88.6% vs 66.7%). The OC training increased the GDPs' knowledge of OC risk factors by 21.9%, None of the GDPs answered "do not know" after training, however, 10.4% (15 out of 177) GDPs still showed insufficient answers distributed in all regions.

3.3.6 The comparison OPMD types listed by GDPs before and after OC training

GDPs with sufficient knowledge on OPMD types increased after OC training. Specifically, only 21.5% GDPs (38 out of 144) had sufficient knowledge before training, however, this percentage increased to 80.56% after training (51.06% increase). None of the GDPs responded with blank or I don't know answer after training, however, 19.4% (28 out of 144) GDPs still showed insufficient answers and these were evenly distributed in all regions (**Table 3.7**).

Table 3. 6 GDPs' knowledge of OC major risk factors

OC risk factors	Group	WK		WP		WJ		JKT		BA		All Regions		P-Value
		N	%	N	%	N	%	N	%	N	%	N	%	
Before Training														
IDK	Insufficient group	0	0	3	15	7	11.1	4	14.8	0	0	14	7.9	<0.05
1 RF		1	4.4	0	0	12	19.1	5	18.5	2	4.6	20	11.3	
2 RF		6	26.1	1	5	10	15.9	8	29.6	0	0	25	14.1	
3 RF	Sufficient group	12	52.2	14	7	26	41.3	3	11.1	2	4.6	*57	32.2	
>3 RF		4	17.4	2	10	8	12.7	7	25.9	40	90.9	*61	34.5	
ALL		23	100	20	100	63	100	27	100	44	100	177	100	
After Training														
IDK	Insufficient group	0	0	0	0	0	0	0	0	0	0	0	0	<0.05
1 RF		0	0	1	5.7	4	7.3	1	5	1	2.5	7	4.9	
2 RF		0	0	1	5.6	5	9.1	1	5	3	7.5	8	5.6	
3RF	Sufficient group	0	9.1	5	27.8	9	16.4	4	20	3	7.5	*22	15.3	
>3RF		10	90.9	11	61.1	37	67.3	14	70	35	87.5	*107	73.3	
ALL		10	100	18	100	55	100	20	100	40	100	144	100	

IDK= I don't know; RF = Major Risk Factors (Smoking, Alcohol drinking, Betel quid chewing);1RF = answer 1 of major RF, 2RF= answer 2 major RF, 3RF= answer 3 major RF; >3RF= answer more 3 RF. B = Before training response, A= After training response.

There were 2 groups; group insufficient (who answered = I don't know, 1 and 2 risk factors) and group sufficient (who answer 3 and >3 risk factors). Those sufficient and insufficient knowledge group GDPs were tested using a chi-square test with P Value <0.05 and the results were significantly different between two groups either before or after OC training.

Significantly different

Table 3. 7 The comparison of GDPs' knowledge on recognizing OPMD type before and after OC training conducted

OC risk factors	Group	WK		WP		WJ		JKT		BA		All Regions		P-Value
		N	%	N	%	N	%	N	%	N	%	N	%	
Before Training														
IDK	Insufficient group	9	39.1	10	50	22	34.9	10	37	0	0	51	28.8	<0.05
1 OPMD		5	21.7	9	45	30	47.6	16	59.3	11	25	71	40.1	
2 OPMDs		8	34.8	1	5	8	12.7	0	0	0	0	17	9.6	
3 OPMDs	Sufficient group	0	0	0	0	0	0	0	0	2	4.6	2	1.1	
>3 OPMDs		1	4.4	0	0	3	4.8	1	3.7	31	70.5	36	20.3	
ALL		23	100	20	100	63	100	27	100	44	100	177	100	
After Training														
IDK	Insufficient group	0	0	0	0	0	0	0	0	0	0	0	0	<0.05
1 OPMD		0	0	1	5.7	0	6	3	15	1	2.5	11	7.6	
2 OPMDs		0	0	2	11.1	10	18.2	3	15	2	5	17	11.8	
3 OPMDs	Sufficient group	3	27.3	7.5	4	10	18.2	4	20	3	7.5	24	16.7	
>3 OPMDs		8	72.7	11	61.1	29	52.7	10	50	34	85	92	63.9	
ALL	**Total**	11	100	18	100	55	100	20	100	40	100	144	100	

IDK= I don't know; 1OPMD = answer 1 OPMD; 2 OPMDs= answer 2 OPMDs; 3 OPMD= answer 3 OPMDs; >3 OPMDs= answer more than 3 OPMDs type.

GDPs who answered I don't know, 1 or 2 OPMDs were considered to have insufficient knowledge, while GDPs who listed 3 or more OPMDs were considered to have sufficient knowledge. Those sufficient and insufficient knowledge group GDPs were tested using a chi-square test (with P-value<0.05) separately before and after training.

Significantly different

3.3.7 COE steps performed by GDPs in 5 different regions before OC training conducted

There are 8 steps of a COE to done by a GDPs for a complete examination. The number of GDPs who claimed to perform COE steps with the majority with GDPs performing a gingival examination (90.98%), followed by a buccal (82,49%), labial (76.27%) and lips (76.27%) examination. Very few GDPs performed bimanual and floor of the mouth examination (22.03%) and tongue examination (54.24%) (**Table 3.8**).

Table 3. 8 COE steps done by GDPs before OC training

COE Steps	No N (%)	Yes N (%)
1. Perform extra-oral examination	38	139
	21.47	78.53
2. Perform lips examination	42	135
	23.73	76.27
3. Perform labial examination	51	126
	23.73	76.27
4. Perform buccal examination	31	146
	17.51	82.49
5. Perform gingival examination	16	161
	9.04	90.96
6. Perform palatal examination	43	134
	24.29	75.71
7. Perform tongue examination	81	96
	45.76	54.24
8. Perform bimanual and FOM examination	138	39
	77.97	22.03

3.3.8 GDPs' perceptive in performing COE and identifying pathologic changes in oral mucosa

The degree of confidence in performing a COE was reported by all enrolled GDPs. The response used a Likert's scale divided into 4 categories as follows: very confident (VC), confident (C), not confident (NC), and really not confident (RNC). The COE steps were tested using Chi-square with P-value <0.05, separately done before and after training to further compared.

GDPs confidence in conducting COE steps were different and significantly improved after OC training. Before training was given, as high as 84% GDPs felt they were not confident both in performing bimanual and FOM examination and identifying pathologic change in FOM. This number drastically changed after giving OC training with GDPs' responses to both questions was 90% feeling confident and 6% very confident. A drastic change pre- and post-training was also seen in their perceived ability to perform a tongue examination (59% vs 80%) (**Table 3.10**).

Only 6% GDPs felt really not confident in undertaking both extra-oral examination and identifying pathologic changes in extra oral and lips examination respectively. This condition shifted drastically into none of them (0%) felt really not confident after given OC training (**Table 3.9**)

Table 3. 9 GDPs' perceptive in performing COE and identifying pathologic changes in oral mucosa (Part-1)

COE Steps	Training	VC		C		NC		RNC		P-Value
		N	(%)	N	(%)	N	(%)	N	(%)	
1. Performing extra-oral examination	Before	5	3%	95	54%	66	37%	11	6%	NS
	After	9	6%	133	92%	2	1%	0	0%	<0.05
2. Identifying extra oral pathologic change on extra-oral	Before	1	1%	97	55%	68	38%	11	6%	NS
	After	8	6%	132	92%	4	3%	0	0%	<0.05
3. Perform lip examination	Before	2	1%	108	61%	55	31%	12	7%	NS
	After	11	8%	131	91%	2	1%	0	0%	<0.05
4. Identifying pathologic change on the lips	Before	2	1%	91	51%	83	47%	1	1%	<0.05
	After	9	6%	130	90%	5	3%	0	0%	<0.05
5. Perform labial examination	Before	2	1%	91	51%	84	47%	0	0%	NS
	After	9	6%	131	91%	4	3%	0	0%	<0.05
6. Identifying pathologic change on labial mucosa	Before	3	2%	82	46%	91	51%	1	1%	NS
	After	7	5%	132	92%	5	3%	0	0%	<0.05
7. Perform buccal mucosa examination	Before	2	1%	100	56%	74	42%	1	1%	<0.05
	After	10	7%	131	91%	3	2%	0	0%	<0.05

VC = very confident, C = confident, NC = not confident, RNC = really not confident

Significantly different

Table 3. 10 GDPs' perceptive in performing COE and identifying pathologic changes in oral mucosa (Part-2)

8. Identifying pathologic change on buccal mucosa	Before	2	1%	84	47%	89	50%	2	1%	<0.05
	After	8	6%	128	89%	8	6%	0	0%	<0.05
9. Perform gingival examination	Before	3	2%	115	65%	59	33%	0	0%	<0.05
	After	13	9%	127	88%	4	3%	0	0%	<0.05
10. Identifying pathologic change in gingiva mucosa	Before	3	2%	99	56%	75	42%	0	0%	<0.05
	After	10	7%	126	88%	8	6%	0	0%	<0.05
11. Perform palatal examination	Before	4	2%	93	53%	80	45%	0	0%	NS
	After	6	4%	136	94%	2	1%	0	0%	<0.05
12. Identifying pathologic change in palatal	Before	4	2%	86	49%	86	49%	1	1%	NS
	After	4	3%	132	92%	8	6%	0	0%	<0.05
13. Perform tongue examination	Before	2	1%	70	40%	105	59%	0	0%	<0.05
	After	12	8%	127	88%	5	3%	0	0%	<0.05
14. Identifying pathologic change in tongue	Before	2	1%	60	34%	115	65%	0	0%	<0.05
	After	9	6%	127	88%	8	6%	0	0%	<0.05
15. Perform ventral a bimanual palpation and FOM examination	Before	0	0%	28	16%	149	84%	0	0%	<0.05
	After	8	6%	129	90%	7	5%	0	0%	<0.05
16. Identifying pathologic change in FOM	Before	0	0%	28	16%	149	84%	0	0%	<0.05
	After	8	6%	129	90%	7	5%	0	0%	<0.05

VC = very confident, C = confident, NC = not confident, RNC = really not confident.

3.3.9 OC training performance and GDPs' perceived awareness of OC after training

Training performance and GDPs awareness of OC after training was assessed via a Likert's scale. Respectively, 52% (75 out of 144) and 48% (69 out of 144) GDPs strongly agreed or agreed that OC training provided adequate education for OC (Table 3.11) Furthermore, 65% and 35% of GDPs strongly agreed or agreed, respectively, that OC training is important for GDPs (Table 3.10). Further, GDPs responded with strongly agree (46%) and agree (53%) that OC training should be conducted regularly (Table 3.11).

The GDPs strongly agreed (43%) or agreed (57%) that their awareness has increased after receiving OC training (3.12). The GDPs' confidence in ability to detect OC was increased by OC training (24% strongly agree and 74% agree). The GDPs responded to always perform a complete examination (18% strongly agree and 77% agree). 65% and 32% of the GDPs responded agree and strongly agree respectively to always educate patient of OC risk factors, however, 3% (N=4) of them choose neither (Table 3.12).

Table 3. 11 Training performance after training conducted

Training performance	Strongly Agree N (%)	Agree N (%)	Neither N (%)	Disagree N (%)	Strongly Disagree N (%)	Total N (%)	P-Value
Training has provided adequate knowledge of OC	75	69	0	0	0	144	<0.05
	52	48	0	0	0	100	
I believe this training is important for GDPs	94	50	0	0	0	144	<0.05
	65	35	0	0	0	100	
This training should be conducted regularly	66	77	1	0	0	144	<0.05
	46	53	1	0	0	100	

Significantly different

Table 3. 12 GDPs' awareness of OC after training conducted

GDPs' awareness	Strongly Agree N (%)	Agree N (%)	Neither N (%)	Disagree N (%)	Strongly Disagree N (%)	Total N (%)	P-Value
My awareness of OC increased	62	82	0	0	0	144	<0.05
	43	57	0	0	0	100	
My confidence in my ability to detect OC has increased	35	107	2	0	0	144	<0.05
	24	74	1	0	0	100	
I will always perform a complete oral mucosal examination	26	111	6	1	0	144	<0.05
	18	77	4	1	0	100	
I Will always educate my patients of OC risk factors	46	93	4	1	0	144	<0.05
	32	65	3	1	0	100	

Significantly different

3.3.10 Additional information

Only 2.84% (n=5) GDPs from 5 selected provinces reported the ability to perform oral mucosal biopsy, 1.69% (n=3) claimed to have ability in applying toluidine blue staining procedure, and 2.26% (n=4) the use of an adjunct clinical tool in OC early detection (**Table 3.13**).

Most (64.97%) GDPs who enrolled in this study choose to refer without giving any medication if they suspected patients with OC or OPMD (**Table 3.14**). The other respondents prescribed gargle (21.47%), antibiotic (3.39%), and analgesic (7.91%). Finally, 2.26% GDPs had no knowledge about what to prescribe to patients with suspected OC or OPMD in oral cavity.

Table 3. 13 GDPs' ability to perform biopsy and adjunct tool

NO	**Additional Information**	**Yes (%)**	**No (%)**
1	Have ability to perform oral mucosal biopsy	5 2.84	171 97.16
2	Have ability to perform toluidine Blue staining	3 1.69	174 98.31
3	Have ability to use adjunct tool Velscope®	4 2.26	173 97.74

Table 3. 14 What will be prescribed by GDPs in initial meeting for patient with suspected OC or OPMD

GDPs location	I don't know N (%)	Analgesic N (%)	Antibiotic N (%)	Gargle N (%)	Only refer N (%)	All N (%)	P-Val (Simple regression)
WK	0	7	0	5	11	23	<0.05
	0	30.4	0	21.7	47.8	100	
WP	0	2	2	4	12	20	<0.05
	0	10	10	20	60	100	
WJ	3	4	3	17	36	63	<0.05
	4.8	6.4	48	27	57.1	100	
JKT	0	0	1	5	21	27	<0.05
	0	0	3.7	18.5	77.8	100	
BA	1	1	0	7	35	44	<0.05
	2.3	2.3	0	15.9	79.6	100	
All	4	14	6	38	115	177	
	2.3	7.9	3.4	21.8	65	100	

3.4 Discussion

In the study we have clearly demonstrated that increased education is critical for Indonesian GDPs that appeared to have inadequate knowledge, awareness, and confidence in performing COE as part of a complete examination. This study was able to point which COE steps the GDPs tended to avoid, and the possible barriers for GDPs.

Initial approached to the Faculty of Dentistry and Dental Association in 5 selected Indonesian regions to conduct a hands-on training of OC was very appreciated as they were aware that most of the GDPs have inadequate OC knowledge and skill. It is also shown with the number of GDPs who consent to participate in this study was considerably high.

More than half (50.28%) of the GDPs who enrolled in this study worked in community service or primary health care. The community service is the front-line health facility that provided by Indonesian government, where most of the middle-down economic people primary seek for their health as government subsidise the payment. Indonesian community services often lack good infrastructure, financial and human resources (285, 286). The training has achieved the target to encourage COE for OC screening in primary care setting (1, 23, 287). The dental practice and hospital are the second choice for people as they are much more expensive rather than community service. The number of patient who come to community service could reach 50-100 or more people in a day (286, 288). In the WJ region, the training collaborated with the Dental Association branch of Bandung which is affiliated with a group of GDPs who work in community service. This condition is exceptional to WJ region as not every community service has this particular affiliation. For more remote areas, such as WP and WK, community service also has a vital role in oral health. Most of the GDPs from JKT region (62.96%) and BA (40.91%) work in dental practice. Only few numbers of GDPs work in dental hospital or general hospital and work more than one institution. Dental partitioners should remain aware of early sign and symptoms of cancer (49, 94).

In relation to OC curriculum training that was offered at university during their dental studies, the majority GDPs respondents in the present study felt to have received insufficient training on OC. This should become a 'strong alarm' that needs to be taken into consideration. The Indonesian Faculty of Dentistry needs to improve OC curriculum to allow GDPs to have adequate knowledge, skill, attitude, and confidence in a routine OC examination. This curriculum improvement on OC should be nation-wide learning. Dental practitioners should remain aware of early sign and symptoms of cancer (49, 94) as visual and tactile examination or conventional oral examination could detect oral carcinoma early enough (289, 290, 94). Dental school should give adequate OC knowledge and skill before the GDPs independently work on their own.

As part of OC prevention, educating patients regarding OC risk factors particularly the major risk factors are absolutely important. Patients need to get adequate information about OC risk factors especially from dental practitioners (49, 97, 94). However, our study surprisingly revealed high rates of GDPs admitted to skip educating patient of OC risk factors. GDPs neglecting to explain OC risk factors to patients in all selected region, could have a big impact on OC in Indonesia. People will be unaware of the cause of OC and keep living in the carcinogenic environment, creating many further cancer cases in the future.

More than half of total GDPs from 5 studied regions confessed to not perform screening in high risk patients. Many GDPs work in the front-line community service, however, although GDPs undertook screening, they reported not sufficiently confident to undertake OC prevention. Furthermore, the number of GDPs population ratio in Indonesia was 3.4 per 100,000 people(286, 291, 288). This limited numbers of GDPs national wide in Indonesia is already a problem in oral health system, as well as limited GDPs who work in the primary care(285, 288). This limited number of human resources might be the main reason why GDPs skip to perform a routine complete examination as they have to serve an abundance of patients.

Indonesian government needs to change the public oral health system to increase oral health index both dental and oral diseases.

Similar to performing OC screening in high risk patient, the percentage of GDPs who inadequately performed complete mouth examination was high. This is alarming, as in a concurrent study 43.17% of Indonesian with OPMD live in WP region, strongly associated with BQ chewing habit. Nonetheless, the GDPs skipped to perform complete examination. This contradictory response result in bias and invalid screening results in high risk patients as they skip to do a complete mouth examination and left the patient improperly screened.

Almost a quarter of GDPs choose not to refer to specialist when they observed OPMD or OC, potentially due to confusion as to where they have to refer. Remote regions in Indonesia have highly limited of cancer health facilities and cancer related clinicians. This situation could be understood, nevertheless, this issue should emerge into surface and get Indonesian government attention to resolve. There were a small number of GDPs from WJ, BA and JKT who also decided not to refer patients with OPMD or OC. This is very difficult to accept as those regions are not considered remote and the provided health facilities could be considered better rather than WK and WP regions. The reason could be related with lack of OC knowledge where best to refer for further treatment that could result in diagnoses delayed. Patients very common to come at late stage of OC due to their lack of knowledge but also another barrier for early detection comes from GDPs who do not know how to manage suspected patient with OC and OPMD, one of the management is how to do referral (15, 258, 95, 94).

Oral medicine specialist was the number one GDPs' choice to refer their patient suspected with OC or OPMD. However, the number of oral medicine specialist in Indonesia (OMS) is highly limited, only less than 80 OMS who should serve approximately 260 million Indonesians (292). This limited number of OMS due to lack of OMS educational institution, that only 3 institution across Indonesia hold OMS program such as University of Indonesia in

JKT, University Padjadjaran in WJ, and University of Airlangga in Surabaya. All of the institutions are concentrated in Java island (292).

No OMS work in WP, either do other specialist such as oral surgery, and oral pathologist (292). OC related health facilities such as pathology clinic are extremely limited, all biopsy specimen need to be sent to the other islands such as Sulawesi and Java. This is very devastating both for patient and clinicians as it is time consuming and expensive.

GDPs from JKT also reported their confusion to whom they have to refer their patients for further treatment. This is very surprising as the number of OMS working in JKT is more than 10 distributed in dental teaching hospital, general hospital, and private practice. Nonetheless, the GDPs still are confused where to refer. GDPs from BA is exceptional, all enrolled GDPs seems to know where to refer the patients, with most sending patients suspected to have OC or OPMD to OMS, with only 2 OMS working in BA (292). OMS need undertake more effort promoting their role in the early detection of OC and OPMD.

In general at national level, the ratio of Indonesian dental specialist was only 0.3 (286, 291), hence the government need to improve dental health system in particular to the number of human resources.

More than a quarter of GDPs in the present study reported insufficient knowledge about the most important aspect in preventing OC. These GDPs work at community service as front-line in OC screening program, yet they could not educate the public on OC properly.

Some regions, such as BA demonstrated sufficient knowledge of risk factors. However, we need to look further into their working place background as these were at dental teaching hospital staff and academic staff.

OC training increased GDPs' knowledge of OC risk factors. Oral diseases still considered unnecessary by GDPs, particularly OC knowledge (293), this undeniable situation needs to be changed together with government by creating national OC awareness.

Training with regard to types of OPMD's clearly increased knowledge in the present study. GDPs should always remain alert of early sign and symptom of OC by properly recognising and distinguishing OPMD that appear in the oral cavity(294, 91). It will be difficult for GDPs to decide whether to refer or just neglect if the GDPs have inadequate OPMD knowledge.

There 8 steps of COE by visual and tactile examination is important to undertake a complete examination and should be part of a routine examination in GDPs' daily practice. The present study shows that gingival examination undertaken, however, bimanual and floor of the mouth examination were the least. Therefore, most GDPs skipped bimanual and floor of the mouth examination and about half of them neglecting tongue examination. This is a worrisome finding, as those two areas are high risk area where OPMD could potentially develop into carcinoma(102, 295, 101).

GDPs' confidence in performing COE likely directly related they undertake incomplete examinations. The drastic increase number of GDPs who confidence in performing COE steps after training conducted is reassuring, as they will be able to engage in prevention.

Furthermore, the GDPs' subjective perception about their awareness, confidence to detect OC, to perform complete examination, and to educate patients increased after receiving OC training.

3.5 Conclusion

GDPs have an important role in OC screening, however, most of the GDPs in Indonesia appear not to have adequate knowledge of OC risk factors as well as inadequate knowledge, awareness, and confidence in performing COE as a complete examination that should be inseparable from their routine oral examination. The present study of local Indonesian GDPs from 5 different regions clearly indicates Indonesian GDPs knowledge, awareness, and confidence in OC screening and how OC training positively increases their knowledge and confidence of early screening. This study also identified the most likely obstacles related to why GDPs tend to perform incomplete COE, and what specific step the GDPs tended to avoid. Furthermore, it is important to approach Indonesian government to improve national curriculum on OC learning so that GDPs could have sufficient knowledge and confidence of OC before their graduation. The strategy to prevent OC in Indonesia should also be established by creating a national OC program, and the current OC hands-on training could possibly be used as a training model to improve Indonesian GDPs' awareness of OC.

Chapter 4: Oral Premalignant Disorders Detection Among Indonesians; The Prevalence and Risk Factors

Abstract

Assessment of oral potentially malignant disorders (OPMDs) is a key aid for early detection of oral cancer (OC) and the consequent reduction of cancer-related morbidity and mortality. Despite the high number of Indonesians who are exposed to well-known risk factors such as smoking and betel nut chewing, the prevalence of OPMD in the population is still unknown. Our aim was to investigate the prevalence of OPMD and associated risk factors in Indonesia. Apart from that, we also aimed to investigate several OC cofounding factors such as OC risk knowledge, oral health (OH) behaviour, ethnicities and socioeconomic status (SES).

Methods: This cross-sectional study was undertaken in multiple geographical areas within five Indonesian provinces, namely West Java (WJ), Jakarta (JKT), West Papua (WP), West Kalimantan (WK) and Banda Aceh (BA) regions. Individuals attending community health services, dental hospitals and selected villages were recruited. Volunteer respondents answered a previously validated extensive questionnaire that included socio-demographic factors, oral hygiene practices, diet, adverse oral habits and frequency of dental visits. An oral examination was undertaken using standardized WHO methodology. Data were analysed using ANOVA, Chi-Square, and logistic regression to assess association between risk factors and mucosal disease.

Results: A total of 973 respondents were enrolled (WJ 35.5%; JKT 13.3%, WP 18.3%, WK 9%; BA 23.9%). Smoking (14.8%), BQ chewing (12.6%) and alcohol drinking (4%) varied geographically. An OPMD was detected in 14.3% respondents and 0.2% had history of OC. Leukoplakia was the most common OPMD (8.6%), while the prevalence of erythroplakia/erythroleukoplakia was 0.6%, and oral submucous fibrosis (OSMF) 1.9%. Overall, there was a strong correlation between the prevalence of OPMD and risk factors for

OC. In particular, high use of BQ chewing in WK and WP, related to high prevalence of OPMD. The knowledge of OC risk factors, OH behaviour, ethnicities and low-income SES group were significantly associated with OPMD.

Conclusion: A previously underestimated high prevalence of OPMD in Indonesia strongly correlates with OC risk behaviour. There is an urgent need for primary prevention of OC programs in Indonesia.

Key words: OPMD, oral cancer, prevalence early detection, risk factors.

4.1. Introduction

OC is an increasingly global problem, being the 6th most common cancer worldwide and accounting for 3% of all cancers. In some developing countries such as India, Pakistan, Sri Lanka, and Taiwan , OC is the most common type of malignancy (296, 22). Whilst incidence numbers reported by WHO are the best available, these figures are estimates that should be used with caution. Data collection in some countries is problematic: for example, epidemiological studies on OC with sufficient number of respondents and geographically diverse areas have not been undertaken in Indonesia. This does not allow to draw an accurate picture of the prevalence or incidence rate of OC in Indonesia. Additionally, only few South East Asia (SEA) countries including Singapore, Thailand, Philippines, Vietnam and Malaysia have population-based cancer registries, while Indonesia has not developed this public health information system yet (7).

Several risk factors of OC have been recognized such as tobacco, alcohol, and betel quid (BQ) use. In Western countries, the major risk factors of OC are cigarette smoking and alcohol drinking, whereas in Asian countries, the major risk factors are smoking and betel quid (BQ) chewing (296, 72). Similar risk factors have also been recognised for OPMD. In Indonesia, the use of smokeless tobacco and betel nut in various forms is very popular, which could play a role in the development of OPMD. In particular, betel quid chewing is known to induce OSMF, which is associated with an increased risk of OC development (103).

According to data from *Badan Pusat Statistik Indonesia* (Indonesian Department of Statistic Centre), the number of Indonesian population in 2017 was about 261.890.900, and estimated to be 289.732.100 in 2027 .It has been indicated in Indonesian basic health research undertaken both in 2010 and 2013 that one-third of Indonesian population are smokers (about 87.3 million people). This makes Indonesia the country with the biggest number of smokers in the world (288). Betel nut chewing habit is known as “Menginang” in bahasa Indonesia, which

comes from the Javanese words *tempat kinang* (meaning betel nut's storage). Betel nut chewing habit has been a heritage culture passed down through many generations (7, 160). These lifestyles potentially bring Indonesian at high risk of developing malignancy and could have a remarkable impact on prevalence and incidence rates of OPMD and OC. Unfortunately, no accurate data exist on the prevalence of OPMD in Indonesia.

Early transformation of oral mucosal to malignancy is almost always thought to be precede by a clinically visible as OPMD lesion, which allows clinician to screen and treat effectively at early intraepithelial stages of oral carcinogenesis (23, 297). Nevertheless, most OC are diagnosed at a late stage which leads to a complex treatments, costly and poor prognosis (23). The early detection of cancer is of critical importance because survival rates markedly improve when the oral lesion is identified at an early stage. Therefore, detection of OPMD is key, and this can be effectively achieved by population screening (298). According to Lingen MW et al (2008): "Screening, in medicine, is a strategy used in population to detect a disease in individuals without any signs or symptoms of that disease or examination of a group of asymptomatic individuals to detect those with a high probability of having or developing a given disease. The purpose of this is to identify disease in a community early, thus enabling earlier intervention and management in the hope to reduce the mortality and suffering from the disease. Given the accessibility of the oral cavity, conventional oral examination (COE) is the most common method used for OC screening" (29). It is critically important for Indonesian people to have oral screening not only to enable early management but also to detect the prevalence rate and the factual risk factors for OPMDs and OC.

This present study focuses on oral malignant and OPMD detection. It was conducted in selected diverse areas in Indonesia utilizing visual and physical clinical oral examination namely (COE). The results of this study will provide valuable information on the number of oral malignant and OPMD cases, risk factors, and awareness in Indonesia. This research has

the potential to result in a wide range of benefit such as; providing accurate and valid data regarding OPMD among Indonesian, thus encouraging government to create national programs for oral malignant awareness; to increase awareness of Indonesian people by early detection of oral malignancy and OPMD, and thus decrease both morbidity and mortality of OC cases; to provide information for public, policy regarding oral malignant, OPMD, and risk factors.

Thus, this research has the potential to make enormous impact within Indonesia. This project is long overdue and with support, it can profoundly change the health and live of many Indonesians. Moreover, this study is likely to result in Indonesia leading significant change in health policy and outcomes both in the local and geographic region as well as globally.

4.2 Material and methods

This was a descriptive and qualitative cross-sectional study using questionnaire and a physical assessment. The physical assessment of COE used a step-by step guideline from the British Columbia OC prevention program (BCCA) in 2008 (41) and using a review of nomenclature and classification of potentially malignant disorders of the oral mucosa (259). The detail methods for this study are prescribed in section 2.3. While not falling in any OPMD category, non-healing oral ulcerations were also recorded.

4.3 Results

4.3.1 General assessment of OPMD or OC among Indonesians

Respondents enrolled to this study were 973 with a mean age of respondents was 42 (17 – 82). Female was predominant (76.9%) compared to male (23.1 %) respondents and 49% of respondents were un-employed (**Table 4.1**). 30% of respondents had some knowledge of OC; of these, 17.8 % learned about OC from a dentist and 12.2% from the Government (**Table 4.2)**. 14.5% of respondents were smokers, 12.6% were betel nut chewers and 3.9% were alcohol drinkers (**Table 4.3**). 139 (14.3 %) of the 973 had OPMD, and 2 respondents (1.6%) presented with chronic ulceration diagnosed after biopsy as OC (**Table 4.4**).

Table 4. 1 Respondents' data, examination places and selected region for COE

n=973 (100%)	Variable	Frequency	Percentage
Gender	Female	748	76.9
	Male	225	23.1
Occupation	Government Employee	192	19.7
	Non-Government Employee	302	31.0
	Un-Employed	479	49.2
Examination Places	Community Service (rural)	291	29.9
	Community service (urban)	259	26.6
	Villages (rural)	129	13.3
	Village (urban)	294	30.2
Region	Aceh, BA	251	25.8
	Bandung, WJ	337	34.6
	Special district of Jakarta, JKT	126	12.9
	Pontianak, WK	86	8.8
	Sorong, WP	173	17.8

Table 4. 2 Respondents' reason for meeting dentist and oral cancer information

n=973 (100%)	Variable	Frequency	Percentage
Reason for seeking dentist	Consultation	450	46.3
	Dental pain	343	35.3
	Missing teeth	45	4.6
	Periodontal disease	75	7.7
	Other	60	6.2
Healthcare professional sought when having oral problem	Dentist	624	64.1
	Medical doctor	34	3.5
	Nurse	26	2.7
	Treat themselves	164	16.9
	Other	125	12.9
Diagnosed OC previously	No	966	99.3
	Yes	7	0.7
Diagnosed OC in extended family	No	960	98.7
	Yes	13	1.3
Diagnosed OC in Friends	No	959	98.6
	Yes	14	1.4
Diagnosed OC in someone else known from TV, news, internet etc	No	847	87.1
	Yes	126	13.3

Table 4. 3 Respondents' risk factors of oral cancer and oral hygiene

n=973 (100%)	Variable	Frequency	Percentage
Smoking	No	832	85.5
	Yes	141	14.5
BQ chewing	No	850	87.4
	Yes	123	12.6
Alcohol drinking	No	935	96.1
	Yes	38	3.9
Eat vegetables	No	370	37.8
	Yes	603	62.2
Eat Fruits	No	584	60.0
	Yes	389	40.0
Brush teeth daily	No	62	6.4
	Yes	911	93.6
Brush teeth information	Dentist	364	37.4
	Family	78	8.0
	Teacher at school	79	8.1
	TV	57	5.9
	Other	395	40.6

Table 4. 4 Presence of OPMD, other lesion, distance travelled and insurance of respondents

n=973 (100%)	Variable	Frequency	Percentage
OPMD or OC	No	834	85.7
	Yes	139	14.3
Another Lesion*	Recurrent aphthous stomatitis	20	
	Frictional keratosis	28	
	Ulcer traumatic	5	
	Benign growth[#]	5	
	OPMD or OC	199	
	Normal	719	
Easy to travel to dental Facilities	No	208	48.2
	Yes	765	78.6
Health Insurance	Government Insurance	403	41.4
	Non-Government Insurance	27	2.8
	Self-Payment	292	30
	Other types	251	25.8

*Three patients had another lesion found besides OPMD, therefore, they have more than one lesion. [#]The five patients with a benign growth had an exophytic mass covered by normal mucosa that have been present for a considerable time. These lesions were subsequently biopsied.

4.3.2 Common type of OPMD

Leukoplakia was the most common type of OPMD and accounted for 67.6% of all OPMD (94 out of 139). The second common type was OSMF (15.8%), followed by leukoplakia combined OSMF (7.9%). The least common type was erythroplakia, it was found only in one person (0.7%), while erythro-leukoplakia had a higher prevalence (4.3%). Two persons (1.4%) had a large ulcer on their tongue which have been diagnosed with OC previously (Table 4.5).

Table 4. 5 Type of OPMD or OC

OPMD/OC	N	(%)	F	M	Age (SD)
Erythroplakia	1	0.1	1	0	26.8 ± 0.0
Erythro-leukoplakia	6	0.6	5	1	49.8 ± 9.1
*Leukoplakia	94	9.7	46	48	44.9 ± 15.3
Leukoplakia + OSMF	11	1.1	11	0	49.0 ± 13.0
OSMF	22	2.3	2	1	47.2 ± 14.0
Lichen Planus	3	0.3	20	2	46.8 ± 9.2
Ulcer (OSCC)	2	0.2	2	0	45.7 ± 14.6
Total OPML	139	14.3	87	52	44.3 ± 12.5
Normal	834	85.7	661	173	42.5 ± 15.5

4.3.3 Common site of OPMD

The observed OPMD mostly appeared on single site of oral mucosal regardless of the occurrence on left or right side. The most common affected site was buccal mucosa (71.2%), 6.7 % were found on lateral tongue and 4.3% on gingiva. We found 2 (1.6%) respondents with OPMD on all of their oral mucosal surfaces. Only 20 (14.4%) out of 139 OPMD occurred on multiple sites of oral mucosa.

Table 4. 6 Site of OPMD or OC

Site of affected oral mucosal	Site	Type	Frequency (n)	Percentage (%)
Single site	Gingiva	L	6	4.3
	Hard Palate	L	1	0.7
	Buccal	E	3	2.2
		L	72	51.8
		E-L	1	0.7
		L-OSMF	4	2.9
		LP	3	2.2
		OSMF	16	11.5
	Buccal		***99***	***71.2***
	Labial	L	2	1.4
	Lateral tongue	L	6	4.3
		U	2	1.4
		OSMF	1	0.7
	Lateral tongue		***9***	***6.7***
	Full Mouth	OSMF	2	1.4
Multiple sites	Buccal + FOM	E	3	2.2
	Buccal + Dorsal tongue	L	1	0.7
	Buccal + Gingiva	L	2	1.4
	Buccal + Hard Palate	L	1	0.7
	Buccal + Lateral Tongue	L	5	3.6
	Gingiva + FOM	L	1	0.7
	Gingiva + Lateral Tongue	L	1	0.7
	Labial + Buccal + Ventral	L	1	0.7
	Lateral Tongue + FOM	L	1	0.7
	Buccal + Lateral Tongue + Ventral	OSMF	1	0.7
	Buccal + Soft Palate	OSMF	1	0.7
	Labial + Buccal	OSMF	1	0.7
	Labial + Buccal + FOM	OSMF	1	0.7
	Multiple sites		***20***	***14.4***
			139	**100**
L (Leukoplakia), E (Erythroplakia), E-L (Erythro-leukoplakia), OSMF (OSMF), U (Ulcer), LP (Lichen planus), L-OSMF (Leukoplakia and OSMF)				

4.3.4 The major risk factors and OPMD or OC

Table 4.7 presents the association between the actual risk factors and OPMD or OC cases among Indonesian shown that BQ chewing (n=59) was the most frequent risk factor compared to smoking tobacco and alcohol drinking, however, alcohol drinking was the most causative risk factor, bearing the highest Odds ratio (OR=12.2).

Table 4. 7 The actual risk factors and OPMD or OC

Risk factors	**OPMD**		**OR (95%CI)**	**P-Value**
	Yes	**No**		
BQ (Yes)	59	64	8.9	<0.05
BQ (No)	80	770	(5.8, 13.5)	
Smoking (Yes)	53	88	5.2	<0.05
Smoking (No)	86	746	(3.5, 7.9)	
Alcohol Drinking (Yes)	24	14	12.2	<0.05
Alcohol Drinking (No)	115	820	(6.2, 24.3)	

Significantly different

4.3.5 Geographical region and OPMD or OC

There was 60 (6.17%) of 973 respondents who had OPMD lived in WP and 79 of 973 (8.12%) of OPMD spread diversely amongst the other 4 regions (**Table 4.8**).

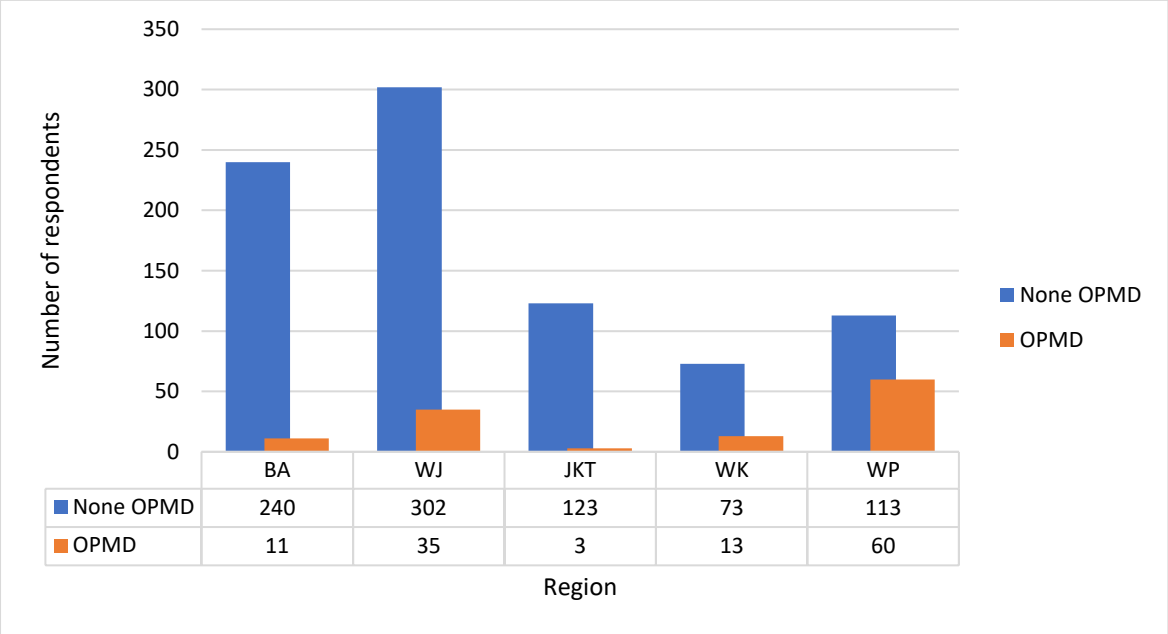


Figure 4. 1 Regional variation in number of oral mucosal disease (OPMD or OC)

Table 4. 8 Region and OPMD or OC

Level A	Level B	Odds Ratio	95% CI	P-Value
WP	BA	19.8	(9.5, 41.2)	<0.05
WP	WJ	5.4	(3.3, 8.7)	<0.05
WP	JKT	22.3	(7.7, 64.6)	<0.05
WP	WK	1.7	(0.9, 3)	<0.05
WP	ALL REGIONS	4.9	(3.3,7.2)	<0.05
Odds ratio for level A relative to level B				

Significantly different

A comparison of the presence of OPMD's across the five regions showed that people living in WP had 22.3, 17.8, and 5.4-fold higher risk of having OPMD compared to people living in JKT, BA, and WJ respectively, but only 1.7 times higher risk of OPMD compared to people living in WK. This analysis of respondents showed that the highest OPMD rate occurred in people from WP and the least number of OPMD occurred in people from JKT (Table 4.8 and Figure 1).

4.3.6 BQ Chewing and OPMD or OC

The duration and frequency of BQ consumption in those with OPMD cases showed that respondents who had consumed BQ for more than 15 years were more likely (2.5-fold) to develop OPMD compared to those who were BQ consumers for less than 15 years (Table 4.9). Respondent who chewed BQ regularly had 2.5-fold increased risk of OPMD occurrence compared to occasional users (**Table 4.10**).

The various ingredients of BQ consumed in different region of Indonesia significantly differed and respondents who consumed BQ containing betel stem inflorescence (BQ-SI) had a risk of OPMD almost 4 times higher (OR=4.2) compared to respondents who consumed BQ containing betel leaf (BQ-L). Interestingly, BQ-SI was only consumed in WP, while BQ-L was consumed in BA, WK and WJ. The addition of tobacco to BQ-L chewing increased the risk (OR=10.2) compared to consuming BQ-L without tobacco. It is also shown that chewing betel leaf alone did not cause OPMD in the oral cavity (**Table 4.11**).

BQ in fabricated package was associated with an increased risk of OPMD (3.6-fold) compared to BQ non-package that was prepared ad hoc from fresh areca nut, leaf/stem inflorescence and other spices. (**Table 4.12**)

Table 4. 9 The association of duration of BQ consumption with OPMD or OC

Duration of Consumption	OR (95%CI)	P Value	Frequency (%)	Mean Age (SD)
>15 years	2.5 (1.2, 5.2)	<0.05	67 (54.5)	48.2 (15)
<15 years	0.4 (0.2, 0.8)	<0.05	56 (45.5)	48.7 (16)
Total			***123 (100)***	

Significantly different

Table 4. 10 The association of frequency of BQ consumption with OPMD or OC

Frequency of Consumption	OR (95%CI)	P-Value	Frequency (%)	Mean Age (SD)
Regular Use	2.5 (1.4, 4.5)	<0.05	62 (50.4)	47.3 (18)
Occasional Use	0.2 (0.06, 0.4)	<0.05	44 (35.8)	50.1 (15)
Total			***123 (100)***	

Significantly different

Table 4. 11 The association of various BQ ingredients with OPMD or OC

Level A	Level B	Odds Ratio	95% CI	P-Value
BQ + Tobacco	BQ-L	*10.2	(1.7, 62.3)	<0.05
BQ-SI	BQ-L	*4.2	(1.5, 11.6)	<0.05
BQ-SI +To	BQ-L	1.3	(0.1, 10.2)	NS
Betel Leaf	BQ-L	0.2	(0.02, 2.4)	NS
Other	BQ-L	0.2	(0.019, 1.9)	NS
Not Chewing BQ	BQ-L	0.2	(0.09, 0.6)	<0.05
BQ-SI	BQ-L + Tobacco	0.4	(0.06, 2.8)	NS
BQ-SI	BQ-L + Tobacco	0.1	(0.01, 1.5	NS
Betel Leaf	BQ-L + Tobacco	0.0	(0.002, 0.3)	<0.05
Other	BQ-L + Tobacco	0.0	(0.001, 0.3)	<0.05
Not Chewing BQ	BQ-L + Tobacco	0.0	(0.004, 0.1)	<0.05
BQ-SI + Tobacco	BQ-SI	0.3	(0.04, 2.1)	NS
Betel Leaf	BQ-SI	0.1	(0.006, 0.5)	<0.05
Other	BQ-SI	0.0	(0.005, 0.4)	<0.05
Not Chewing BQ	BQ-SI	0.1	(0.03, 0.1)	<0.05
Betel Leaf	BQ-SI + Tobacco	0.2	(0.01, 3.1)	NS
Other	BQ-SI + Tobacco	0.1	(0.009, 2.5)	NS
Not Chewing BQ	BQ-SI + Tobacco	0.2	(0.03, 1.1)	NS
Other	Betel Leaf	0.9	(0.04, 17.5)	NS
Not Chewing BQ	Betel Leaf	1.0	(0.1, 8.9)	NS
Not Chewing BQ	Other	1.2	(0.1, 9.7)	NS

Odds ratio for level A relative to level B, NS = Not significant

BQ-L = areca nut, slaked lime, and Betel Leaf.

BQ-SI = areca nut, slaked lime, and Betel stem inflorescence (only consumed in WP).

Others = other ingredients apart from areca nut, slaked lime, betel leaf, betel stem inflorescence and tobacco.

Significantly different

Not significantly different

Table 4. 12 BQ package and OPMD or OC

BQ Package	OR	P-Value	Frequency (%)	Mean of Age (SD)
Yes	3.6 (1.5, 8.5)	<0.05	89 (72.4)	50.5 (15)
No			34 (27.6)	42.8 (15)
Total			***123 (100)***	

Significantly different

4.3.7 Smoking and OPMD or OC

The duration of smoking habit (less than 5 years, 5-15 years and more than 15 years) was not significantly related with OPMD (**Table 4.13**). However, in respondents who consumed 10 cigarettes a day the risk of having OPMD was doubled (OR=2.7) compared to those who consumed less than 10 cigarettes or more a day (**Table 4.14**).

The occurrence of OPMD was not associated with the various types of cigarettes consumed overall. The prevalence of OPMD between non-smokers and the various types of cigarette smoked showed non-smokers respondents had 0.3 and 0.2-times lower risk of having OPMD compared to kretek (original Indonesian cigarette) and filtered cigarette smokers, respectively (**Table 4.15**).

Table 4. 13 Duration of smoking and OPMD or OC

Level A	Level B	Odds Ratio	95% CI	P-Values
5-15 years	<5 years	1.1	(0.41, 3.02)	NS
>15 years	<5 years	1.1	(0.42, 2.66)	NS
None smokers	<5 years	0.2	(0.1, 0.47)	<0.05
>15 years	5-15 years	1	(0.42, 2.15)	NS
Non-smokers	5-15 years	0.2	(0.09, 0.38)	<0.05
Non-smokers	> 15 years	0.2	(0.12, 0.34)	<0.05
Odds ratio for level A relative to level B				

Table 4. 14 The number of cigarettes consumed daily and OPMD or OC

Level A	**Level B**	**Odds Ratio**	**95% CI**	**P-Value**
>10	<10	2.7	(1.33, 5.49)	< 0.05
None smokers	<10	0.3	(0.18, 0.52)	<0.05
None Smokers	>10	0.1	(0.06, 0.20)	<0.05
Odds ratio for level A relative to level B				

Table 4. 15 The type of cigarette and OPMD or OC

Level A	**Level B**	**Odds Ratio**	**95% CI**	**P-Value**
Filter	Kretek	1.2	(0.5, 2.9)	NS
*Non-smokers	Kretek	*0.3	(0.1, 0.6)	<0.05
*Non-smokers	Filter	*0.2	(0.1, 0.3)	<0.05
Odds ratio for level A relative to level B				

Significantly different

Not significantly different

4.3.8 Alcohol drinking and OPMD or OC

Alcohol drinkers had 12.2-fold increased risk of having OPMD compared to the non-drinkers (**Table 4.7**). In relation to duration of drinking alcohol beverages, we found no significant correlation between duration of consuming alcohol with the occurrence of OPMD in oral mucosa. Furthermore, the amount of alcohol beverage consumed in a day was not significantly correlated to OPMD either (Table 4.16). In addition of various type of alcohol drinking, the different types of brands of alcoholic beverages did not correlate with risk of oral mucosal diseases (Table 4.18). However, in comparison to the non-drinker versus various brand type of alcohol drinkers, respondents who drank arak putih, vodka, mixed brands, and beer, compared to non-drinker respondents had 25.2, 21.9, 15.5, and 7.6-times higher risk of having OPMD, respectively (Table 4.18). All types of alcohol drinking could cause OPMD to oral mucosa (**Table 4.18**)

Table 4. 16 Duration of drinking alcohol and. OPMD or OC

Level A	Level B	Odds Ratio	95% CI	P-Value
5-15 years	<5 years	1.2	(0.09, 15.84)	NS
>15 years	<5 years	0.5	(0.12, 2.38)	NS
Not drink	<5 years	0.1	(0.02, 0.21)	<0.05
>15 years	5-15 years	0.4	(0.04, 5.03)	NS
Not drink	5-15 years	0.1	(0.005, 0.49)	<0.05
Not drink	>15 years	0.1	(0.05, 0.28)	<0.05
Odds ratio for level A relative to level B				

Table 4. 17 The amount of alcohols consumed per day and OPMD or OC

Level A	Level B	Odds Ratio	95% CI	P-Value
2-5 bottles	1 bottle	2.2	(0.38, 12.71)	NS
Not Drink	1 bottle	0.09	(0.04, 0.21)	<0.05
Not Drink	2-5 bottles	0.04	(0.009, 0.21)	<0.05
Odds ratio for level A relative to level B				

Significantly different

Not significantly different

Table 4. 18 The different brand of alcohol beverages and OPMD or OC

Level A	Level B	Odds Ratio	95% CI	P-Value
Cap tikus	Arak Putih	4.1	(0.17, 98.77)	NS
Vodka	Arak Putih	1.2	(0.07, 20.52)	NS
Beer	Arak Putih	3.3	(0.55, 19.82)	NS
Mix	Arak Putih	1.6	(0.09, 28.69)	NS
Vodka	Cap tikus	0.3	(0.007, 11.32)	NS
Beer	Cap tikus	0.8	(0.04, 15.14)	NS
Mix	Cap tikus	0.4	(0.0099, 15.89)	NS
Not drink	Cap tikus	6.2	(0.38, 100.46)	NS
Beer	Vodka	2.9	(0.22, 37.99)	NS
Mix	Vodka	1.4	(0.046, 42.75)	NS
Mix	Beer	0.5	(0.04, 6.52)	NS
Vodka	*Not drink	*21.6	(1.93, 247.09)	<0.05
Arak Putih	*Not drink	*25.2	(5.33, 119.18)	<0.05
Beer	*Not drink	*7.6	(2.999, 19.36)	<0.05
Mix	*Not drink	*15.5	(1.37, 176.24)	<0.05
Odds ratio for level A relative to level B, NS = Not significant				

Significantly different
Not significantly different

4.3.9 Knowledge of risk factors and OPMD or OC

Respondents' responded to question regarding their knowledge of OC risk factors before OC screening. It appears that people who have knowledge of risk factors were more likely not to have OPMD (OR=0.18) rather than those who do not have knowledge (Table 4.19). Only 17.78% were educated by dentist, and as high as 82.22% admitted that dentists did not give any information about OC (Table 4.19). However, respondents that had been educated by dentists had a significantly reduced risk (OR=0.5, 95%CI 0.27, 0.85) of OPMD whereas those who learned about OC from other sources did not. (**Table 4.19**).

Table 4. 19 Knowledge of risk factors and OPMD or OC

Knowledge of OC	**OPMD**		**OR (95%CI)**	**P-Value**
	Yes	**No**		
Have Knowledge (Yes)	7	191	0.2 (0.08, 0.39)	<0.05
Have Knowledge (No)	132	643		
Knowledge from Dentist (Yes)	14	159	0.5 (0.27, 0.85)	<0.05
Knowledge from Dentist No)	125	675		
Knowledge from Government (Yes)	15	105	0.8 (0.47, 1.49)	0.37
Knowledge from Government (No)	124	729		

Significantly different
Not significantly different

4.3.10 Oral health behaviour and OPMD or OC

Respondents who brush their teeth properly (OR=0.6) had a reduced risk of OPMD compared to those who improperly brush their teeth (Table 4.20). Properly brushing teeth was described as practising at least twice a day (in the morning and before going to bed), the teeth were brushed for 2, and all of teeth surfaces are cleansed thoroughly. In addition, respondents who brush their teeth twice a day could lower the risk of OPMD compared to those brushing their teeth once a day (**Table 4.21**)

Table 4. 20 Oral Health Behaviour and OPMD or OC

Oral health behavior		**OPMD or OC**		**OR (95%CI)**	**P-Value**
		Yes	**No**		
Brush teeth	Yes	132	779	1.3 (0.6, 3.5)	0.2
	No	7	55		
Proper brush teeth	Yes	71	524	0.6 (0.43, 0.89)	<0.05
	No	68	310		

Table 4. 21 Frequency of tooth brushing per day and OPMD or OC

Level A	**Level B**	**Odds Ratio**	**95% CI**	**P-Value**
Twice	Once	*0.6	(0.33, 0.98)	<0.05
Thrice or more	Once	0.7	(0.39, 1.38)	NS
Thrice or more	Twice	1.3	(0.84, 2.05)	NS
Odds ratio for level A relative to level B, NS = Not significant				

Significantly different
Not significantly different

4.3.11 Ethnicity and OPMD or OC

The risk of development of OPMD in various ethnicities in the survey. It shows that Papua (WP region) and Dayak ethnic (WK region) have 23.6 and 21.3-fold higher risk rather than Aceh ethnic (BA region). Javanese, Balinese and East Nusa Tenggaranese (ENT) have 1.1 times more likely to have risk than Aceh ethnic (**Table 4.22**). Whereas Sulawesi and Maluku ethnic showed 7 times higher risk than Aceh ethnic. Some ethnicities were detected in current survey such as Sulawesinese, Malukunese, North Sumatra ethnic, West Sumatra ethnic, Balinese, and ENT were not the indigenous to the 5 selected OC screening regions. However, they were immigrant and have been living in those 5 selected regions.

Table 4. 22 Ethnicity and OPMD or OC

Level A	Level B	Odds Ratio	95% CI	P-Value
Dayak	Aceh	*21.3	(8.81, 51.64)	<0.05
Papua	Aceh	*23.6	(11.11, 50.2)	<0.05
North and West Sumatra	Aceh	1.5	(0.51, 4.61)	NS
Java, Bali, ENT	Aceh	*2.6	(1.32, 5.29)	<0.05
Sulawesi and Maluku	Aceh	*7.0	(2.76, 17.83)	<0.05
Papua	Dayak	1.1	(0.51, 2.41)	NS
North and West Sumatra	Dayak	*0.1	(0.024, 0.22)	<0.05
Java, Bali, ENT	Dayak	*0.1	(0.06, 0.25)	<0.05
Sulawesi and Maluku	Dayak	*0.3	(0.13, 0.85)	<0.05
North and West Sumatra	Papua	*0.1	(0.024, 0.18)	<0.05
Java, Bali, ENT	Papua	*0.1	(0.07, 0.19)	<0.05
Sulawesi and Maluku	Papua	*0.3	(0.14, 0.64)	<0.05
Java, Bali, ENT	North and West Sumatra	1.7	(0.66, 4.5)	<0.05
Sulawesi and Maluku	North and West Sumatra	*4.6	(1.47, 14.16)	<0.05
Sulawesi and Maluku	Java, Bali, ENT	*2.7	(1.26, 5.58)	<0.05

Odds ratio for level A relative to level B, NS = Not significant

Significantly different
Not significantly different

4.3.12 Socioeconomic status (SES) and OPMD or OC

SES was assessed in relation to location of COE examination, occupation, health insurance, and ease of travel to dental health facilities.

The location of COE examination was either in the community service (CS) or selected villages (VI), both CS and VI located either at rural or urban area. Respondents who were screened at urban-CS places had a lowered risk (OR=0.43) of OPMD or OC compared to those screened at rural-CS (Table 4.23). Whereas, respondents screened at urban-VI also had less risk of the case compared to those screened at rural-VI (**Table 4.23**). Respondents who were screened at the rural-CS had a 2.6 times risk of OPMD compared to urban-VI. Thus, respondents who were examined at the rural area had higher risk of OPMD or OC rather than the respondents screened at urban area, whether it was screened in CS or VI (**Table 4.23**).

The occupation of respondents was grouped into government employee, non-government employee and un-employed. The total number of un-employed respondents were 479 (Female = 422, Male = 57). The non-government employee respondents had a 3.3-fold higher risk of OPMD, or OC compared to government employees. Low income association with the occurrence of OPMD or OC was clearly shown (**Table 4.23**).

Health insurance was grouped into government insurance, non-government insurance and self-funded. The Indonesian government insurance is strictly given to those who are in need with many terms and conditions. The respondents who had non-government insurance could be considered economically capable as they could purchase the insurance. Respondents who had non-government insurance had less risk (OR=0.3) of having OPMD or OC compared to those who had government insurance.

Traveling to the dental health facilities was measured from subjective respondents' response whether they felt that this travel was easy or not. Interestingly there was no

significant difference to the response questioning if it was easy or not to reach dental facilities with the occurrence of the OPMD or OC in oral cavity (**Table 4.23**)

Table 4. 23 SES and OPMD or OC

SES Variables	Level A	Level B	Odds Ratio	95% CI	P-Value
Location of COE	Rural-VI	Rural-CS	0.9	(0.53, 1.5)	NS
	Urban-VI	Rural-CS	0.2	(0.1, 0.33)	<0.05
	Urban-CS	Rural-CS	0.4	(0.27, 0.68)	<0.05
	Urban-VI	Rural-VI	0.2	(0.1, 0.41)	<0.05
	Urban-CS	Rural-VI	0.5	(0.27, 0.85)	<0.05
	Urban-CS	Urban-VI	2.3	(1.19, 4.38)	<0.05
Occupation	Non-government employee	Government employee	3.3	(1.8, 5.9)	<0.05
	Un-employed	Government employee	1.5	(0.81, 2.69)	NS
	Un-employed	Non-government employee	0.5	(0.3, 0.67)	<0.05
Health Insurance	Non-Government	Government	0.3	(0.2, 0.55)	<0.05
	Yourself	Government	0.8	(0.5, 1.164)	NS
	Yourself	Non-Government	2.3	(1.30, 4.09)	<0.05
Travel to dental health facilities	Easy	Not easy	1.6	(0.99, 2.63)	NS

Significantly different
Not significantly different

4.3.13 The relation between all risk factors with OPMD or OC

The multiple regression was carried out to analyse the relation between all risk factors with OPMD or OC. The analysis shows that smoking, betel quid chewing, and ethnicity are the most significant risk factors association with OPMD, or OC followed by type of health insurance, type of alcohol, location of examination and occupation respectively, as shown in the Table 4.24. Whilst, gender, alcohol drinking, and brushing teeth daily were found to be not significant (**Table 4.24**).

Table 4. 24 All risk factors and OPMD or OC

Risk factors	Coefficient	SE Coefficient	95% CI	P-Value
Constant	0.629	0.191	(0.25, 1.006)	3.3
Gender	-0.02	0.0273	(-0.07, 0.04)	0.5
Age	0.001	0.000	(-0.00003, 0.003)	0.06
Occupation	-0.029	0.015	(-0.06, -0.0007)	0.05
Health insurance	-0.030	0.008	(-0.05, -0.01)	0.0005
Smoking	0.184	0.034	(0.12, 0.3)	<0.0001*
Betel quid chewing	0.304	0.0338	(0.2, 0.4)	<0.0001*
Alcohol drinking	-0.078	0.121	(-0.3, 0.2)	0.5
Knowledge of oral cancer	-0.068	0.028	(-0.1, -0.01)	0.02
Proper brushing teeth	-0.004	0.042	(-0.09, 0.08)	0.9
Type of alcohol	-0.098	0.035	(-0.2, -0.03)	0.006
Ethnicity	0.029	0.007	(0.02, 0.04)	<0.0001*
Location Of examination	-0.025	0.009	(-0.04, -0.006)	0.01
*The most significant risk factors associated with OPMD and OC development compared to all significant risk factors for OPMD or OC that were found in this current study.				

Significantly different
Not significantly different

4.4 Discussion

The present study has shown for the first time that the prevalence of OPMD in Indonesia was surprisingly high (14.3%). Risk factors, knowledge of risk factors, OH behaviour, ethnicity and SES were significantly associated with incidence of OPMD. The association between those variables will be further discussed in detail.

4.4.1 Prevalence and risk factors

In the present study, 139 (14.3%) of the 973 respondents included in this opportunistic and targeted screening were considered clinically to have OPMD lesions. This OPMD prevalence found was the highest compared to other similar oral screening performed in Guam, Spain (299) and in Japan (300). It has previously been studied that the prevalence of lesions, as well as the observed prevalence of smoking and drinking, known risk factors for oral cancer, observed in the community dental health services probably closely represents the prevalence of the diseases in the general population (11). This opportunistic screening in a general dental practice setting may be a realistic alternative to population screening (301).

In the present study, the most common affected site of OPMD was buccal mucosa (71.2%). Previous studies showed that approximately 70% of leukoplakias are found on buccal mucosa, lip vermilion, and gingiva, and yet lesions on buccal mucosa rarely show dysplastic or malignant change compared to lesions seen on the floor of the mouth, lip vermilion and gingivae (102). OPMD found in the floor of the mouth (FOM) together with other sites were found in 6 respondents (4%). The lateral tongue and floor of the mouth are known to be high risk sites of OPMD because OPMDs in these locations are more likely to develop into malignancy. In the present study, we found lateral tongue and floor of the mouth sites were the second and third most common sites for OPMD. Two respondents with a large single ulcer on lateral border of the tongue were diagnosed with oral squamous cell carcinoma (OSCC) and

subsequently treated. Our results suggest that the risk of developing OC from OPMD is not negligible in the Indonesian population and requires urgent management.

A strong association was observed between some risk factors and OPMD. The highest number of risk factor was smoking cigarettes (14.5%), followed by betel nut chewing (12.6%) and alcohol consumption (3.9%) with people who regularly drink alcohol having an increased likelihood of having OPMD. Leukoplakia was the most common type OPMD found in the survey most likely due to high number of smoking habit related. Thus, in addition to smoking and BQ chewing, alcohol consumption is a significant risk factor for OPMD in Indonesia.

4.4.2 Regional variation of OPMD

Sixty (44.1%) out of the 139 respondents who had OPMD were living in WP and the other 55.9% of OPMD spread diversely amongst other 4 regions. The highest OPMD occurred in people from WP where there is a strong culture in BQ chewing passed down through many generations as well as drinking traditional made alcohol beverage. This could explain why the OPMDs were found with high prevalence in West Papua with conversely, people who live in JKT had the lowest risk of developing OPMD, with a better understanding about OC risk factors, good social economic status and healthy nutrition. Previous studies have also reported that high SES had a decreased risk of having OC (302, 303). Consistently, people living in WJ had a relatively low rate of OPMD as well. Thus, it is possible to speculate that individuals living in urbanised regions are at a lower risk of developing OPMD compared to those living in rural or remote areas. This may also be confounded by the fact that most of the people living in JKT, WJ and BA are muslims and are therefore less likely to consume alcoholic beverages (304).

4.4.3 The association between BQ chewing and OPMD or OC

BQ chewing was the most frequent risk (n=59) factor detected in this current survey with 8.9-fold increased risk of OPMD. Many epidemiological, clinical and laboratories studies have reported a strong correlation between BQ chewing and OPMD or OC development, specifically OSMF and leukoplakia development in oral cavity (157, 249, 262, 305, 142).

Further, consuming BQ for more than 15 years was shown to be associated with a 2.5-time higher risk of having OPMD compared to a timeframe of less than 15 years. One of OPMD caused by BQ chewing is OSMF a chronic disease known to increase with time and is proportional to the duration of BQ use (142). In remote regions, it is common to consume BQ from an early of age, and some reported that this habit commences in individuals as young as 10 years old (157). Respondents in the present study who chew BQ regularly everyday were shown more likely have OPMD rather than occasional users. Consistently, the development and severity of OSMF is known to correlate to BQ use in a dosage, frequency, and time dependent manner (157, 262).

The various ingredients of BQ are also suspected to differentially influence OPMD development. Most BQ are composed of areca nut, slaked lime and betel leaf or betel SI, and some additional spices and tobacco. Taiwanese and Melanesian commonly chose betel SI (flower or pods part) rather than betel leaf (155). In the present study, only people living in WP consumed BQ-SI and they are Melanesian in ethnicity. This custom of BQ-SI chewing exists in WP due to the proximity to Papua New Guinea, and it is strongly possible they share both culture and ethnicity. The Papua island (West Papua and Papua) is part of the New Guinea island known as the second largest island in the world, after Greenland. The New Guinea island has two parts, the land of Papua, which is part of Indonesia, and Papua New Guinea (PNG) which is a separate country. The indigenous population of Papua and PNG are racially of Melanesian heritage (306).

Furthermore, the logistic regression analysis showed that BQ-SI chewers had 4.2 times higher risk of having OPMD compared to BQ-L chewers. Both betel leaf and betel SI alone have precisely been shown to contain extensive polyphenolic as antimutagenic compounds (155, 307, 262). We also showed that none of betel leaf chewer had OPMD in the oral cavity. Safrole is the major phenol in betel SI and may act as carcinogen (307). Petti and Scully have previously postulated that excessively high and monotonic intake of polyphenols could have different effects, being cytotoxic to the oral mucosa. Further research is needed to assess whether BQ-SI or the combination with different slaked lime causes higher risk of OPMD. The addition of tobacco to BQ-L chewing could increase the risk (OR=10.2) compared to consuming BQ-L alone. Several studies have shown that tobacco could indeed increase the frequency of onset of OSMF and accelerate the OPMD development into cancerous lesion (165, 142). One study in Pakistan found that adding tobacco into BQ increased the severity of clinical grading of OSMF in a statistically significant manner (157).

BQ in fabricated package was associated with a 3.6-time higher risk of OPMD compared to non-package BQ prepared by the users themselves. This “home-made”, self-prepared BQ was composed of fresh areca nut, leaf/SI, and additional spices and possibly possesses more polyphenols and other antimutagenics compared to fabricated BQ packages process by which BQ package are fabricated with heating or freeze-drying, are likely to result polyphenols damage (157).

4.4.4 The association between smoking cigarette and OPMD or OC

Cigarettes smokers had 5.22-time increased risk of OPMD compared to the non-smoker's respondents. Tobacco has been proven as one of major risk factors associated with OPMD and OC (15, 102, 295). In SEA countries such as Indonesia, smoking and BQ chewing are the most common risk factors associated with OC (7).

Further logistic regression analysis showed that differences in the duration of smoking did not correlate with OPMD prevalence. However, the number of cigarettes consumed (more or less than 10 a day) was shown to have had a significant impact (OR=2.7) on the presence of OPMD.

The occurrence of OPMD was not significantly associated with the different type of cigarettes consumed. However, when comparing non-smokers to individuals smoking different types of cigarettes, non-smokers respondents had 3.8 and 5.3-times lower risk of having OPMD compared to kretek and filter cigarette smokers, respectively.

There are two major types of cigarette in Indonesia, which are kretek and filter. Kretek is characterised by the use of a significant amount of clove, the sun-dried flower buds of the clove tree (*Syzygium aromaticum, syn. Eugenia aromaticum*), added to the tobacco since the early 1880s. The clove content of the kretek cigarettes varies typically between 20% and 40% (w/w). Kretek are mostly made from domestic tobaccos especially from Java, Bali, Sumatra, and Lombok (308). There are more than 20 tobacco varieties blended in a kretek. Kretek itself comes from the word that imitates the sound produced by object when it's burned, the cracking noise made by clove when it's burned :"kretek kretek'(309, 308). Conversely, filter cigarettes do not contain clove but only tobaccos with lower tar and nicotine level compared to kretek (308). Six in vitro and in vivo studies reported that Indonesian kretek cigarettes unexpectedly showed no toxicity and exhibited a reduced of pulmonary inflammation. When assessed in vitro the smoke from kretek showed less toxic to the cell and less mutagenic (total particulate

matter) compared to American-blended cigarettes. In vivo experiments showed kretek cigarette smoke had a lowered toxicity in the respiratory tract (310-312, 308). These cytotoxic studies of kretek seems to be consistent with our current clinical study in the oral cavity. Thus, it could be hypothesised that similar to the evidence found in the respiratory toxicity, Indonesian kretek cigarette less cytotoxic to oral mucosal cell lines.

4.4.5 The association between alcohol drinking and OPMD or OC

In the current study, we found that alcohol drinking was the most causative factor of OPMD. The respondents who consumed alcohol had 12.2-fold risk of OPMD compared to non-alcohol drinkers. Studies have reported alcohol strongly correlated with OC (15, 102, 295, 103) and the risk of OC increased with greater alcohol intake, and considered no safe limit intake to cancerous change (313).

Whilst alcohol drinkers were at a significantly higher risk of developing OPMD, the duration of this habit did not show a significant correlation with the occurrence of OPMD. All types of alcohol drinking brands could cause OPMD to oral mucosal. Arak putih (tuak Dayak) is one of Indonesian traditional homemade alcohol beverage, which contains alcohol typically near 27-34% (314). The alcohol is fermented from rice. Most of arak putih consumers in this study are from Suku Dayak, Pontianak, WK region. On the other hand, the beer brand consumed in this study is Saguer, this beverage is also traditionally made, and the level of alcohol could possibly reach 70% or more. Because of the high concentration of alcohol in the saguer, some news reported social problem in WP. Most respondents who consumed beer (Saguer) were from WP. Saguer itself originally come from North Sulawesi's culture and adapted by people from surrounding islands including WP. Saguer is made from fermentation of various palms to make sugar (314).

4.4.6 The association between knowledge of OC and OPMD or OC

Inadequate knowledge of OC is a common obstacle for OC awareness and early screening programs (315, 316, 18). The respondents who had knowledge of OC risk factors had a significantly lower rate of OPMD (OR=0.2) compared to those who do not have knowledge with 82.2% admitting that dentists did not give any information about OC. The OC knowledge provided by Government were given through Indonesian national television and local newspaper were insignificantly reduced risk of OPMD. Indonesian national television has a very lower rate of viewers compared to commercial television that belongs to individual. Even though, both are aired nationally. The local newspaper is not the best media either to educate oral cancer information because the readers seems to prefer digital platform such as media socials or digital news.

The lack of knowledge about signs and symptoms of OC leads to a lack of awareness of the need for early screening. Providing written information in the primary care service has proven to be successful to immediately increase knowledge levels of OC, thus resulting in increased intentions likelihood of OC screening (315, 316).

4.4.7 The association between oral health behaviour and OPMD or OC

Surprisingly, 6.7% of respondents claimed to never brush their teeth and those who confidently and properly brush their teeth (OR=0.6) were at a lower risk of OPMD. Additionally, the respondents who brush their teeth twice a day could lower the risk of OPMD compared to those who brush their teeth once a day. This number should be taken into consideration when setting proper intervention strategies for raising oral health awareness. A study in Iran reported that the good behaviour in properly maintaining oral health significantly correlated with the behaviour in avoiding oral disease risk factors, such as tobacco use and poor diet nutrition with those who carefully watched their oral health status mostly aware of

their oral health in general (293). However, unfortunately, the association between oral health status and non-communicable disease commonly is still neglected (293).

4.4.8 The association between various Indonesian ethnicity and OPMD

Many study have reported that OC in men is prominent in western and southern Europe, south Asia, Melanesia, southern Africa and Australia/New Zealand (7, 317). In women, it is prevalent in south-central Asia, Melanesia and Australia/New Zealand (7, 317). Each year, there are 197 000 deaths, worldwide, from cancer of the mouth and pharynx, with the highest mortality from mouth cancer occurring in Melanesia and south-central Asia (317). There is growing evidence of intra-country ethnic differences; such that, a significantly higher number of deaths from OC have been recorded in men from the Indian subcontinent in the UK, than in the indigenous UK population, and OC appears to be most prevalent in areas with a high Asian population (7, 317). Similarly, we have shown that Papua (Papua Island) and Dayak (WK) ethnicities have 23.61 and 21.33-fold higher risk than Aceh ethnic (BA). Javanese, Balinese and East Nusa Tenggaranese (ENT) groups are slightly more likely to have OPMD than Aceh ethnic, whereas Sulawesi and Maluku ethnic shows 7.01 times of higher risk to Aceh ethnic.

4.4.9 The association between SES and OPMD or OC

Several factors have been used for SES measurement in relation to OC development (318). In the current study, we measured SES by the location of COE examination, occupation, health insurance, and whether it was easy or not to travel to the dental health facilities. Some studies reported that OC risk is associated with low SES significantly related to lifestyle risk factors (318, 302, 303)

The location of COE examination was in the community service (CS) and selected village (VI), both CS and VI located at rural and urban area. CS is the front-line health facility provided by Indonesian government, where most of the middle and lower SES people primarily seek health service as government subsidise the payment (285, 286). Respondents who were screened at urban areas had a lowered risk (OR=0.43) of OPMD or OC compared to those screened at rural areas. This is most likely due to people who living in a rural area may commonly have a lack of hygiene, poor nutrition, bad health facilities, and poor living conditions that may attribute to causation of OC by complex interactions (302).

Non-government employee had 3.3-fold higher rate of OPMD compared to government employee respondents. The low and unstable income received by non-government employee such as fishermen, traders, and farm workers are the reason for poor living conditions and difficulty in buying good nutrition food. The un-employed respondents were insignificantly associated with OPMD, and most of the unemployed respondents (Total n=479, Female =422, Male =57) were home-stay mothers who depend on their husband's income. This predominant home-stay mother is un-employed but may not necessary mean they had no family income.

The respondents' income also can be assessed by whether they were covered by government insurance or purchased the insurance by themselves. The Indonesian government insurance is strictly only given to those who are in low income status. The respondents who had non-government insurance could be considered economically capable as they can purchase insurance or were covered by their working company. Those respondents who had non-government insurance had a lower risk (OR=0.33) of OPMD compared to those who had government insurance.

The ability to access dental health facilities is also a factor thought to be associated with SES. Most low-income population who live in remote areas had a difficulty in accessing dental health facilities, that impacts on OC development (302, 303). However, in the current study

analysis showed no significant difference whether it was easy or not to reach dental health facilities with the occurrence of OPMD.

The demographic difference of OC screening can also contribute to SES measurement in this survey. People living in WP and WK regions were found to have the highest risk of OPMD among all respondents and these are considered very remote areas. WP and WK region were difficulty to be reached from capital city of Jakarta. Limited small domestic flights are available, and another long-distance trip by boat or car is required to reach the area. Thus, the socioeconomic status would greatly different compared to WJ, JKT and BA region.

4.4.10 The relation between all risk factors with OPMD or OC

Smoking, betel quid chewing, and ethnicity were found to be the most significant risk factors for OPMD or OC development, while alcohol drinking was found to be insignificant. This can be understood, that not only because of the substance in smoking (15, 102, 295) and BQ chewing (157, 249, 262, 305, 142) have been proven to associate with OPMD or OC but also, in the present study, a large number of people who was smoking (14.5%) and BQ chewing (12.6%), while alcohol drinker only 3.9% from total number of respondents, resulting the huge gap in 95% CI for respondents who was alcohol drinkers (Table 4.3). Thus, in the current study, alcohol drinking was found to be not significant compared to all significant risk factors to cause OPMD or OC. Ethnicity itself has also been reported to associate with OPMD or OC (7, 317), and in the current study we observed the same with ethnicity significantly related to OPMD or OC.

4.5 Conclusion

The prevalence of OPMD among Indonesians were of surprisingly high (14.3%), with a female predominance. This finding is in contrast with a number of studies reporting male predominance in OPMD prevalence. Leukoplakia was found to be the most common type of OPMD followed by OSMF, leukoplakia combined OSMF, erytho-leukoplakia, and erythroplakia respectively. The most commonly affected sites of OPMD were the buccal mucosa, lateral border of the tongue, and gingiva. Lateral tongue and floor of the mouth are known to be high risk sites of OPMD.

There was a strong correlation between the prevalence of OPMD and risk factors for OC being smoking cigarettes (14.5%), BQ chewing (12.6%) and alcohol drinking (3.9%). The different ingredients of BQ could well be significantly associated with different rate of OPMD as those who chewed BQ with betel stem inflorescence (OR=4.2) were found to have a higher risk of OPMD compared to those who chewed BQ with betel leaf. The addition of tobacco to BQ chewing (OR=10.2) could further increased the risk of OPMD compared to respondents who consumed BQ without tobacco.

The cofounding risk factor such as knowledge of OC risk factors, OH behaviour, ethnicities and low-income SES group were significantly associated with OPMD occurrence.

Our results suggest an urgent need for primary prevention of OC programs in Indonesia. Based on the results, I would suggest creating an educational program to cease risk factor habits associated with oral cancer. In particular to cease smoking and betel quid habits which found significantly the most impactful causing OPMD in this study. The target region for the educational program would be West Papua, West Kalimantan and West Java. The health community centre or primary health community would be the best place to educate the people.

Chapter 5: Characterization of phenolics and arecoline constituent profile in betel quid from four regions of Indonesia

Abstract

Betel quid (BQ) is a chewing mixed package, that mainly contains areca nut (AN), betel leaf (Leaf) or betel stem inflorescence (SI), and slaked lime. BQ chewing is common in South East Asia and has been strongly associated with malignant and potentially malignant diseases of the oral cavity. Alkaloids such as arecoline are often accounted for the carcinogenic potential of BQ, however the chemical composition of BQ has not been studied in detail. Interestingly, BQ also contains phenolic compounds, which are commonly known for their health benefits including anticancer effects.

Aims: we investigated the total phenolic content (TPC), antioxidant activity (by mean of ferric reducing antioxidant power, FRAP), radical scavenging activity (DPPH test), phenolics profile and arecoline content in different components of BQ which were AN, Leaf or SI, Husk, and blended BQ (BQ mix). BQ mix were a blending form contained AN, Leaf or SI and slaked lime. Samples were imported from 4 major regions of Indonesia, namely: Banda Aceh (BA), North Sumatra (NS), West Kalimantan (WK) and West Papua (WP). The highest TPC, FRAP, and DPPH values were detected in AN samples compared to other BQ components, while samples from WP region were of higher values compared to the other regions.

Results: High performance liquid chromatography - Mass Spectrometry (LC-MS) analysis showed that Husk contains the widest range of polyphenols, including hydroxybenzoic acids, hydroxycinnamic acids, flavanols, flavonols and stilbenes. Catechin and epicatechin were the main polyphenols detected in BQ, and they were present at the highest concentrations in WP-AN sample. Arecoline was detected in all AN and BQ mix samples and was

significantly correlated with catechin and epicatechin, and significantly negatively correlated with *p*-hydroxybenzoic acid.

Conclusion: The current study is the first to extensively characterise the chemical composition of BQ and provides insight for the interactions of BQ alkaloids and phenolics in the development of oral submucous fibrosis and oral cancer.

Keywords: *phenolics, arecoline, BQ, oral cancer, alkaloid, HPLC*

5.1 Introduction

The key risk factors of oral cancer have been recognized and include tobacco, alcohol, and betel quid (BQ) consumption (22, 102). In Western countries, the major risk factors of oral cancer are cigarette smoking and alcohol drinking, whereas in Asian countries such as Indonesia, the major risk factors are smoking and BQ chewing (296, 72). These same risk factors are also associated with the development of precursor lesions, overall known as oral potentially malignant disorders (OPMD). Indonesia has a poor cancer registry with limited studies conducted on the prevelance of oral cancer and OPMD, and their relation with BQ chewing (319). Our recent epidemiological study, conducted in five different regions of Indonesia (Banda Aceh, West Java, Jakarta, West Kalimantan, and West Papua), showed that of 974 participants, 11.12% had oral potentially malignant disorders (OPMD) (320), including submucous fibrosis. This previously underestimated prevalence of OPMD has major health implications as these conditions have a high potential to develop into oral cancer.

AN, the endosperm of the fruit of the Areca palm (*Areca catechu)*, is considered the key carcinogenic ingredient of BQ due to its high alkaloid content (321). Four alkaloids, namely arecoline (most potent), arecaidine, guvacine, and guvacoline were reported to stimulate fibroblasts to produce collagen, thought to represents the main pathogenic mechanism of oral submucous fibrosis (OSMF), a potentially malignant condition that found common in Indonesia (321, 137). The mutagenic effects of constituents and extracts of the AN have been studied extensively on cells (322). However, AN is always consumed in a BQ package that contains additional components, most commonly betel leaf or betel stem inflorescene (SI) and slaked lime, which can affect the carcinogenic potential of AN extracts (323, 324, 176). Therefore, it is important to investigate the chemical composition of all BQ ingredients.

Polyphenols are very important plant constituents and they exert antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals (325). However, polyphenols also possess pro-oxidant effect under certain conditions, such as high concentrations, high pH and the presence of redox-active transition metals (326-328). The pro-oxidant activity of catechins at non-cytotoxic levels have been used as chemosensitizer for the treatment of cancer (326). However, at high concentrations, certain polyphenols, such as catechins could exhibit cytotoxicity to health cell (329-331). Thus, moderate amounts of polyphenols could protect against diseases associated with oxidative stress such as cancer, coronary heart disease, inflammation via mechanisms like antioxidant activity and neutralisation/modulation of human/bacterial/viral proteins/enzymes (332, 333).

The major polyphenols have been found in AN were catechin, flavonoids, flavan-3,4-diols, leucocyanidins and hexahy-droxyflavans (334). Polyphenols are likely to contribute to the toxicity of AN (335). Previously Chavan and Shinghal reported that catechin and epicatechin were identified from the *Mangalore* variety of AN (218). Further study reported catechin as the most abundant compound in AN from Malaysia (189).

AN is consumed at different stages of ripeness, in raw form or after processing in many forms (155). It has been reported that arecoline was the major alkaloid found in the immature nut (335) and polyphenols was also found in a higher amount in immature nut (155). Alkaloids content in AN is affected by multifactor such as geographical location, maturity of areca fruit, processing method and storage (335). A study by Senica et al investigated the change of polyphenols content in elder leaves (*Sambucus nigra* L.), flowers, and berries induced by different altitudes and locations (336). The result showed that polyphenols content influenced by the altitude of plant's growing site (336).

Presently, there are limited studies investigating polyphenols and alkaloid composition of different components of BQ, and their potential correlation. The current study aims to fill this gap by exploring the polyphenols and alkaloid constitutes of areca nuts (AN) the endosperm of the fruit of *Areca catechu*, betel leaf of *Piper betle* (Leaf), betel stem inflorescence of *Piper betle* (SI), areca husk of *Areca catechu* (Husk) and fully packaged betel quid (BQ mix) originated from four key BQ consuming regions in Indonesia (BA, NS, WK, and WP). The location where BQ were imported can be seen in **Figure 5.1.** The current study also aims to establish correlations between different chemical compounds in BQ, which could inform further studies that investigate the interactions between alkaloids and phenolics in the development of OPMD and oral cancer.

Figure 5. 1 Betel quid imported from four key regions of Indonesia.
Banda Aceh (BA), North Sumatra (NS), West Kalimantan (WK) and West Papua (WP). Image was produced using Google Earth (US Department of state geographer, 2018 Google, Image landsat/Copernicus).

5.2 Materials and methods

5.2.1 Experimental design and sample preparation

Raw BQ components were collected from four regions of Indonesia; Banda Aceh (BA), North Sumatra (NS), West Kalimantan (WK), and West Papua (WP). The four regions were selected to represent the west, middle, and east part of Indonesia whose people perform a BQ quid chewing habit. BQ components were bought from local traditional market from each selected region. A range of variation of BQ components within the selected region is highly possible, however we focused to study the most common BQ components as being AN, Leaf/SI, Husk, and BQ mixture. For BA, NS and WK regions, dried AN, Leaf, Husk, and BQ mix samples were analyzed, and the BQ mix contains dried AN, Leaf and lime at the ratio of 80.5:12.5:7 by weight (262) (**Figure 1.10**). For WP region, dried AN, SI, Husk, and BQ mix samples were analyzed, and different from other regions, the BQ from WP region contains, betel inflorescence stem (SI) that commonly named as flower, instead of betel leaf (155). The outer cap colour differences of AN is a manifestation of the ripeness of AN, where the unripe AN looks green (shown by An from WP and WK regions), while the ripe AN looks yellow-orange colour (155) shown by AN from BA and NS (**Figure 1.10**).

Extraction of polyphenols from BQ samples were performed following published method with modifications (263). Seeds and husks of AN were manually separated. All samples were freeze-dried for 72 hours using FD3 Freeze Drier (Dynavac Engineering, Australia), and finely grinded using electronic grinder (Multigrinder II, model EM0405, Sunbeam, Auckland, New Zealand). 5g of each sample powder was weighed into a 50mL centrifuge tube prefilled with nitrogen gas (99.99% Grade 4.0, Coregas, Yennora NSW, Australia), added with 50mL methanol as extraction solution. Extractions were carried out at 20ºC for 48hr under shaking condition at 150rpm (Incubator shaker ZWYR-240, LABWIT scientific, Australia). After the extraction, the samples were centrifuged at 6500rpm for 10

minutes at 20ºC. The supernatants were passed to a clean tube and evaporated at 40ºC to remove the solvent entirely using Hei-VAO Value rotary evaporator (Heidolp Instruments GmbH & Co.KG, Schwabach, Germany), then redissolved in 10mL of LC grade methanol. The final extract was flushed with nitrogen gas and sealed with parafilm to avoid oxidation and storage at 4ºC in darkness until analysis. The extract stock solution was filtered using 30mm x 0.45µm nylon syringe filter (Thermo Fisher Scientific, VIC, Australia) to gain a clear solution before further analyses.The detail methods in determining TPC, antioxidant activities, identification of BQ components using HPLC, LC-MS and GC-MS can be seen in the section 2.4.

5.3 Results

5.3.1 Total phenolics content (TPC) and antioxidant activities in betel quid samples

The highest TPC was found in AN group from WP followed by AN from BA, NS, and WK respectively. The TPC value of WP-AN was almost 2-fold that of nut from other regions as can be seen in **Table 5.1** indicating that WP-AN contains the most abundant phenolics among nut group.

There was a considerable reduction of TPC and antioxidant activities (FRAP and DPPH scavenging activities) when AN was in a BQ mixture with slaked lime (AN: betel leaf/SI: slaked lime = 80.5:12.5:7 by weight). In the betel leaf/SI group alone, WK-Leaf had the highest phenolics and antioxidant activities, while the least phenolics content was found in NS-Leaf.

Table 5. 1 Total Phenolics content and antioxidant activities in AN, betel Leaf/ betel stem inflorescence, BQ mixture and husk

Samples	TPC mg GAE g-1 DM	FRAP mM Fe2+ eq./g sample DM	DPPH mg TE /g sample DM
BA-AN	122.27±8.09***c***	62.59±8.41***c***	277.43±9.69***c***
NS-AN	111.72±10.5c*d*	60.78±3.83*c*	280.06±2.21*c*
WK-AN	108.84±8.81*d*	56.61±7.34*c*	267.91±19.26*c*
WP-AN	216.71±18.06*a*	107.77±7.33*a*	422.53±1.11*a*
BA-Leaf	26.710±1.94*f*	18.24±1.62*ef*	55.08±5.13*fg*
NS-Leaf	19.91±2.4*f*	15.39±1.5*ef*	42.67±6.49*g*
WK-Leaf	63.93±4.78*e*	31.99±3.79*d*	111.28±13.41*d*
WP-SI	59.00±3.79*e*	21.61±3.28*e*	71.73±13.64*e*
BA-BQ mix	6.70±0.69*g*	4.91±0.43*h*	16.45±3.19*hi*
NS-BQ mix	10.57±3.36*g*	6.01±1.18*g*	26.86±8.72*h*
WK-BQ mix	27.69±3.84*f*	12.78±1.78*fg*	62.52±8.51*ef*
WP-BQ mix	155.09±11.13*b*	94.60±8.14*b*	303.34±0.72*b*
BA-Husk	3.13±0.26*g*	1.66±0.06*h*	3.63±0.64*i*
NS-Husk	5.14±0.54*g*	2.76±0.15*h*	9.62±0.82*i*
WK-Husk	3.05±0.24*g*	1.61±0.03*h*	4.69±0.48*i*
WP-Husk	5.36±0.20*g*	2.45±0.21*h*	8.29±0.96*i*

Data are expressed as mean ± standard deviation of triplicate experiments (n=3) with confident interval significantly (P<0.05). Mean values in the same column followed by the same letter do not differ significantly. An *a* letter besides mean values refer to the highest value, and its value decreases following the descending letter in the same column.

BA = Banda Aceh, NS = North Sumatra, WK = West Kalimantan, WP = West Papua.

AN= Areca Nut, SI= stem inflorescence *BQ Mix* = Areca nut + betel leaf/betel stem inflorescence stem + slaked lime. TPC = total phenolic content. FRAP = ferric reducing antioxidant power. DPPH = 1,1-diphenil-2- picrylhydrazyl

The value is significantly higher compared to another mean in the same column

The value is significantly lower mean values in the same column

5.3.2 Polyphenols content in betel quid components

Our study shows that phenolic acids were mostly detected in husk, leaf and SI samples, but traced in nuts and BQ mixtures. The highest amount of phenolic acids detected were protocatechuic acid, *p*-hydroxybenzoic acid and syringic acid, while gallic acid, caffeic acid, p-coumaric acid, ferulic acid and sinapic acid were detected at low concentrations. The highest protocatechuic acid content was observed in NS-Husk followed by WK-Husk and BA-Husk respectively. The highest p-hydroxybenzoic acid was observed in WP-SI, followed by NS-Leaf, BA-Leaf, and WK-Leaf respectively. NS-Leaf has the highest concentrations of syringic acid (**Table 5.2 and Figure 5.2**).

Flavanols, mainly catechin and epicatechin, were the most abundant polyphenols detected in nearly all samples except WK-leaf. Higher flavanols concentrations were observed in nuts compared to leaf, husk and SI. Epicatechin gallate was also detected in BA-Leaf at high concentrations, but not in other leaf samples. Flavonols, including quercetin-3-galactoside, quercetin-3-glucuronide, quercetin-3-rhamnoside, kaempferol-3-glucoside and quercetin were also detected in the leaf and husk samples mainly from BA, but low or absent in samples from NS, WK and WP. Resveratrol was the only stilbene detected in BQ mainly in leaf and husk, but absent or at low concentrations in nut samples.

Table 5. 2 Phenolic acids in BQ samples originated from different Indonesia regions (mg/g DM)

Polyphenols	BA-AN	BA-LEAF	BA-HUSK	BA-BQ MIX	NS-AN	NS-LEAF	NS-HUSK	NS-BQ MIX	WK-AN	WK-LEAF	WK-HUSK	WK-BQ MIX	WP-AN	WP-SI	WP-HUSK	WP-BQ MIX
Phenolic Acids																
gallic acid	ND	ND	ND	ND	ND	0.039 ± 0.06	13.42 ± 4.31	ND	ND	ND	ND	ND	ND	ND	ND	ND
5-(hydroxymethyl)furfural	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
protocatechuic acid	ND	ND	0.25± 0.037	ND	ND	ND	6.99 ±0.223	ND	ND	ND	0.40±0.001	ND	ND	ND	ND	ND
p-hydroxybenzoic acid	ND	0.37± 0.082	0.114 ± 0.09	0.005 ±0.001	ND	0.949 ±0.75	ND	ND	ND	0.065 ±0.014	0.43 ±0.002	ND	ND	1.01± 0.02	0.33 ± 0.065	ND
caffeic acid	ND	ND	0.004 ±0.002	ND	ND	0.01 ±0.002	0.006 ±0.001	ND	ND	0.0037 ±0.001	ND	ND	ND	ND	ND	ND
caftaric acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
syringic acid	ND	0.37±0.015	0.324±0.27	ND	ND	0.86 ±0.04	0.28 ± 0.002	ND	ND	ND	0.02 ±0.005	ND	ND	0.011± 0.006	0.094 ± 0.003	ND
p-coumaric acid	ND	ND	0.022 ±0.002	ND	ND	ND	ND	ND	ND	0.004 ±0.00	ND	ND	ND	ND	ND	ND
ferulic acid	ND	ND	0.0005 ±0.0	ND	ND	ND	ND	ND	ND	0.005 ±0.002	0.015 ±0.003	ND	ND	0.005 ±0.001	0.005 ± 0.002	ND
sinapic acid	ND	ND	0.074 ±0.003	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.015 ±0.0005	ND	0.008 ± 0.003	0.015 ±0.0004
Anthoxanthins and stilbenes																
procyanidin B1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Catechin	9.699±0.96	0.364±0.02	0.276 ±0.21	0.717 ±0.102	8.51±1.03	0.469 ±0.12	0.379 ±0.03	1.107 ± 0.2	2.335±1.3	ND	0.059 ±0.012	1.16 ±0.21	24.18 ±4.36	0.65 ±0.086	5.18 ±0.39	15.95 ±0.16
procyanidin B2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Epicatechin	1.462±0.218	0.860±0.089	0.063 ±0.035	0.064 ±0.007	1.02 ±0.68	0.40 ±0.01	0.78 ±0.026	0.061 ±0.0	0.23 ±0.06	ND	0.11±0.007	ND	1.045 ±0.135	0.96 ±0.12	0.123 ±0.03	1.05 ±0.069
Polydatin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
epicatechin gallate	ND	0.588±0.016	ND	ND	ND	ND	0.082 ± 0.015	ND	ND	ND	ND	ND	ND	ND	ND	ND
Quercetin-3-galactoside	0.036 ±0.0005	0.009 ±0.0001	0.047 ±0.008	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Quercetin-3-glucuronide	ND	0.006 ±0.0001	0.014 ±0.002	ND	ND	ND	ND	ND	ND	ND	0.036 ±0.003	ND	ND	ND	ND	ND
Quercetin-3-glucoside	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Kaempferol-3-glucoside	ND	ND	80.66 ±2.33	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.08 ± 0.003	ND
Quercetin-3-rhamnoside	ND	0.005 ±0.0003	0.028 ±0.003	ND	ND	ND	ND	ND	ND	ND	0.034 ±0.004	ND	ND	ND	ND	ND
Resveratrol	ND	0.15 ±0.002	0.004 ±0.001	ND	ND	0.008 ±0.003	0.007 ± 0.001	ND	ND	ND	0.018 ±0.004	ND	0.003 ±0.001	ND	0.008 ± 0.0004	0.002 ±0.0003
Quercetin	ND	0.007±0.0002	ND	0.05 ±0.008	ND	ND	0.007 ±0.0003	ND	ND	ND	0.012 ±0.001	ND	0.11 ±0.016	0.01 ±0.001	0.06 ±0.004	0.03 ± 0.002
Kaempferol	0.003 ±0.001	ND	ND	0.015±0.01	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.01 ± 0.0	ND

*Data are expressed as mean ± standard deviation of triplicate experiments (n=3) with confident interval significantly (P<0.05).

BA = Banda Aceh, NS = North Sumatra, WK = West Kalimantan, WP=West Papua. AN= Areca Nut, BQ Mix = AN + betel leaf/betel inflorescence stem + slaked lime. SI= stem inflorescence, ND = Not Detected

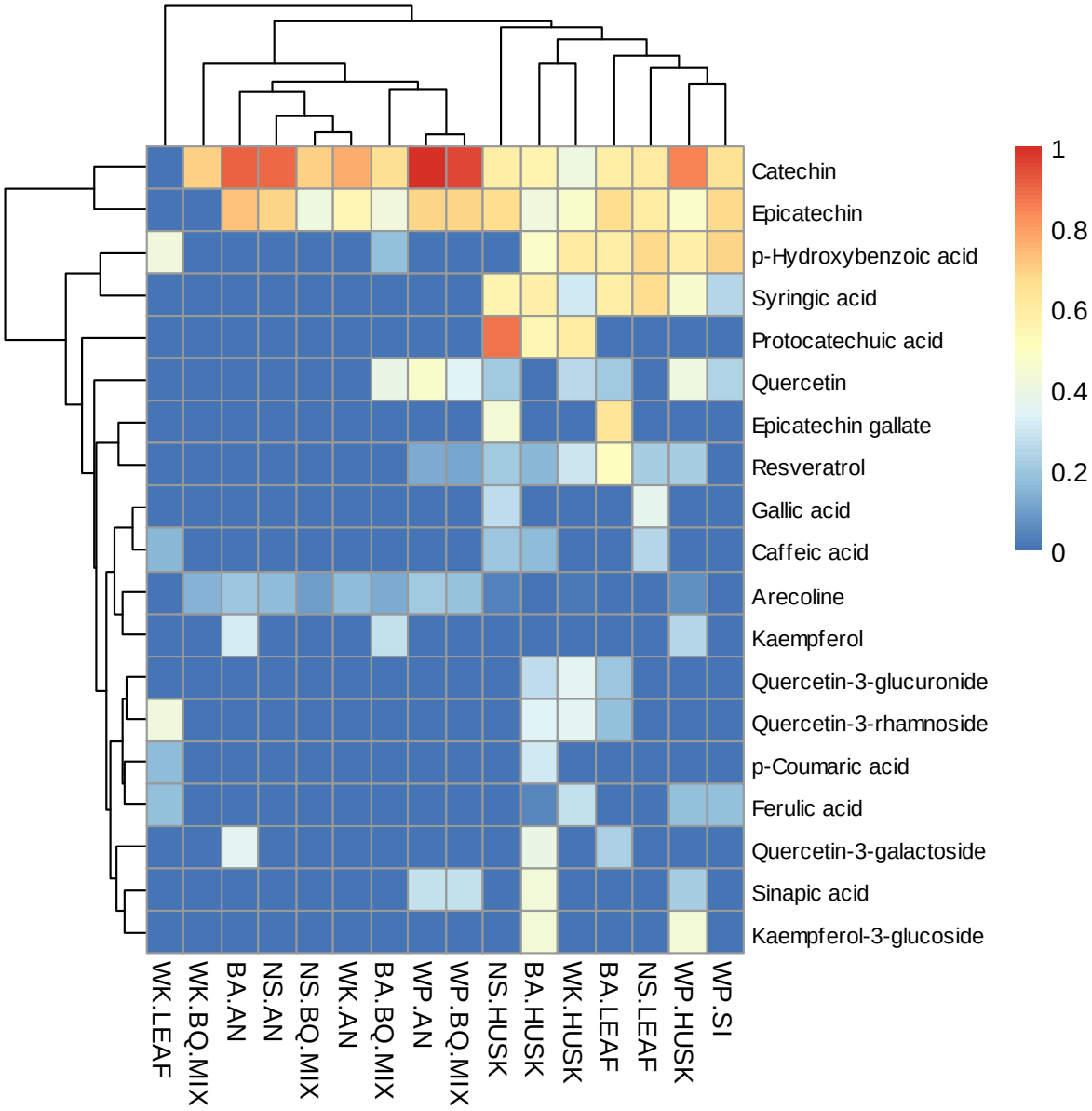


Figure 5. 2 Heat map showing arecoline and polyphenols identified in the sample.

Red colour shows the highest concentration, yellow colour shows moderate concentration and blue colours shows the lowest concentration of constituents.

BA= Banda Aceh, NS= North Sumatra, WK= West Kalimantan, WP = West Papua.

AN= Areca Nut, SI= stem inflorescence *BQ Mix*= Areca nut + betel leaf/betel stem inflorescence + slaked lime

5.3.3 Alkaloid content in betel quid components

Only arecoline analytical standard showed a stable peak using the current method, while arecaidine, guvacine and guvacoline showed unstable peaks for standard curve. Therefore, arecaidine, guvacine, and guvacoline could not be identified and quantified with this method.

The highest arecoline concentration in the current study was observed in WP, followed by BA, WK, and NS respectively in a nut group alone (**Table 5.3**). Interestingly, in the mixture group, the concentration of arecoline in BQ from WP and WK dropped only 0.38 and 1.3 mg/g DM, respectively, compared to arecoline concentration in their AN. However, BQ mixtures from the other 2 regions showed a considerable reduction of arecoline concentration to more than 50% from their nut. No arecoline was detected in betel leaf and stem inflorescence samples. Low concentrations of arecoline was detected in husk from most regions, where husk from BA was the only husk which does not contain arecoline.

Table 5. 3 Identification and quantification of arecoline by HPLC analysis

Samples	Arecoline (mg/g DM)
BA-AN	6.10±0.99ab
BA-Leaf	ND
BA-Husk	ND
BA-Mix	2.58±0.39d
NS-AN	4.11±0.22c
NS-Leaf	ND
NS-Husk	0.37±0.04fg
NS-Mix	1.54±0.23e
WK-AN	4.4±0.27ac
WK-Leaf	ND
WK-Husk	0.16±0.09
WK-Mix	3.11±0.15d
WP-AN	6.92±1.64a
WP-SI	ND
WP-Husk	1.01±0.05ef
WP-Mix	5.57±0.66b
*Data are expressed as mean ± standard deviation of triplicate experiments (n=3) with confident interval significantly (P<0.05). ND = Not Detected. An *a* letter besides mean values refer to the highest value, and its value decreases as following the letter descending.	

The value is significantly higher compared to another mean values

The value is significantly lower mean values

5.3.4 Association between BQ polyphenols and arecoline contents

Principal component analysis (PCA) was performed to systematically analyse the variations among different BQ samples in their phenolics and arecoline content (**Figure 5.3**). Agglomerative hierarchical clustering (AHC) was further used to classify samples into different groups (**Figure 5.4**). A total of 42.45% of variation was explained by PC 1 (24.11%) and PC 2(18.34%). Arecoline, catechin, quercetin, kaempferol and epicatechin mainly contributed to positive aspect of PC 1, while sinapic acid, quercetin-3-galactoside, quercetin-3-rhamnoside, quercetin-3-glucuronide, sinapic acid, *p*-coumaric acid and ferulic acid contributed to negative aspect of PC 1 and positive aspect of PC 2. Protocatechuic acid, caffeic acid, syringic acid, *p*-hydroxybenzoic, gallic acid, epicatechin gallate and resveratrol contributed to the negative aspects of PC 1 and PC 2. Catechin, epicatechin on PC 1 were significantly positively correlated with arecoline, while *p*-hydroxybenzoic on the negative aspect of PC 2 was significantly negatively correlated with arecoline (**Table 5.4**).

Samples were well separated long PC 1 vectors (mainly arecoline, catechin and epicatechin) into two groups, while samples on the negative side of PC 1 would further be divided into three groups based on vectors of PC 2 (**Figure 5.3).** The samples on the positive aspect of PC 1 are mainly nuts and BQ mix samples with relatively high concentrations of catechin, epicatechin and arecoline, while samples on the negative aspect of PC 1 were similar for their low catechin, epicatechin and arecoline concentrations, but different in their concentration of other polyphenols. NS-leaf and NS-husk samples were well separated from others due to the abundant of phenolic acids, while BA-was distant from most samples due to its relatively high concentrations of flavonols.

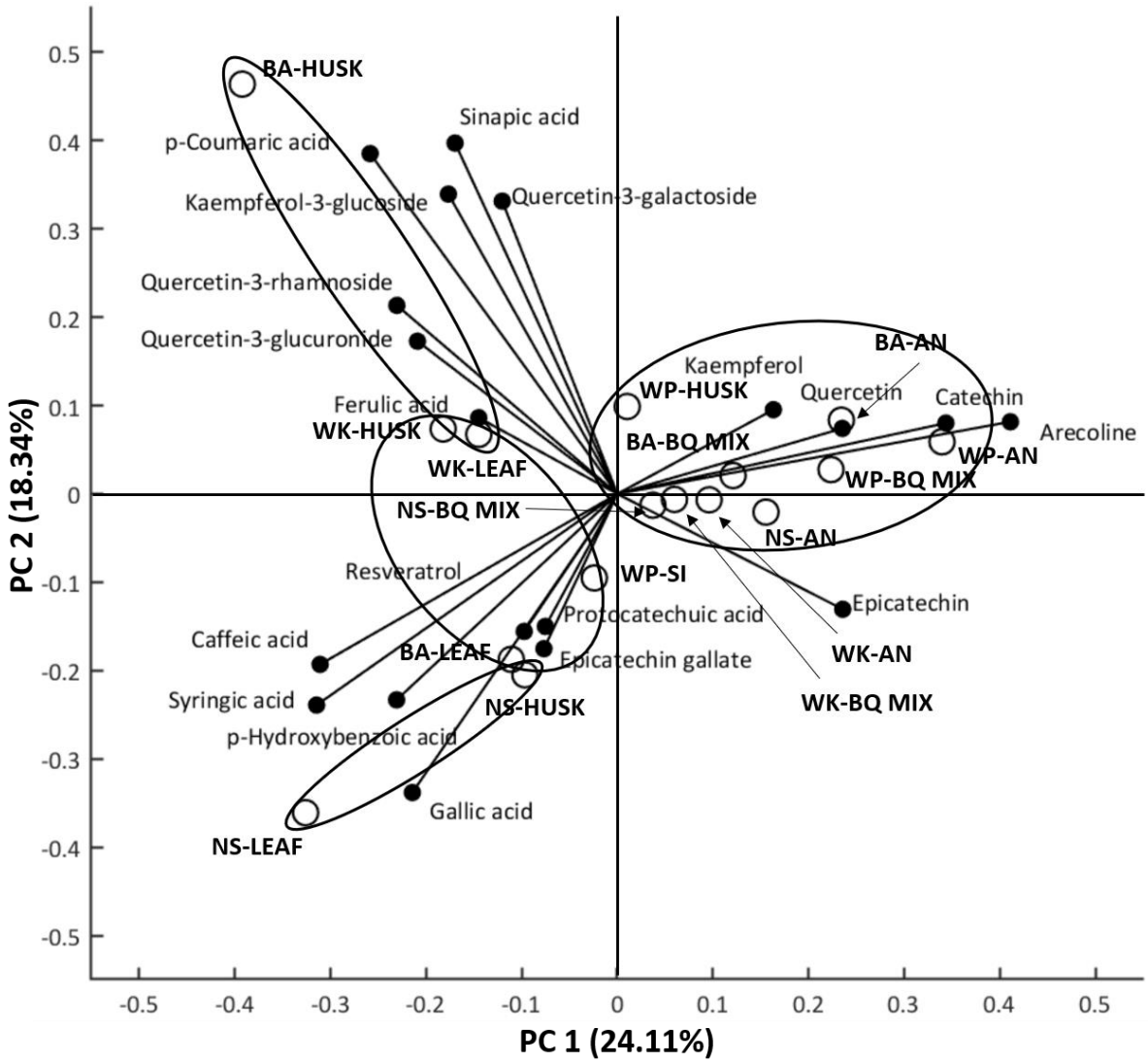


Figure 5. 3 Principal component analysis (PCA) of betel quid phenolics and arecoline content among betel quid samples from 4 key regions of Indonesia.

PC 1 explained 24.11% of total variance, while PC 2 explained 18.34% of total variance.

BA = Banda Aceh, NS = North Sumatra, WK = West Kalimantan, WP = West Papua.

AN= Areca Nut, SI= stem inflorescence *BQ Mix* = Areca nut + betel leaf/betel stem inflorescence + slaked lime

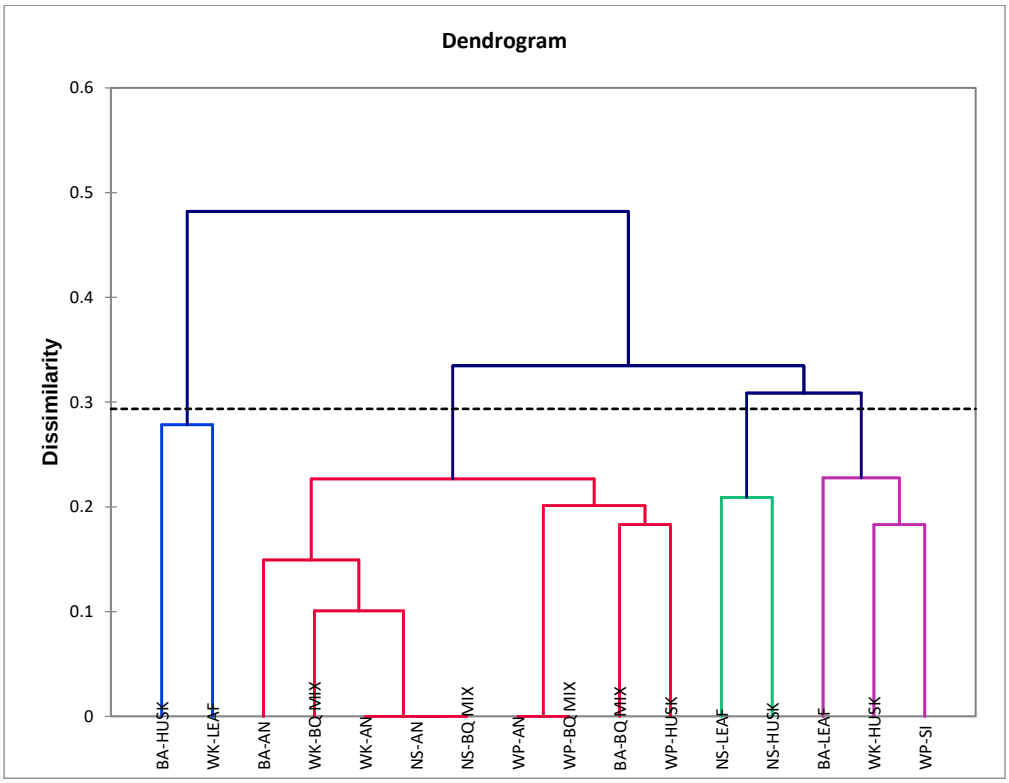


Figure 5. 4 Agglomerative hierarchical clustering analysis for the betel quid samples based on phenolics and arecoline content.

BA= Banda Aceh, NS= North Sumatra, WK= West Kalimantan, WP = West Papua.

AN= Areca Nut, SI= stem inflorescence *BQ Mix*= Areca nut + betel leaf/betel stem inflorescence + slaked lime

Table 5. 4 Correlation (Pearson) between arecoline and phenolic compounds

Phenolics acid compounds	**r-value**	***P*-value**
Ferulic acid	-0.407	0.166
P-hydroxybenzoic acid	**-0.555**	**0.026**
Epicatechin	**0.515**	**0.041**
Syringic acid	-0.481	0.059
Quercetin-3-rhamnoside	-0.427	0.099
Kaempferol-3-glucoside	-0.276	0.301
Catechin	**0.828**	**< 0.0001**
Quercetin	0.427	0.099
Caffeic acid	-0.456	0.076
Gallic acid	-0.300	0.259
P-coumaric acid	-0.288	0.280
Epicatechin gallate	-0.270	0.313
Quercetin-3-glucuronide	-0.344	0.193
Quercetin-3-galactoside	0.024	0.931
Sinapic acid	-0.080	0.767
Resveratrol	-0.294	0.269
Kaempferol	0.312	0.239
Protocatechuic acid	-0.2224	0.404

The phenolic acid compound is significantly correlated with arecoline

The phenolic acid compound is insignificantly correlated with arecoline

5.4 Discussion

We performed the first systematic thorough chemical characterisation of the individual constituents of BQ. Our data show, for the first time, that BQ has distinct chemical features that are mixture, and region specific. Interestingly, the alkaloid and polyphenols content as well as antioxidant activity of individual ingredients change significantly when these are combined into a BQ mixture.

The results of the present study as well as our previous epidemiology research (320), indicate that BQ from WP was of particular interest. All forms of BQ from WP contain the highest concentrations of arecoline, phenolic acid, anthoxanthins, and stilbenes which may help explain the high prevalence of OSMF and oral cancer in that region. Further research is required to better elucidate the association between the identified arecoline and phenolics from four regions of Indonesia with the development of OSMF.

The highest arecoline concentration in the current study was observed in AN from WP, followed by AN from BA, WK, and NS respectively. We highlighted the results from mixture group, where the concentration of arecoline in WP-BQ mix and WK-BQ mix drops only 1.35 and 1.3 mg/g DM respectively. However, BQ mixtures from other 2 regions show a considerable reduction of arecoline concentration to more than 50% from their nut with addition of slaked lime and leaf/SI. This shows that adding slaked lime not only decreases the concentration of polyphenols but, also, of arecoline. This might be due to extreme increases of pH up to 10 by slaked lime as most of alkaloids are only stable in the range of pH 4 – 7 (177). This extreme pH has been shown to damage the alkaloid (337, 338) that might lead to decreased of arecoline concentration. However, the immature AN from WK and WP were interestingly less affected in losing arecoline after added slaked lime, potentially due to chemical and biological process in the mixture component (339). It could indicate that polyphenols and arecoline have a synergic protection from pH change that preserve the inclination number of

arecoline i.e. the more phenolics in AN, the less arecoline damage by slaked lime in BQ mixture. However, further research is needed to clarify this hypothesis. Our current study using methanol extraction solvent showed arecoline quantification between 0.37 – 6.9 mg/g DM. The various concentration of arecoline identified in the present study may be due to the origin of BQ geographical and climates growing site, and its degree of maturity (340). While all BQ components were raw without being processed by sun drying, roasting, or boiling before consumed. It was reported that raw AN is more harmful than the processed AN to be comsumpted by chewers (Rao and Das, 1989). There was no storage process that can harm the chemical in the BQ as we processed all the sample freshly and extracted using methanol solvent and further storage of extract in airtight tube filled with N_2O_2 to avoid oxidation.

No arecoline was detected in betel leaf and SI samples. This finding was consistent with other studies, which reported high polyphenols contents and absence of alkaloids in betel leaf and SI (341, 155, 307). Low concentrations of arecoline was detected in husk from most regions, except BA-Husk. No previous study has investigated alkaloid and phenolics composition of husk, thus the present study is the first to assess the chemical composition of areca husk.

It has been suggested that regular and frequent polyphenols intake may be helpful to protect against oral cancer (332). However, polyphenols can also act as pro-oxidant at high concentrations under certain conditions (327, 328). In the present study, we found polyphenols mainly catechin and epicatechin in high amount, especially in WP-AN sample. Polyphenols induce cell apoptosis by triggering reactive oxygen species (ROS) through two mechanisms (326), direct formation of labile aroxil radical or a labile radox complex with a metal cation (342) and indirect activation of intracellular production of ROS by NADPH oxidases(343). ROS may also interact with cellular macromolecules leading to DNA damage, lipid peroxidation and protein oxidation, either alone or in the presence of transition metals. Cell

culture study showed catechin-induced DNA damage when treating human leukemia HL-60 cells with epigallocatechin-3-gallate (EGCG) at 50µM(329). Further, *in vivo* studies show the dose-dependent hepatotoxicity of EGCG at single dose from 750-1500 mg/kg, where 1500 mg/kg treatment reduce survival rate by 85% (331). Consistently, increasing numbers of hepatoxicity cases in humans have been associated with intake of green tea polyphenols, mainly EGCG (344) and in some cases, associated with liver inflammation and necrosis (345). Despite the results of a recent study that EGCG transiently inhibits both cell proliferation and migration of oral cavity cancer cells (346), there is limited evidence showing the pro-oxidant effect of catechins and cytotoxicity to oral cells, which warrants further investigation.

Catechin as the main flavanol present in BQ can also act as antioxidant that has been shown to prevent cell mutation by inhibiting the production of metalloproteases, leading to potentially reducing invasion and migration, inducing apoptosis and growth arrest in both oral cancer and oral leukoplakia cell lines (332). The lower concentrations of catechin and other flavanols in mixture compared to nut observed in the current study may likely due to the dilution effects of other BQ components. Furthermore, addition of lime can increase pH to 10 and damage the polyphenols (177). Further, the polyphenols compounds in AN have been proven to possess many pharmacological activities (347, 348). These include antiparasitic effects, anti-depressive effects, anti-fatigue effects, antioxidant effects, antibacterial and antifungal effects, antihypertensive effects, anti-inflammatory and analgesic effects, anti-allergic effects, the promotion of digestive functions, suppression of platelet aggregation, regulatory effects on blood glucose and lipids, etc (347, 348, 333).

The large variations in BQ polyphenol contents of different Indonesian regions may be due to various reasons, such as environment conditions, germination, variety of AN, degree of ripeness (347, 155). The AN from WP and WK were immature, while AN from BA and NS were mature. The immature AN showed an overall higher polyphenols content compared to

mature AN. BQ components were grown in the different geographical and climates condition being from east, middle and west region of Indonesia. Thus, in the present study polyphenols contents were affected by the geographical, climate, variety of AN and degree of ripeness.

In the current study, alkaloid and flavonoid (catechin and tannin) components were simultaneously identified in AN. The alkaloids are considered to be the most potent component to cause OSMF, while flavonoids have a synergistic role (321). Flavonoids could decrease collagenase, stabilize the collagen fibrils to resistant degradation by collagenase (137).

Other polyphenols identified were ferulic acid and resveratrol that have been previously reported in AN (348, 235), however, in the present study ferulic acid was not detected in any nuts samples and resveratrol was only detected in nuts from WP. Further polyphenols such as quercetin and kaempferol were also reported in AN (348, 235), these compounds were detected in the current study and interestingly observed in leaf and husk samples as well. Previous studies on BQ mainly reported polyphenols in the nuts (218, 349, 348, 235, 207, 208), but few study reported in husk, leaf and SI. Therefore, the phenolics that were identified such as quercertin and resveratrol in husk, leaf and SI reported in the current study explain the antioxidant capacity of these samples and provided insights for future research investigating antioxidants in BQ.

Sarode, S. C., Mahuli, A., Sarode, G. S. and Mahuli, S. (176) hypothesized that only AN alone cannot trigger OSMF, without including other necessary required factors such as slaked lime and inflammation. These factors possibly result in conversion to phenotypically altered fibroblasts, which leads to increased fibrosis in the oral mucosa causing OSMF. Previous research has shown that powdered slaked lime applied to the chewed AN and betel inflorescence at the corner of the mouth in Papua New Guinea, causes the pH change to 10, at which reactive oxygen species (ROS) are produced from BQ in vitro (177). This high pH change and excessive polyphenols can alter the polyphenols to act as pro-oxidant (326). Thus,

we hypothesise that high concentration of polyphenols in AN, especially catechin and epicatechin, together with high pH altered by slaked lime, are the main factors triggering OSMF. Further, our study showed that adding slaked lime into BQ mixture decreased by more than 70% all the antioxidant potency (TPC, FRAP and DPPH scavenging activities), compared to AN without slaked lime. However, only WP-BQ mixture showed a slight inclination of polyphenols and arecoline concentration. In this case, consuming WP-BQ mixture results in having BQ with high concentration of polyphenols, arecoline and high pH due to added slaked lime. This evidence why people living in WP consuming WP-BQ mixture are more likely to have OSMF in the oral mucosal as shown in our previous study (320). Further *in vitro* and *in vivo* studies need to be conducted to clearly explain the association of Indonesian BQ with pathogenesis of OSMF.

The observed high TPC value of WP-AN compare to AN from other regions could reflect the different amount of immature nuts from this region, as unripe ANs commonly contain more polyphenols compared to ripe AN (155). This theory is consistent with our current study that the unripe AN from WP and WK showed the highest total phenolics content. Further, we also found that FRAP and DPPH antioxidant activities are higher in unripe nut rather than in the ripe AN from BA and NS. The TPC value of nut may also depend on the region where areca catechu is grown, and its processing method (340). In present study, we could recognize unripe AN by its green in colour, while the ripe AN looks yellow (**Figure 1.10**) (155). In some countries, such as Taiwan, Guam, and China, the unripe AN is mostly consumed (155), similar to WP and WK. Whereas in BA and NS, the ripe AN is favoured (320).

An interesting finding was that the phenolics content value and antioxidant activities were dropped in the BQ mixture featuring slaked lime (AN: betel leaf/SI: slaked lime = 80.5:12.5:7 by weight). A previous study also reported betel quid without slaked lime had

higher TPC, antioxidant and cytoprotective activities rather than betel quid with slaked lime (189). It may be that slaked lime raises the pH up to 10 leading to phenolics damage (177). TPC in BQ mixture from BA, NS, and WK dropped by 94.5%, 90.5%, and 74.5% compared to TPC of AN alone, respectively. These results were consistent with FRAP and DPPH tests as well. Interestingly, total phenolics content in WP-BQ mixture only dropped by 28,4% from the amount in AN alone. This might be due to the degree of immaturity of WP-AN and the form of slaked lime. The excessive amount of phenolics could lead to cytotoxicity instead of exerting protective action (332). The results of our recent survey supports the theory that consuming excessive polyphenols in BQ could be detrimental to, rather than protective for oral health (332). Our previous study showed that 60 out of 139 participants (43.17%) who had OPMD were living in WP region and mostly BQ consumers (320). This correlates in this current study with BQ containing the most abundant phenolics levels. Thus, we suggest consuming BQ containing ripe AN is better compared to consuming BQ containing unripe AN, as ripe AN contains less arecoline, known to trigger OSMF development (137), and contains lower polyphenols content (350, 335).

WK-leaf had the highest antioxidant activities and the widest range of phenolic acids among betel leaf/stem group alone. WP-SI was observed to have the highest concentration of phenolic compounds, such as *p*-hydroxybenzoic acid. Previous study reported that betel SI may contain polyphenols such as hydroxychavicol, eugenol, isoeugenol, eugenol methyl ester and safrole (341). Safrole as the major polyphenols in betel inflorescence is also considered to be a carcinogen (307). However, safrole was not profiled in the present study. The values of FRAP and DPPH were consistent with TPC results in most samples, indicating that FRAP and DPPH attributes of the samples were due to the presence of polyphenol compounds.

Some people living in Taiwan, China, Guam, and Papua New Guinea include the husk of outer AN pericarp as part of the BQ mixture (155). This might increase the development of

OSMF, as our study revealed that husk from WK, NS, and WP contains 0.16, 0.37, 1.1 mg/g DM of arecoline respectively. Further, an improper management of waste of husk could also possess potential hazardous to environment as it contains carcinogenic properties. The recent study suggested the use of areca husk as self-assembly of oleylamine modified nano-fibrillated cellulose from areca husk fibers into giant vesicles formed that can have applications in storage and delivery of drugs in topical applications (351). Thus, our findings describing the chemical composition of areca husk is important to avoid or manage future risks. Further study is required to clarify how husk can hazardous the environment. There betel husk should be carefully managed to avoid entering human food chain.

5.5 Conclusion

BQ compounds have distinct chemical features that are mixture, and region specific. Interestingly, the alkaloid and phenolic content as well as antioxidant activity of individual ingredients change significantly when these are combined into a BQ mixture. We observed that BQ compounds from WP was distinctive from other regions, and that AN and BQ mixture from this region contained the highest TPC, antioxidant activities, polyphenols and arecoline content.

Catechin and epicatechin are the main polyphenols detected in BQ, while WP had the highest concentrations in nut sample. The high amount of catechin and epicatechin could change the effect of antioxidant into pro-oxidant leading to raise the potential of developing OSMF.

Arecoline was detected in all ANs and mixture samples and was significantly correlated with catechin and epicatechin, and significantly negatively correlated with *p*-hydroxybenzoic acid. Phenolic acids and flavanols content in betel quid mixture were low, likely due to the addition of lime, which also led to lower antioxidant activity and radical scavenging capacity in the mixture.

The ripeness degree of AN is directly related to the amount of both polyphenols and arecoline. The unripe AN contained a higher concentration of polyphenols and arecoline compared to ripe AN. Thus, immature WP-AN had the highest amount of polyphenols and arecoline. Suggesting that consume the ripe AN could lower the potential in developing OSMF.

These data provide a background for understanding the distinct pathophysiological and pro-carcinogenic effects of different BQ mixtures and may inform health policies in Indonesia.

Chapter 6: Metabolomic Profile of Indonesian Betel Quid using Gas Chromatography Mass Spectrometry

Abstract

High pressure liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS) hve been commonly used for the analysis of areca nut alkaloids samples in previous researches. This study aims to investigate the possibility of using gas chromatography mass spectrometry (GC-MS) to analyse the alkaloids present in betel quid originated from four different regions of Indonesia, Banda Aceh (BA), North Sumatra (NS), West Kalimantan (WK), and West Papua (WP). Further, our study profiled the plant metabolites present in different components of betel quid, including areca nuts, betel leaf, stem inflorescence and betel quid as whole originated from each region. Three types of alkaloids (arecoline, arecaidine, and guvacoline) were successfully identified using GC-MS. However, guvacine could not be analysed due to low volatility. This currently also profiled the metabolites in different BQ components and identified high concentrations of benzenoid and terpense, in stem inflorescence samples, especially the carcinogenic constitute, safrole. Interestingly, high concentrations of sesquiterpenes, such as gamma-cadinene, spatulenol, alpha-copaene, linalool, alpha-gurjunene, gamma-amorphene were also identified in stem inflorescence.

Keywords: areca nut, arecoline, arecaidine, guvacoline, GC-MS, sesquiterpenes

6.1 Introduction

Areca nuts contain four types of alkaloids, namely arecoline, arecaidine, guvacine, and guvacoline (**Figure 6.1)**, that have been reported to stimulate fibroblasts to produce collagen, thought to be the main pathogenic mechanism of oral submucous fibrosis (OSMF) (321, 137). The mutagenic effects of these compounds and the extracts of betel quid have been studied extensively both *in vitro* and *in vivo* (322, 157, 249, 262, 305), which confirm their association with oral cancer. In areca nuts consuming countries, such as Indonesia, areca nuts are usually consumed as betel quid package that may contain the leaf of *Piper betel* or the stem inflorescence of *Piper betel* L. and slaked lime (321). The addition of slaked lime, namely calcium hydroxide, could facilitate hydrolysis of arecoline to arecaidine shown to be responsible for amplified fibroblastic proliferation, increased collagen formation, and therefore increased risk of oral cancer (323, 324, 176). Betel stem inflorescence also contains other carcinogenic compounds, such as safrole (352-354).

Indonesia has been one of the top 10 largest producing countries of areca nut with as many as 36,200 million tons produced in 2001 (155). The betel quid consuming habits at different regions of Indonesia is quite different, with people in West Papua consuming more betel quid than that of Java island and Sumatra island (320). More importantly, the betel quid consumed at different regions of Indonesia are quite different (320). However, the detail chemical composition of these betel quid, including alkaloids and other potential carcinogenic compounds, has not been previous studied. Currently, the most commonly used method to analyse areca nut alkaloid is high pressure liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LC-MS) (218, 349, 189, 207, 208). Despite some researchers attempting gas chromatography mass spectrometry (GC-MS) to profile alkaloids in other plant materials (355, 356), there is no research attempting to use GC-MS to analyse betel alkaloids. Additionally, GC-MS based metabolomic could be used to profile plant

metabolites present in betel quid, which help researches to identify other compounds potentially related to oral submucous fibrosis.

In the current study, we established and investigated the possibility of using GC-MS to analyse the alkaloids presented in the betel quid originated from four different regions of Indonesia, Banda Aceh (BA), North Sumatra (NS), West Kalimantan (WK), and West Papua (WP). Further, our study profiled the plant metabolites present in different components of betel quid, including areca nuts, betel leaf, betel stem and betel quid as whole, for samples originated from each region. The current study developed a method for the measurement of three main alkaloids presented in areca nuts as well as screening the natural molecules present in different betel quid compounds that may be relevant to oral cancer.

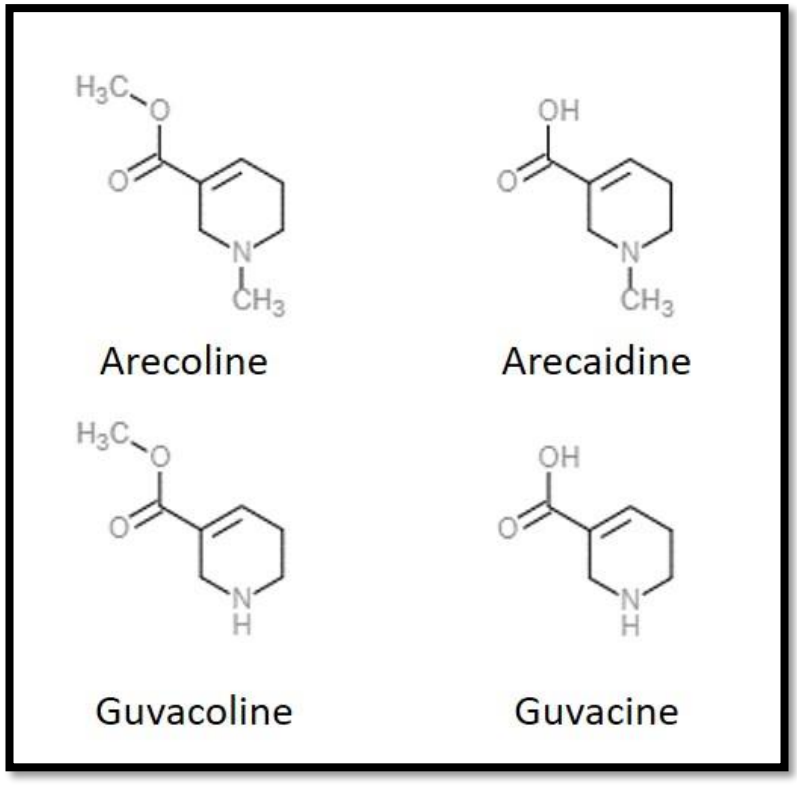


Figure 6. 1 Chemical structure of betel quid alkaloids

6.2 Methods and Experimental Design

The GC-MS analysis program used was modified from a published method on alkaloid (270). Briefly, an Agilent Technologies 6850 series II (GC; Agilent Technologies, Santa Clara, CA) connected to an Agilent Technologies 6973 mass selective detector (MSD) and coupled with an Agilent PAL multipurpose autosampler was used for volatile analysis. The instruments were controlled using Agilent G1701EA MSC ChemStation software in conjunction with Agilent PAL autosampler Control software B.01.04 for ChemStation. The GC was fitted with a J&W DB-5ms column (30 m x 0.25 mm, 0.25 µm film df), with helium as carrier gas (ultrahigh purity, BOC Australia, North Ryde, NSW, Australia) at a constant column flow rate of 1ml/min. The GC inlet was fitted with a borosilicate glass split inlet liner (volume 935 µl, Agilent Technology) and held at 250 ^{o}C. 1 µl of sample was injected to inlet in split mode at split ratio of 30:1. The column temperature initiated at 100 ^{o}C and increased to 180 ^{o}C at 15 ^{o}C/min, held at 180 ^{o}C for 1 min, then increased to 300 ^{o}C at 5 ^{o}C/min and held for 6 mins, and post run at 100 ^{o}C for 2 mins. The MS source, quadruple and transfer line were held at 230 ^{o}C, 150 ^{o}C, and 280 ^{o}C, respectively. The MS was operated in scan/sim mode (35-350 m/z) with positive EI of 70 eV. The sim target ions for alkaloids quantification include: 53, 55, 69, 73, 82, 96, 126, 127, 140, 141, 155 m/z. The standard solution of arecoline, arecaidine, guvacoline and guvacine were analyzed using the same method as samples. All standard solutions were spiked with internal standard and analysed as sample to generate standard curves (R^2>0.99). Alkaloids were identified by comparing the mass spectra and retention indices with the pure standard, while other metabolites in the sample were identified using NIST library in ChemStation and Webbook database as well as the standard solutions obtained. Sesquiterpenes in the betel samples were analysed using MassFinder software with its build-in terpenes library (Hochmuth Scientific Consulting, Hamburg, Germany) by comparing terpene RI values and mass spectra to those of the experimental samples in accordance to our previous researche

(357). All compounds were quantified based standard curves using target ions. The concentration of alkaloids was presented as mg/g dry mass (DM), while other metabolites were semi-quantified and expressed as ug/g dry mass as internal standard correspondent. Blank and internal standard were checked regularly for the sensitivity of GC-MSD system. The detail of this method can be seen in the section 2.5.

6.3 Results

6.3.1 Alkaloids

A representative GC-MS chromatogram of all four alkaloids standards is showed in **Figure 6.2**. All four standards have clear differences in retention time and mass spectrum (**Figure 6.3**). The quantification ions and quality control ions used for each alkaloid were m/z 140, 96, 155 for arecoline (**Figure 6.2A**), m/z 96, 141, 140 for arecaidine (**Figure 6.2B**), m/z 82, 126, 53 and 141 for guvacoline (**Figure 6.2C**), and m/z 82, 53, 127 for guvacine (**Figure 6.2D**). Extremely long tailing was observed for guvacine with unstable peaks, therefore, it could not be identified and quantified with this method. This unstable peak was likely due to the high melting point of guvacine (306-309^{o}C), which exceeded the highest analysis temperature used in the current method (300^{o}C). Thus, this method is not suitable for the identification and quantification of guvacine. Slight tailing was also observed for other three alkaloids standards, which were likely due to the alkaloids salt used in the current study. All commercial alkaloids standards were only available in salt form and therefore the current study added high concentrations of ammonia to alkaloids salt solution to release free alkaloids as standards, which was in accordance to similar research (270).

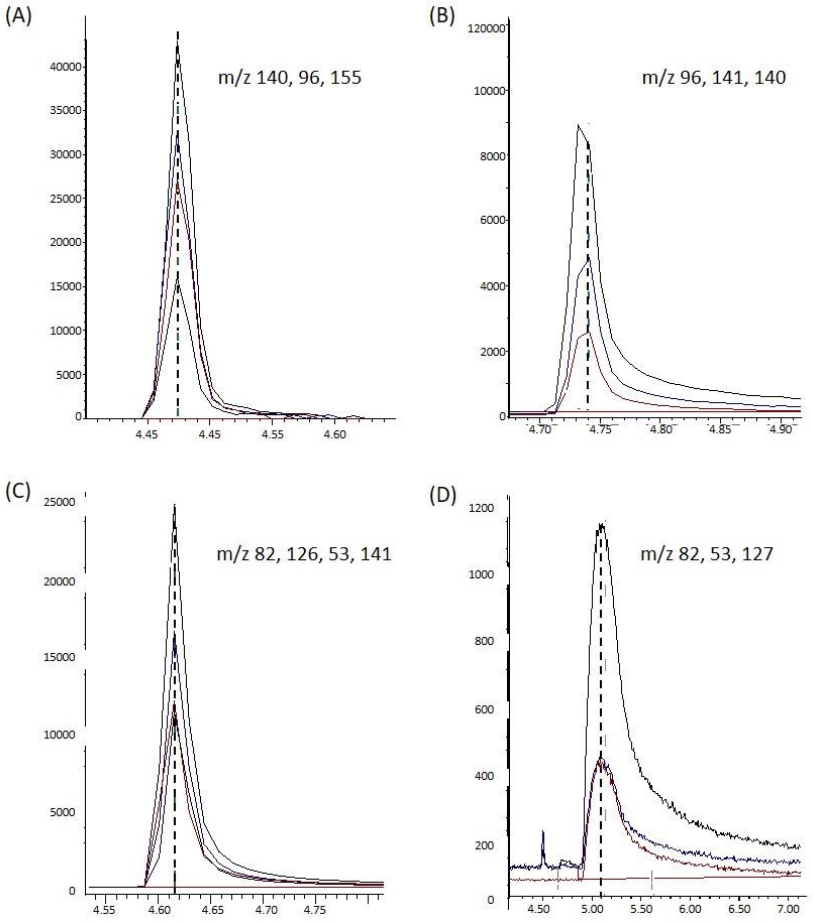


Figure 6. 2 Peaks of alkaloids; A. Arecoline, B. Arecaidine, C. Guvacoline, D. Guvacine

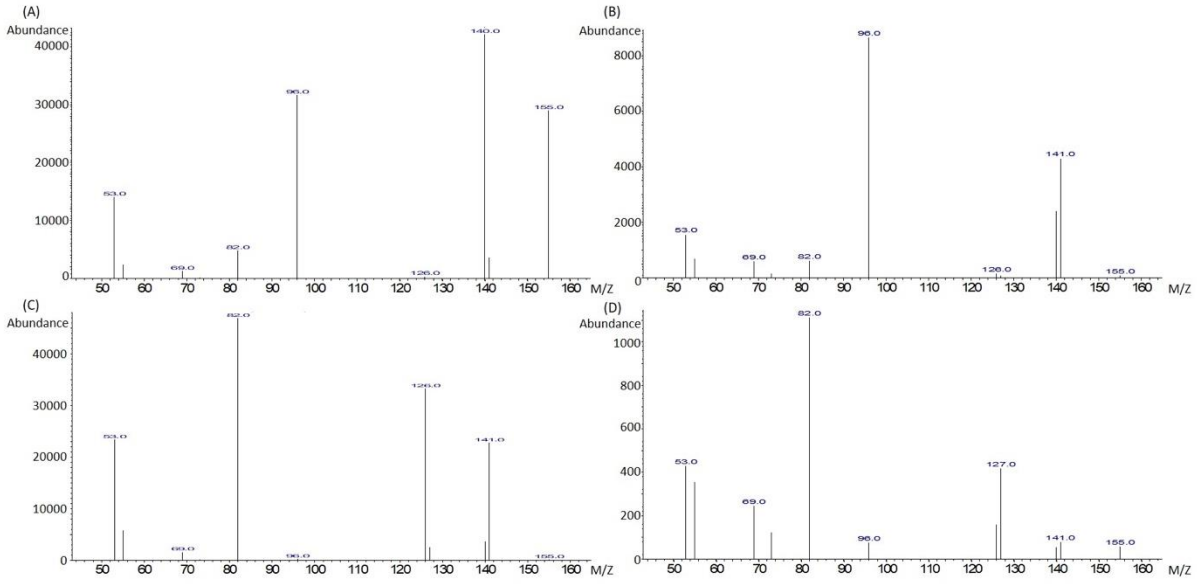


Figure 6. 3 Mass spectrum of target ions of arecoline (A), arecaidine (B), guvacoline (C), guvacine (D)

To ensure the accuracy of the alkaloid analysis, standard curve for each of the three alkaloids were established using serial dilutions of each standard. The actual concentration of alkaloid in each standard were adjusted based on the molecular weight of the alkaloid and the alkaloid salt. Each of the alkaloid standard was quantify based on the target quantification ion, m/z 140, 82 and 96 for arecoline, guvacoline and arecaidine, respectively, and standard curves with R^2>0.99 produced for each standard (**Table 6.1 and 6.2**). Alkaloids were further quantified in all betel quid samples, where arecoline was found in all samples, except in WP-SI and WK-Leaf **(Table 6.1 and 6.2)**. In nut samples, arecoline was found at the highest concentration in WP-AN (157.9 mg/g DM), followed by BA-AN (94 mg/g DM), WK-AN (69.4 mg/g DM), and NS-AN (48.5 mg/g DM). A similar variation in arecoline content was also observed in betel quid mixture samples among the four regions except at lower concentrations. Areca husk from the WP region also showed the highest arecoline (66.1 mg/g DM) compared to husk from another regions (with range <6 mg/g DM). Arecoline was detected in the betel leaf samples from BA and NS at a very low concentration, 1.3 and 1.6 mg/g DM respectively. Arecaidine was only detected in NS-Mixture sample at low concentration (3.9 mg/g DM), while guvacoline was detected in NS-Mixture and BA-Mixture at low concentrations, 1.4 and 0.7 mg/g DM respectively, but not in other samples.

Table 6. 1 Composition of alkaloids in betel quid from BA and NS regions determined by GC-MS (mg/g DM)

Peak No	RI	RIdb	Chemicals	Target Ion	QC Ion	Retention Time (min)	Standard Curve Equation	Banda Aceh				North Sumatra			
								Husk	Leaf	AN	Mix	Husk	Leaf	AN	Mix
1	1237.16	1236	Arecoline	140	96,155	4.493	y = 180.59x - 0.4982	0.23± 0.01	0.03±0. 02	4.15± 0.56	6.98± 0.06	0.24±0. 03	0.05± 0.02	2.4±0.0 9	4.7±0.6
2	1251.25	1250	Guvacoline	82	126,53	4.606	y = 98.138x + 2.7381								0.04±0. 01
3	1279.43		Arecaidine	96	141,14 0	4.832	y = 370.98x + 12.19					23.25±2 .9			0.45±0. 1

Table 6. 2 Composition of alkaloids in betel quid from WK and WP regions determined by GC-MS (mg/g DM)

Peak No	RI	RIdb	Chemicals	Target Ion	QC Ion	Retention Time (mins)	Standard Curve Equation	West Kalimantan				West papua			
								Husk	Leaf	AN	Mix	Husk	SI	AN	Mix
1	1237.16	1236	Arecoline	140	96,155	4.493	y = 180.59x - 0.4982	0.05± 0.01		3.22± 0.96	5.4±0.3 3	3.12±0. 14		6.2±0.3 2	6.9±0.8 6
2	1251.25	1250	Guvacoline	82	126,53	4.606	y = 98.138x + 2.7381								
3	1279.43		Arecaidine	96	141, 140	4.832	y = 370.98x + 12.19								

6.3.2 Metabolomic analysis

In addition to the 3 alkaloids detected in betel quid samples, 92 metabolites were semi-quantified in the betel quid samples, where benzenoids, terpenes and esters were the dominating chemical groups. A heatmap was created to illustrate the variations in these compounds among different betel samples (**Figure 6.4** and **Table 6.3**).

6.3.2.1 Benzenoid

Twenty benzenoids were identified in betel samples in the current study, where safrole was one of most abundant benzenoids detected. The highest concentration of safrole was measured in the WP-SI sample (761.6 mg/g DM). Safrole was undetected in BA-AN, NS-AN, WK-AN, and at a very low concentration in WP-AN. Safrole was also detected in leaf and husk samples from BA, NS, and WK regions with low to moderate concentrations. Further, safrole was detected in all betel mixture samples with WP mixture of the highest concentration, more than 10-fold higher compared to the be betel quid mixture from other regions. Eugenol was also found in the betel leaf and betel SI samples, where WP-SI contained higher concentration of eugenol compared to BA-Leaf, NS-Leaf, and WK-Leaf. Eugenol was not detected in areca nut samples. Additionally, high concentrations of 3-Allyl-6-methoxyphenol and its derivative 3-Allyl-6-methoxyphenol-acetate were observed in betel SI and leaf samples, with WP-SI having the highest concentration in both compounds. Other benzenoids, such as, 5-Hydroxymethylfurfural, was found in the husk samples from BA, NS and WP regions, while 1,3,5-benzenetriol was only detected in betel leaf samples, but not in SI sample from WP.

6.3.2.2 Terpene

Monoterpenes, sesquiterpenes and diterpenes were all detected in different betel samples. 4 types of monoterpenes were detected including, D-limonene, linalool, cis-β-terpineol and 4-terpineol, where D-limonene was the most abundant monoterpene and mostly detected in the nut samples from all region. Other monoterpenes were mainly detected in leaf and SI samples. 21 sesquiterpenes were detected in betel samples, such as α-copaene, α-gurinene, β-gurinene, β-ylangene, (E)-β-caryophyllene, α-humulene, spathulenol etc., with the majority detected in betel leaf and SI samples. For most sesquiterpenes, their concentrations were much higher in SI compared to leaf samples. Only 1 type of diterpene, phytol, was detected, which mainly presented in the leaf samples of NS, WK and low in husk and SI of WP.

6.3.2.3 Others (acid, aldehyde, alcohol, ester)

In addition to the major benzenoids and terpenes detected in betel samples, other compounds including 11 acids, 4 alcohols, 2 aldehydes and 26 esters and 2 amines were detected. Most acids detected were long-chain fatty acids with a minimum of 12 carbons except for lactic acid. The husk samples from BA and NS were most abundant in acids, while other samples contain much less amount of acids. Alcohols were mainly detected in the areca husk, leaf and SI samples, but less abundant in areca nuts of all regions except NS, where moderate level of 2,3-butanediol were detected. Similarly, aldehydes were mainly detected in husk and SI samples, but low in leaf and nut samples. Majority of the esters detected were fatty acids derived esters, except for methyl nicotinate, which was mainly detected in nuts samples, but relatively low or absent in husk, leaf and SI samples from all regions. Amines were mainly detected in leaf samples, but low or absent in other betel samples.

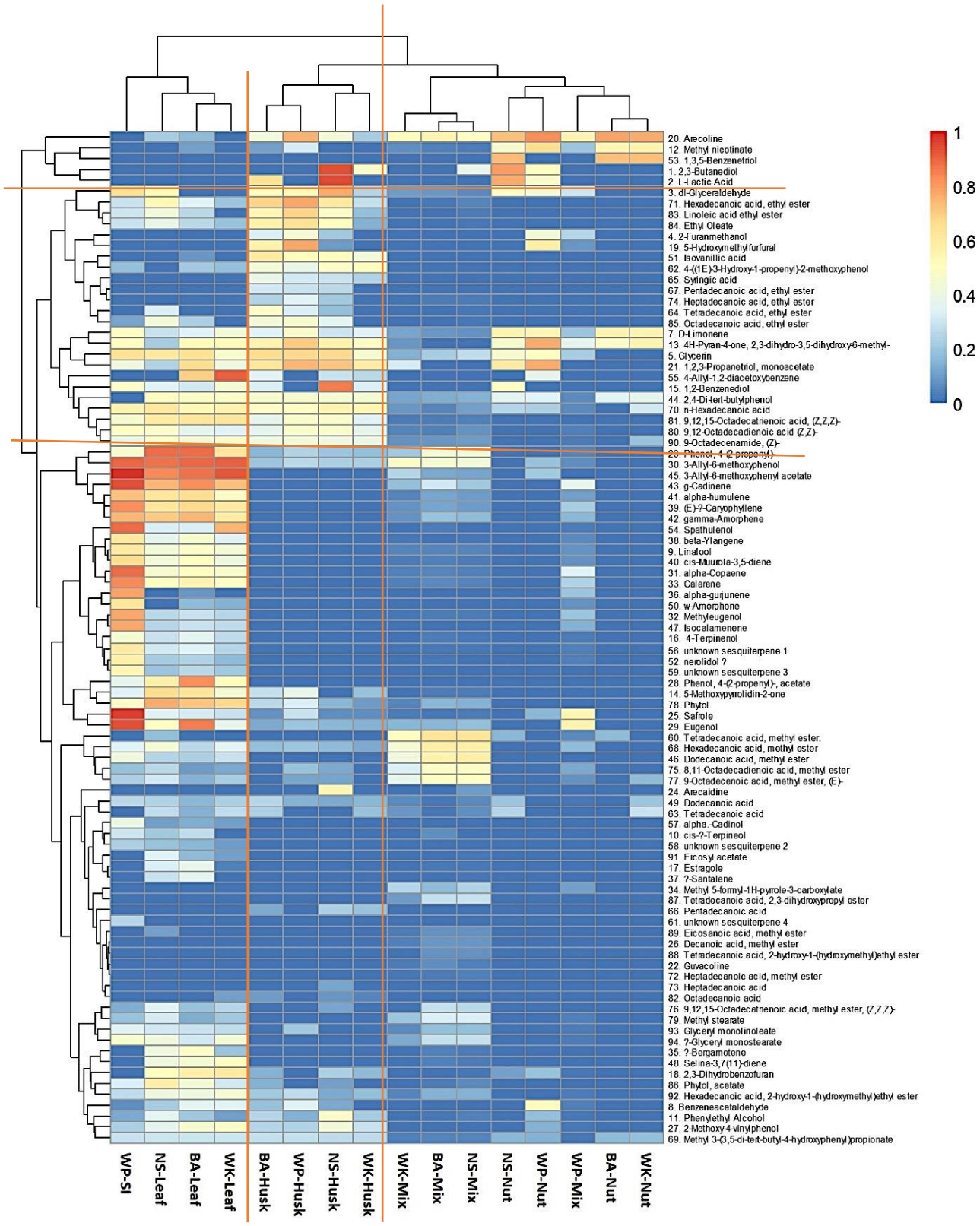


Figure 6. 4 Heat Map of metabolites presented in betel quid.

The heatmap was constructed based on the relative concentrations of the metabolites detected in the betel quid samples as detailed in Supplementary Table 1. BA = Banda Aceh, NS = North Sumatra, WK = West Kalimantan, WP = West Papua. AN= Areca Nut, SI= stem inflorescence *BQ Mix* = Areca nut + betel leaf/betel stem inflorescence stem + slaked lime.

6.3.3 Principal component analysis

A principal component analysis was performed to analyse the variations in the chemical composition of betel samples, considering all category of compounds analysed above (**Figure 6.5**). PC 1 and PC 2 explained 63.04% of total variance of the samples and PC 1 was mainly contributed by benzenoid, sesquiterpenes, monoterpenes and diterpene on the positive aspect and by alkaloids and esters on the negative aspect, while PC 2 was mainly contributed by alcohols, acids, aldehydes and esters on the positive aspect. WP-SI, and all leaf samples were clearly separated from other samples on the PC 1 aspect, indicating the significant high concentrations of benzenoids, terpenes presented in these samples. While the husk sample from NS was clearly separated from all other samples on the PC 2 aspect, which indicated that alcohols, acids and aldehydes were the key distinguishing metabolites in the husk sample from NS.

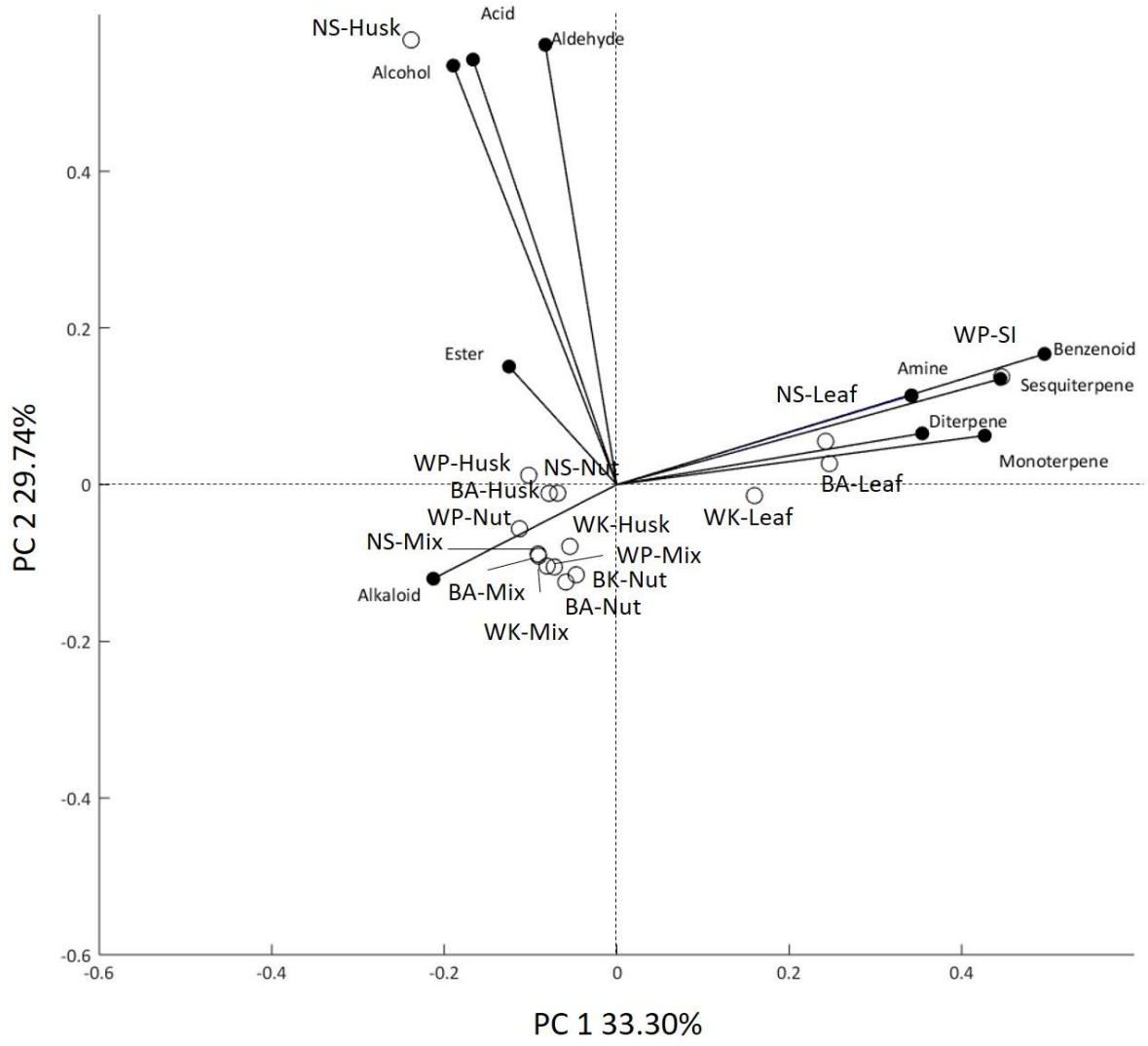


Figure 6. 5 Principal component analysis of chemical composition in betel samples. PC 1 and PC 2 explained 33.30% and 29.74% of total variance, respectively.

BA = Banda Aceh, NS = North Sumatra, WK = West Kalimantan, WP = West Papua.

AN= Areca Nut, SI= stem inflorescence *BQ Mix* = Areca nut + betel leaf/betel stem inflorescence stem + slaked lime.

6.4 Discussion

6.4.1 Alkaloids

It has been confirmed in numerous studies that areca nuts contain four types of alkaloids including arecoline, arecaidine, guvacoline, and arecaidine (**Figure 6.1**)(358-360), and upon human consumption these alkaloids can undergoing nitrosation transformating in the oral cavity into nitrosamines, such as N-nitrosoguvacoline, N-nitrosoguvacine, and 3-methylnitrosaminopropionitrile, which can lead to DNA damage (361-363). In previous research, areca alkaloids were often analysed based on liquid chromatography methods. The HPLC method to analyse all four areca alkaloids was firstly developed by Huang and McLeish (360), who utilized a cation-exchange column and orthophosphoric acid/methanol/water based mobile phase. More recently, reverse phase C18 column was utilized to analyse betel alkaloid in human saliva, however only arecoline could be analysed (364). Importantly, this method used triethylamine based mobile phase, which could interfere mass spectra detection due to adherence to LC system and therefore was only suitable for application with UV detector, but not with mass spectrometer detector. Later, Jantarat et al. 2013 (365) further simplified the method and modified the mobile phase to sodium phosphate buffer-acetonitrile to achieve arecoline quantification on C18 column, nevertheless, this method was not suitable to analyse other alkaloids simultaneously. Further, liquid chromatography was used in conjunction with mass spectrometry to achieve quantification of betel alkaloids and their derivatives in animal model (366). This study continued to use C18 column with formic acid-acetonitrile based mobile phase and time-of-flight ms/ms detector, and was able to detect both arecoline, arecaidine and their derivatives, but not guvacoline and guvacine. The most recent method development on areca nut alkaloids analysis was able to achieve simultaneously quantification of these four alkaloids using UPLC-MS/MS system, where alkaloids were quantified based on targeted m/z ion for each compound (220).

Interestingly, none of the previous study attempted to use gas chromatography to quantify these betel alkaloids, even though there are numerous study use GC-MS to quantify alkaloids in other samples. For example, GC-MS was used to quantify 7 natural alkaloids in the bulb of *Leucojum aestivum* (270) and 13 alkaloids in the leaf of *Leucojum aestivum* (270). The same study was also able to detect 23 and 18 alkaloids from the bulb and leaf of *Narcissus confusus*, respectively. Additionally, the alkaloids content of *Nerium oleander* and *Solanum nigrum* were also characterized using GC-MS (367, 368). The current study tried to adapt the GC-MS parameters used in a previous alkaloid research to analyse the alkaloids in betel quid samples (270) and was able to identify three out of the four betel alkaloids compounds simultaneously without derivatization that LC-MS failed to identify. Even though, all four betel quid have completely separated retention time under the current experimental condition, guvacine could not form a well peak in GC-MS due to high melting/boiling point. Galanthamine trimethylsilyl (TMS) based alkaloid derivatization has been proven efficient in analysing alkaloids compounds with low volatility (270). Therefore, future research may consider converting betel alkaloids, especially guvacine, to TMS derivatives for improved sensitivity and better quantification on GC-MS. This method was not utilized in the current study mainly as we aimed to explore a GC-MS method able to identify betel quid alkaloid quickly, and the derivatization process clearly reduce the efficiency of the analysis especially when handling large quantities of betel samples.

Arecoline was found in all samples at various concentrations, except in WP-SI and WK-Leaf. The different levels of arecoline observed among areca nuts could be due to the maturity of nut, the extraction and quantification methods, storage, and geographical location (220). Arecoline level is usually high in immature areca nut and drops significantly in the mature areca nut (369), which was consistent with our results. WP mixture had the highest concentration of arecoline compared to all samples, even the nut sample from WP. This might

be due to the elevated pH of slaked lime which could damage the arecoline and other alkaloids (177). Interestingly, trace amounts of arecoline were also observed in the betel leaf samples, which has not been reported previously. In contrast, some previous studies reported no alkaloids in betel leaf (217, 247, 189). This could vary among betel production regions due to agronomic parameters as only betel leaf from specific region was found to have arecoline in the current study.

Arecaidine was only detected in NS mixture sample at low concentration, while guvacoline was detected in both NS mixture and BA mixture. Both were not detected in either WP mixture or WK mixture. This could be partially explained by the maturity of areca nut samples. Previous study have demonstrated that arecoline is the main alkaloid in unripe areca nut, while arecaidine and guvacoline mainly present in ripper areca nut (370). Areca nuts are mainly harvested unripen in the WK and WP region, explaining the fact of high arecoline and undetectable arecaidine and guvacoline from the betel samples of those regions. Additionally, the arecaidine detected in NS mixture might also be due to the hydrolysis of arecoline to arecaidine in high pH conditions triggered by the presence of slaked lime (220). A previous study showed that the concentration of arecaidine in Pan masala and Gutka from India ranged from 0.14 to 1.70 mg/g DM, while guvacoline ranged 0.17 to 0.99 mg/g DM, which was consistent with our results (370). The current study further confirmed the presence of arecoline in the areca husk originated from all studied regions, which could indicate that adding husk to BQ might contribute to the higher risk of OSMF and oral cancer development. The habit of adding husk is very common to people living in Taiwan, China, Guam, and Papua New Guinea (155) and as well as the WP region (320).

6.4.2 Metabolomic analysis

6.4.2.1 Benzenoids

Various benzenoids have been detected in the current study, especially safrole, and eugenol, which were most abundant in the SI sample from WP. This was in accordance to previous research on betel SI showing a high concentration of safrole, eugenol, hydroxychavicol, isoeugenol, and eugenol methyl ester (155, 371, 247). Hwang *et al* reported that safrole was the major phenolic compound presented in the betel SI from Taiwan (341). These compounds have various impacts of human consumption with most of them having beneficial effects to human health, such as antioxidant, antimicrobial (372) and anti-inflammatory properties (373). *In vitro* studies also suggested that eugenol may have an anti-carcinogenic effect against breast, liver and fibrosarcoma cancer (372, 374). The antimicrobial and antioxidant property of eugenol are also well known and applied in foods, pharmaceuticals, cosmetics industries and packaging materials (375-377).

In contrast, safrole, which was present at high concentrations in SI, is a possible potential human carcinogen (341, 307). A study using the nuclease P1 version of the ^{32}P-postlabeling technique successfully determined safrole-DNA adduct in the hepatic tissues from patients with hepatocellular carcinoma, and further suggested that betel quid safrole might be involved in the pathogenesis of hepatocellular carcinoma in human beings (353). Chiu-Lan Chen *et al*, suggested that safrole forms stable safrole–DNA adducts in human oral tissue following BQ chewing, which may contribute to oral carcinogenesis (352). Furthermore, cell culture based research suggested that betel SI extract had cytotoxicity effect on cultured gingival keratinocytes by decreasing 20–40% cell numbers over 5 days of incubation (378). This is also consistent to our own study, showing that SI sample from WP to have cytotoxic effect on oral keratinocyte (OKF-6) at 500 μg/ml after incubated for 48 hours, which could be due to the high concentrations of safrole (320). The high concentration of safrole detected in

the WP sample in the current study might also explain the results from our previous epidemiology study, which reported that people living in WP had the highest prevalence number of OSMF associated with BQ chewing(320). Importantly, safrole was not only presented in betel SI but also in areca husk samples, which suggests that adding husk into betel quid may lead to increasing risk of having OSMF and oral cancer.

6.4.2.2 Terpene

Terpenes have been detected in numerous plants and their fruits and these compounds have been demonstrated to have many beneficial effects to human health, such as anti-inflammation, anti-carcinogenesis and neuroprotection effects (379). In our current study, terpenes were mostly detected in betel SI and leaf samples, which was in accordance to previous research showing estragole, linalool, α-copaene, anethole, chavicol, and caryophyllene as the main terpenes presented in betel leaf oil (247). Previous study further confirmed that these terpenes had antibacterial activity against *Mycobacterium smegmatis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Up to date, there are very limited research on betel SI, and our study is the first study to ever profile betel SI using GC-MS. In the current study, even though SI contains the carcinogenic safrole, it also contains high concentrations of terpenes, such as α-copaene, α-gurinene, β-gurinene, β-ylangene, (E)-β-caryophyllene, α-humulene, spathulenol etc. Previous study reported that copaene can increase the antioxidant capacity in human lymphocyte culture and proven to not to cause genotoxic (380). An in vitro study using chemical (DPPH and hydroxyl radicals) and biological (Caco-2 cells) models has been shown that α-humulene has antiproliferative effect (381). D-Limonene, the most detected monoterpene in the present study has been shown to have anti-proliferative and apoptosis-inducing effects (382, 383), and it has been used as a chemo-preventive and chemotherapeutic agent against multiple types of tumors (384, 385). Other components of the

essential oil, such as α-pinene, have been shown to inhibit growth of non-small-cell lung carcinoma cells (386).

6.4.2.3 Others (acid, aldehyde, alcohol, ester)

Ascorbic acid in the betel leaf is also shown to have antioxidant effect (387). The aroma of betel leaf is presented by essential oils, consisting of phenols and terpenes (388, 389). The leaf of *piper betle* produces an aromatic volatile oil containing a phenol called chavicol which has powerful antiseptic properties (388, 389).

6.5 Conclusion

We have successfully established a method using GC-MS to analyse 3 types of alkaloids in BQ components, however, guvacine could not be analysed using this method. This is the first study using GC-MS to profile the volatile compounds in different BQ components.

Arecoline was found in a high concentration in immature areca nut such as areca nut from West Papua and drops significantly in the mature areca nut. The very high concentration of safrole was observed in WP-SI (761.6 mg/g DM). Further, areca husk from the WP region also showed the highest arecoline (66.1 mg/g DM) compared to husk from another regions (with range <6 mg/g DM). Thus, from these finding we conclude that adding immature areca nut, SI and husk into BQ compound could raise the risk of developing OSMF.

WP-SI contains high concentration of benzenoid and sesquiterpense, especially safrole and terpene such as G-cadinene, spatulenol, alpha-copaene, linalool, alpha-gurjunene, gamma-Amorphene. Other detected compounds were mainly fatty acids and esters, which mainly contribute to the fragrance of betel quid.

Table 6. 3 Metabolomic analysis of betel quid using GC-MS (mg per gram of sample as 4-octanol correspondent)

Compound	Chemical	Retention time (min)	Banda Aceh (BA)				North Sumatra (NS)				West Kalimantan (WK)				West Papua (WP)			
			Husk	Leaf	Nut	Mix	Husk	Leaf	Nut	Mix	Husk	Leaf	Nut	Mix	Husk	SI	Nut	Mix
2,3-Butanediol	Alcohol	2.116					5 2.1±59.1		112.5±7.8	4±6.4							9.5±0.9	
L-Lactic Acid	Acid	2.257	29.6±3				595.3±61.7		86.5±16.3								7.5±0.3	
dl-Glyceraldehyde	Aldehyde	2.267	9.8±1				85.5±8.6	1.9±15.8	8.4±0.7		1.6±0.3			0.4±0.1	11.6±0.8	28.4±1.3	6.6±0.7	2.3±0.2
2-Furanmethanol	Alcohol	2.333	2.8±0.3				1.1±0.2								6.3±0.8		5.9±0.5	1.5±0.1
Glycerin	Alcohol	2.71	33.4±1.8	27.7±1.4		1.3±0.1	37.4±3.7	21.7±1.8	6.6±0.8	1.9±0.4	6.6±4.3	7.2±0.7			36.1±1.7	27.6±4.7	9.8±0.6	
D-Limonene	Monoterpene	3.125	2.9±0.7	3±0.7	13.8±3.6		2.2±0.6	2±0.2	14.3±1.3	0.3±01	3±0.3	5.9±1.2	13.7±1.1	0.5±0.2	7.3±0.7	9±1.4	13±1	1±0.1
Benzeneacetaldehyde	Aldehyde	3.238	0.9±0.1	3.5±0.2			0.6±0.1	1.3±0.1				3.1±0.5			3.3±0.7	0.2±0.1	1 .3±0.4	
Linalool	Monoterpene	3.484		7.6±0.3				5.7±0.2				6.1±0.3				2 .6±1		
cis-β-Terpineol	Monoterpene	3.587		1.8±0.1				1.1±0.1								2.3±0.2		
Phenylethyl Alcohol	Alcohol	3.663	1.5±0.2	1.2±0.1			7.7±0.9	3.4±0.1			1.3±0.2				0.7±0.1		0.9±0.1	
Methyl nicotinate	Ester	3.852	0.4±0.2	0.4±0.1	13.2±0.7				6.7±0.4				15.4±4.1		2.6±0.3		32.7±1.8	1±0.1
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Benzenoid	3.88	14.5±1.8	11.3±0.3	7.7±0.7		15.1±2.2	1.2±0.3	6.6±0.6		8.5±1	11.2±0.6	13.7±14.1		46.6±1.8	9.1±0.5	86.5±3.1	3.7±0.4
5-Methoxypyrrolidin-2-one	Amine	4.021	2±0.1	25.6±2.6				34.9±2			1±0.2	6.2±0.2			4±0.3	3±0.2		
1,2-Benzenediol	Benzenoid	4.097	2.8±0.4	2.4±0.1				3.1±0.9	9.2±1.5		2.7±0.4	6.2±1.2				8.1±1.9		
4-Terpinenol	Monoterpene	4.181		3.4±0.2				2.1±0.1								6.2±0.3		
Estragole	Benzenoid	4.266		3.8±0.1				2.7±0.2										
2,3-Dihydrobenzofuran	Benzenoid	4.332	0.7±0.1	16.7±1.3			1.4±0.3	9.9±0.2			0.8±0.1	19.9±1.3					1±0.1	
5-Hydroxymethylfurfural	Benzenoid	4.389	15.1±2.3				0.4±0.1								92.2±11.2		18.3±1.1	0.3±0.1
1,2,3-Propanetriol, monoacetate	Ester	4.52	23.4±2.5	34.8±2.7			54±2.5	1.5±0.3	21.2±1		19.7±1.6	4.7±0.9		2.5±2	7 .6±1.5	5.3±1.1	82.9±32.9	
Phenol, 4-(2-propenyl)-	Benzenoid	4.615	0.8±0.1	271.7±9.8		5.8±0.2	1.1±0.1	275.3±18.4		5.5±0.2		24.5±1.4		1.2±0.1	1.5±0.2	6.8±0.1		

Safrole	Benzenoid	5.04		2.7±0.2				3.2±0.5				2.1±0.4			2.1±0.2	*761.6±25.6	0.7±0.1	13.4±1.6
Decanoic acid, methyl ester	Ester	5.143								0.3±0.1								
2-Methoxy-4-vinylphenol	Benzenoid	5.172	1.6±0.2	6.9±0.4			5.8±0.7	3.8±0.1			2.1±0.2	8.2±1.4			1.4±0.1	1.4±0.1		
Phenol, 4-(2-propenyl)-, acetate	Benzenoid	5.379		147.9±12.9				17.8±0.7				1 .2±0.7				3.8±0.2		
Eugenol	Benzenoid	5.502		212.3±178.2			0.7±0.1	9±0.8			0.7±0.1	4±0.1		0.8±0.1	1.1±0.2	*487.8±18		14.1±1.6
3-Allyl-6-methoxyphenol	Benzenoid	5.634	1.1±0.1	3 9.5±9.9		6.2±0.4	1.4±0.1	252.8±16.2		5.4±0.2	1.4±0.1	356.5±17.2		8.6±1.3	1.7±0.2	321±274.4		
alpha-Copaene	Sesquiterpene	5.794		9.2±0.2				6.8±0.7				7.1±0.5				285.2±1		3±0.2
Methyleugenol	Benzenoid	5.841		2.1±0.1				1.7±0.1				1.6±0.1				89.2±2.8		1.1±0.1
β-Gurjenene	Sesquiterpene	5.879		14±0.6				9.5±0.8				8.7±0.9				169.1±7.2		1.4±0.1
Methyl 5-formyl-1H-pyrrole-3-carboxylate	Benzenoid	5.983				0.8±0.2				1.4±0.4				1.6±0.3				
α-Bergamotene	Sesquiterpene	6.077		8.4±0.1				5±0.5				1.1±0.2						
alpha-gurjunene	Sesquiterpene	6.124														1 4.6±1.5		1.1±0.1
α-Santalene	Sesquiterpene	6.153		3.6±0.1				2.1±0.3										
beta-Ylangene	Sesquiterpene	6.219		6.8±0.3				3.6±0.3				3.2±0.1				23.1±0.2		
(E)-β-Caryophyllene	Sesquiterpene	6.266		36.6±1.3				24.3±2				2 .1±1				146±4		1.2±0.1
cis-Muurola-3,5-diene	Sesquiterpene	6.341		9.3±0.2				5.4±0.4				4.9±0.1				35.1±0.6		
alpha-humulene	Sesquiterpene	6.624		34.1±1.7				24.2±1.9				8.6±0.3				55.7±1.5		
gamma-Amorphene	Sesquiterpene	6.756		65.9±0.7				48.7±3.8		0.8±0.1		19.4±1				71.4±2.3		
g-Cadinene	Sesquiterpene	6.879		15 .5±5		2.3±0.1		67.9±5.1		1.1±0.1		57.8±2.9		0.9±0.1		5 4.7±8		4±0.2
2,4-Di-tert-butylphenol	Benzenoid	6.916	6.7±0.4	8.5±0.4	3.2±0.1		6.4±0.5	8.8±0.2	3.4±0.2	0.7±0.1	6.1±0.1	8.1±0.3	3.8±0.2		6.7±0.4		3±0.1	
3-Allyl-6-methoxyphenyl acetate	Benzenoid	7.058		259.3±9.5				162.6±6.8		0.5±0.1		446.7±15.8		1.5±0.4		*1158.6±31.9	1.1±0.5	
Dodecanoic acid, methyl ester	Ester	7.058		1.3±0.1		15.4±0.5		1.6±0.2		15.7±2.7		2.1±0.1				5.2±0.1		
Isocalamenene	Sesquiterpene	7.284		2.1±0.2				1.6±0.2				1.8±0.1				95±3.2		

Selina-3,7(11)-diene	Sesquiterpene	7.332		6.4±0.1				5.1±0.2									
Dodecanoic acid	Acid	7.407	1.6±0.1	0.7±0.2			0.6±0.1	1.5±0.3	1±0.2		0.8±0.2	1.4±0.2	1.1±0.2		0.7±0.1	1.6±0.2		
w-Amorphene	Sesquiterpene	7.407		0.8±0.2								0.7±0.1				24.3±0.7		
Isovanillic acid	Acid	7.427	2 .4±0.8	0.4±0.1			1 .4±1.6				5.5±0.2				1 .4±0.4			
nerolidol	Sesquiterpene	7.567		1.5±0.2				1.2±0.2				0.8±0.1				16.9±0.2		
1,3,5-Benzenetriol	Benzenoid	7.775			56.2±15				6 .7±4.9				55.9±9.7					
Spathulenol	Sesquiterpene	8.001		2.9±0.2				2.8±0.2				71.2±58.8				287.7±23 8.6		
4-Allyl-1,2-diacetoxybenzene	Benzenoid	8.407	3.3±0.1	42.9±0.9			2.2±0.2				1.6±0.2	372.4±11. 1					3.6±0.2	
unknown sesquiterpene 1 (161, 120, 105, 204)	Sesquiterpene	8.831						2.4±0.1				1.5±0.3				2 .8±0.6		
alpha.-Cadinol	Sesquiterpene	8.963		0.6±0.1				0.5±0.1				0.4±0.3				4.6±0.4		
unknown sesquiterpene 2 (43, 79, 93, 105)	Sesquiterpene	9.029		0.7±0.1				1±0.2				0.4±0.1				1.3±1.1		
unknown sesquiterpene 3 (109, 91, 159, 220)	Sesquiterpene	9.397		1.7±0.3								1.1±0.1				12.9±4		
Tetradecanoic acid, methyl ester.	Ester	9.652				26.7±1.2		0.9±0.1		27.2±4.8				9.8±0.2				
unknown sesquiterpene 4 (43, 91, 123, 220)	Sesquiterpene	9.68														1.6±1.4		
4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	Benzenoid	9.935	6.1±0.3	1.2±0.1			8.6±1.1				1 .5±0.6	0.9±0.1			4.2±0.1	0.9±0.1		
Tetradecanoic acid	Acid	10.076	1.3±0.3	0.7±0.1				1.9±0.2	1.6±0.2		1±0.2	1.8±0.4	2.1±0.2					
Tetradecanoic acid, ethyl ester	Ester	10.661	8.3±0.9				0.9±0.1	2.8±0.3							2.8±0.2			
Syringic acid	Benzenoid	10.794	4.1±0.4				2.1±0.2				1.5±0.1				2.4±0.2			
Pentadecanoic acid	Acid	11.632	0.6±0.1				1.3±0.3				1±0.1							
Pentadecanoic acid, ethyl ester	Ester	12.236	2.4±0.2				1.7±0.2								2.2±0.2			

Hexadecanoic acid, methyl ester	Ester	12.773	0.9±0.1	1.7±0.1		17.9±0.6	0.8±0.1	6.3±0.6		16.4±2.9		3.5±0.3		6.4±0.1	1±0.2	3.1±0.1		0.8±0.2
Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate	Benzenoid	12.896	2±0.1		0.8±0.2		1.8±0.2	2.2±0.3	0.8±0.1				0.9±0.1			2.1±0.1	0.7±0.1	
n-Hexadecanoic acid	Acid	13.282	8.7±0.4	12.4±1.9			13.7±1.6	22.7±3.5	1.6±0.1		12.8±1.1	15.5±1.1	2.4±0.1		8.3±0.7	14.7±0.6	1.3±0.4	
Hexadecanoic acid, ethyl ester	Ester	13.896	39.5±3.8	3±0.1			22.3±2.4	12.1±0.8			2±1.1	1.1±0.1			89.5±4.4	2.1±0.1		
Heptadecanoic acid, methyl ester	Ester	14.443																
Heptadecanoic acid	Acid	14.952					0.5±0.2											
Heptadecanoic acid, ethyl ester	Ester	15.574	1.4±0.1				1.1±0.2								3.2±0.1			
8,11-Octadecadienoic acid, methyl ester	Ester	15.6		0.4±0.1		8.6±0.3		1.7±0.1		8.5±1.6		0.5±0.1			1±0.2			0.6±0.1
9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	Ester	15.678		1±0.1		2±0.1	0.5±0.1	3.2±0.4		1.7±0.4		1.6±0.2				0.6±0.1		
9-Octadecenoic acid, methyl ester, (E)-	Ester	15.716		0.7±0.1		9.5±0.1	0.5±0.1	2.5±0.3		9.1±1.5		1.2±0.2	0.8±0.2	3.9±0.1	0.5±0.1	1.4±0.1		0.3±0.1
Phytol	Diterpene	15.848				0.7±0.1	0.7±0.1	82.4±5.1		0.7±0.1		35.2±2.6			2.8±0.3	8.1±0.3		
Methyl stearate	Ester	16.121				2.8±0.2		2.5±0.2		2.8±0.5		1.1±0.1						
9,12-Octadecadienoic acid (Z,Z)-	Acid	16.168	3.7±0.1	3.7±0.7			7.1±1	7.8±2.1			3.7±0.5	3.1±0.5			7.2±0.2	9±0.2		
9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	Acid	16.282	3.8±0.3	23.7±4			7.5±0.7	19.2±5.9			3.2±0.5	23.5±1.7			7.1±0.3	6.8±0.7		
Octadecanoic acid	Acid	16.659	0.4±0.4				0.3±0.1				0.2±0.1							
Linoleic acid ethyl ester	Ester	16.697	13.4±1.3	1.7±0.1			8.5±1	5.5±0.6			0.9±0.5				41.7±2.6	2.3±0.1		
Ethyl Oleate	Ester	16.8	15.3±1.3				9.8±1	4.3±0.6			0.6±0.3	1±0.2			28.2±1.5	2.1±0.4		
Octadecanoic acid, ethyl ester	Ester	17.254	5.7±0.5	1.4±0.1			3.3±0.4	5.4±0.4							6.8±0.3	0.5±0.1		

Phytol, acetate	Ester	17.564	0.6±0.1	5.8±0.1				17.6±0.9				2.5±0.1						
Tetradecanoic acid, 2,3-dihydroxypropyl ester	Ester	19.027				1.9±0.2				2.1±0.5								
Tetradecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Ester	19.177								0.3±0.1								
Eicosanoic acid, methyl ester	Ester	19.442								0.3±0.1								
9-Octadecenamide, (Z)-	Amine	19.941	4.8±2.6	6.1±0.6		0.4±0.1	4.8±0.3	8.6±0.5			4.6±0.3	8±1	0.9±0.2	0.3±0.1	5.6±1.2	4.4±0.6		
Eicosyl acetate	Ester	20.733		0.8±0.1				3±0.2				0.5±0.1						
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Ester	22.299	1.2±0.1	4.8±0.3		0.7±0.1	0.5±0.1	5.1±0.2		0.7±0.1	0.9±0.1	6.2±0.3			1.9±0.3	1.8±3.1		
Glyceryl monolinoleate	Ester	24.94		2±0.2		0.9±0.1		2.4±0.4		0.9±0.2		1.8±0.2			1.2±0.2	3.2±0.2		
β-Glyceryl monostearate	Ester	25.015		2.5±0.2		1.9±0.1		5.2±1		2±0.5		5.9±0.3						

Chapter 7: Cytotoxic Effects of Indonesian Betel Quid Components on Oral Keratinocytes and Fibroblasts

Abstract

Betel quid (BQ) chewing habit has been strongly linked to the onset of oral leukoplakia, oral submucous fibrosis and oral cancer. To explore the pathogenicity of individual components of BQ mixtures, we investigated the effects of areca nut (AN), leaf of *piper betle* (Leaf) or stem inflorescence of *piper betle* (SI), areca husk (Husk) and whole BQ Mixture, on the growth of oral keratinocytes (OKF-6) and oral primary fibroblasts (MMF-1). On the basis of their chemical characteristics, BQ from Banda Aceh (BA) and West Papua (WP) regions were selected for *in vitro* testing.

Methods: Fluorescein diacetate assay (FDA) was used as a cell cytotoxicity test of BQ components on OKF-6 and MMF-1 cells.

Results: The current study found that WP-AN exhibited cytotoxic effect on OKF-6 at a concentration of 100 μg/ml, as measured by a 50% reduction of cell viability (IC50), while BA-AN exhibited cytotoxic effect at a higher concentration (500 μg/ml) after 2 days of incubation. WP-SI also showed cytotoxic effect at 500 μg/ml after 2 days incubation. In contrast, the extract of Leaf, BQ Mixture and Husk did not exhibit cytotoxic effect for up to 3 days of incubation. The exposure of BQ components to MMF-1 showed no cytotoxicity at any concentration assessed. Incubation of MMF-1 with BA-AN and WP-AN led to an increase of cell proliferation (1 day) followed by a decline of cell growth (2 days) in a dose and time dependent manner.

Conclusion: The results of our study showed distinctive cytotoxic profiles in stromal and epithelial cells of the oral cavity induced by BQ components.

Key words: cell cytotoxic, oral keratinocytes, oral fibroblast, cell proliferation

7.1 Introduction

Several epidemiological and case control studies in the Indian subcontinent, South-East Asia and South Pacific islands have reported a strong correlation of betel quid (BQ) chewing habit with oral cancer and OPMDs (157, 252, 249, 305, 142, 251). In particular, BQ chewing is associated with an increased risk of oral submucous fibrosis (OSMF) (252, 251). The pathophysiology of these BQ-induced mucosal changes is not well understood, and the data on potential BQ toxicity are heterogenous due to differences in methods of BQ extraction as well as the cytotoxicity assays utilised (252, 250).

The ingredients of BQ differ in different countries, but the major components being used are areca nut (AN), betel leaf or betel stem inflorescence (SI) and slaked lime. Tobacco and other spices are optional and reflect local chewing practices (154-156). Approximately 600 million individuals have been estimated to be AN chewers worldwide (154, 155).

Our phytochemistry study (Chapter 5) on Indonesian BQ components from four different regions has successfully profiled the antioxidant properties and highlighted potentially detrimental effects in all BQ components tested. In the present study, cytotoxicity assays were used to investigate the pathobiological effect of BQ components on oral mucosal cells. We selected BQ components from BA and WP regions as these had the lowest and the highest level of antioxidants, polyphenols, and arecoline, respectively. Specifically, we aimed to investigate whether the pathobiological effect of BQ on oral mucosal cells correlated with its phytochemical profile.

AN is the main component of BQ and is considered to be the major aetiologic factor of BQ-related oral cancer (249, 250). AN extract (ANE) contains mainly tannin and areca alkaloids, such as arecoline, arecaidine, guvacine, and guvacoline, which are cytotoxic but can also work as potential carcinogens. These substances are released from BQ components during the period of chewing BQ and with time can dramatically alter the normal oral mucosa and

induce pathological changes typical of OSMF (251). Previous studies have reported that ANE, arecoline and BQ extracts were cytotoxic to cultured oral keratinocytes and fibroblasts (255-257, 253, 254, 251), and this is partially in contrast with clinical data that imply a pro-carcinogenic effect of BQ mixtures. Surprisingly, the potential cytotoxic effects of individual BQ components has not been investigated so far. A detailed understanding of the effects of single BQ ingredients could well be important to inform targeted preventive and chemo-preventive strategies to reduce the incidence of OSMF and BQ-related oral cancer. Hence, in the present study we aimed to assess the cytotoxic effects of various Indonesian BQs to mimic the pathobiological changes of oral keratinocytes (OKF-6) and primary oral fibroblasts (MMF-1) *in vitro*.

7.2. Material and Methods

The assay was carried out according to the procedure reported by Gonzalez-Reyes et al with some modifications (272, 273). 96-well Costar® microplates (Sigma-Aldrich, Australia #CLS3603-48EA) were used and plated with 100 µl of $2x10^4$ cells/ml of OKF-6 or $1.35x10^4$ cells/ml of MMF1 cell suspension. Cell viability of seeded cells was at least 90% when determined with trypan blue stain. A humidified incubator (Thermo Scientific, Forma direct heat, CO_2 incubator) was used to incubate cells and this was performed at 37 °C in 5% CO2. After 24 hours of seeding, plates were removed from incubator and washed with 100 µl/well PBS and the cells treated with 100 µl/well BQ concentrations as previously prepared (section 2.6.4 and 2.6.5). Three wells/plate were used as controls. Control wells contained cells that were incubated with 100 µl culture medium containing Dublecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, supplemented with 10% (v/v) FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Blank wells were placed in empty wells (without cells), with 100 µl culture medium only. After treating cells with various BQ concentrations, the cells were incubated in a humidified at 37 °C/5% CO2 incubator and incubated for 1 day. After 1 day, control media or test compounds were removed, the cells were rinsed with 100 µl PBS and subsequently 100 µl of FDA solution prepared in serum free medium was added to each well. The plates were incubated at 37 °C/5% CO2 for 40 minutes, then FDA fluorescence was measured by spectrometry at wavelength of 485/20 nm and 528/20 nm, respective excitation and emission, in Synergy HTX Multi-Mode Reader (Bio-Tek, USA). The FDA stained cells were viewed by an inverted microscope (Floid cell imaging station, Life Technologies) with fluorescence/green or white light as required to see whether the cells were correctly stained. The steps were repeated after treated with BQ for 2 days and 3 days of incubation. To validate the result of spectrometry readings in Synergy HTX Multi-Mode Reader, one of BQ cytotoxic concentration (IC50) was selected with its time point (1 time point over 3 days incubation).

Cells were seeded in a 6-well plate in triplicates for 24-hour incubation to attach and after 24-hour, cells were treated with BQ at cytotoxic concentration (IC50) according to previous spectrometry finding. After exposing cell to BQ components at found IC50 and incubated for IC50's time point of incubation (whether the IC50 happen on day 1, day 2, or day 3 of incubation), cells were counted with automated cells counter (Bio-Rad, Australia) and stained previously with trypan blue to count viable cells. And if the results of IC50 consistently similar to spectrometry reading, then the IC50 read by spectrometry reading is correct. The detailed material and methods for cytotoxicity assay were reported in section 2.6.

7.3 Results

With few exceptions, exposure of oral mucosal cells to BQ components from BA and WP regions for over 3 days influenced cell growth in a dose and time-dependent manner (Table 7.1 and 7.2). Notably, all BQ components significantly reduced cell viability, albeit to a variable extent.

7.3.1 Cell viability effects of BQ extracts on OKF-6

BA-AN and WP-ANE both inhibited OKF-6 growth in a dose and time-dependent fashion, with the exception of 100 μg/ml at 2 and 3 days. The IC50 of WP-ANE for OKF-6 was 100 μg/ml (2 days), while ANE from BA region exhibited cytotoxicity at 500 μg/ml (2 days).

BA-BQ Mixture and WP-BQ Mixture (1, 15, and 30 mg/ml) induced mild cytotoxicity during 3 days of incubation. In pilot experiments, BA-BQ Mixture and WP-BQ Mixture (100, 250, 500, and 1000 μg/ml) induced cell proliferation, hence we raised the concentration to 1,15, and 30 mg/ml so as to clearly observe the cytotoxic effect of BQ Mixture on OKF-6.

The cytotoxic effect of WP-SI was significant at 500 μg/ml (2 days), whereas BA-Leaf stimulated OKF-6 growth at all concentration used (100, 250, and 500 μg/ml).

The husk extract from both regions did not exhibit a significant reduction of cell number/viability on OKF-6 at the concentration assessed (1, 22.5, and 45 mg/ml) for up to 3 days. The IC50 of arecoline was reached at 100 μg/ml after 2 days incubation. The reduction of cell viability induced by arecoline was dose and time dependent, as shown in **Table 7.1** and **Figure 7.1.**

Table 7. 1 Cell viability of OKF-6 exposed by BQ component extracts.

BQ Components	Concentration	1 day Cell Viability (%) ± SD	2 days Cell Viability (%) ± SD	3 days Cell Viability (%) ± SD	P-Value
Control	**0**	100	100	100	
BA-AN	**100 µg/ml**	86.6 ± 3.0	68.8 ± 4.2	75.6 ± 7.8	<0.05
	250 µg/ml	77.1 ± 3	53.8 ± 2.0	47.6 ± 4.4	
	500 µg/ml	59.9 ± 3.4	*32.8 ± 1.4	21.8 ± 1.0	
WP-AN	**100 µg/ml**	77.5 ± 5.8	*44.6 ± 4.7	51.1 ± 2.3	<0.05
	250 µg/ml	80.8 ± 3.6	43.3 ± 0.7	31.48 ± 2.1	
	500 µg/ml	58 ± 4.6	24.4 ± 2.1	16.86 ± 0.5	
BA-BQ Mixture	**1mg/ml**	83.5 ± 6.9	95.0 ± 3.1	95.3 ± 7.7	<0.05
	15 mg/ml	86.8 ± 4.6	92.8 ± 4	86.0 ± 8.4	
	30 mg/ml	88.5 ± 1.84	89.6 ± 2.8	89.8 ±3.1	
WP-BQ Mixture	**1 mg/ml**	97.2 ± 4.3	84.4 ± 3	81.3 ± 6.7	<0.05
	15 mg/ml	100.2 ± 2.5	90.3 ± 4.2	82.3 ± 5.2	
	30 mg/ml	91.8 ± 3	83.4 ± 5.0	77.5 ± 1.8	
BA-Leaf	**100 µg/ml**	93.6 ± 8.4	94.7 ± 5.7	101.2 ± 4.3	<0.05
	250 µg/ml	92.3 ± 2.6	88.6 ± 2.8	96.2 ± 4	
	500 µg/ml	91.9 ± 6.3	80.7 ± 4.6	101.1 ± 6.3	
WP-SI	**100 µg/ml**	86.4 ± 5.3	88.33 ± 10.1	90.3 ± 2.1	<0.05
	250 µg/ml	89.8 ± 3.4	70.7 ± 6.9	85.2 ± 4.1	
	500 µg/ml	65.3 ± 1.4	*30.3 ± 0.7	18.1 ± 1.3	
BA-Husk	**1 mg/ml**	93.4 ± 5.8	90.7 ± 2.3	102.2 ± 1.1	<0.05
	22.5mg/ml	89.8 ± 5.0	91.9 ± 7.6	101.1 ± 4.6	
	45 mg/ml	96.9 ± 2.7	86.4 ± 1.2	95.2 ± 5.8	
WP-Husk	**1 mg/ml**	89.1 ± 6.8	78.3 ± 4.7	86.2 ± 3.6	<0.05
	22.5 mg/ml	90 ± 4.9	74.8 ± 10.6	84 ± 3.4	
	45 mg/ml	92.5 ± 2.7	80.2 ± 5	78.8 ± 5.2	
Arecoline	**1 µg/ml**	100.5 ± 7.9	97.4 ± 0.4	80.8 ± 18.2	<0.05
	10 µg/ml	93.9 ± 11.8	51.63 ± 1	48.8 ± 1.5	
	100 µg/ml	80.6 ± 8.4	*39.7 ± 5.5	33.2 ± 1.7	

Tested using Tukey comparison to see significant differences between different concentration and control in each day of incubation.
The significantly different with control value marked with different colours.

Cytotoxic mean values (IC50) at the earliest time and lowest concentration on OKF-6
Significantly different mean values (number of OKF-6) compared to control value
Not significantly different mean values (number of OKF-6) compared to control value

7.3.2 Cell viability effects of BQ extracts on MMF-1

None of the BQ components from any region exhibited cytotoxicity to MMF-1 at the concentration used for up to 3 days and, importantly, arecoline failed to induce cytotoxic effects at all concentration used (1, 10, and 100 μg/ml) for up to 3 days of incubation (**Table 7.2**). Notably, all BQ components induced proliferation of oral primary fibroblasts as shown by the increased cell viability compared to untreated controls. This cell proliferation only occurred in the first 1 day of incubation, and the number of viable cells declined consistently thereafter (**Table 7.2 and Figure 7.2**).

Table 7. 2 Cell viability of MMF-1 treated with BQ component extracts

Legend should briefly explain the table

BQ Components	Concentration	1 day Cell Viability (%) ± SD	2 days Cell Viability (%) ± SD	3 days Cell Viability (%) ± SD	P-Value
Control	**0**	100	100	100	
BA-AN	**100 µg/ml**	123.4 ± 2.8	102.8 ± 7.7	115.2 ± 5.1	<0.05
	250 µg/ml	113.8 ± 4.1	110.9 ± 0.8	110.3 ± 5.6	
	500 µg/ml	116.6 ± 5.7	110.9 ± 4.6	108.9 ± 2.5	
WP-AN	**100 µg/ml**	119.0 ± 1.0	105.7 ± 3.3	94.8 ±3.7	<0.05
	250 µg/ml	120.0 ± 1.1	96.7 ± 13.9	102.4 ± 2.4	
	500 µg/ml	120.4 ±0.2	104.4 ± 2.6	96 ± 3.3	
BA-BQ Mixture	**1 mg/ml**	105.2 ± 5.2	100.7 ± 5.6	104.2 ± 3.9	<0.05
	15 mg/ml	114.5 ± 1.3	98.4 ± 4.5	92 ± 3	
	30 mg/ml	111.2 ± 0.9	97.4 ± 6.1	93.4± 3.0	
WP-BQ Mixture	**1 mg/ml**	112.6 ± 2.7	99.1 ± 2.5	95.8 ± 6.6	<0.05
	15 mg/ml	111.2 ± 0.5	88.4 ± 5.2	86.7 ± 0.7	
	30 mg/ml	103 ± 2.6	85.5 ± 1	82.2 ± 2.9	
BA-Leaf	**100 µg/ml**	114.4 ± 3.9	109.2 ±3.7	103.5 ± 6.6	<0.05
	250 µg/ml	122.4 ± 8.8	116.8 ± 1.6	111.9 ± 5.2	
	500 µg/ml	123.7 ± 6.5	117.7 ± 1.2	121 ± 3.7	
WP-SI	**100 µg/ml**	114.4 ± 4.2	107.8 ± 4.9	110.9 ± 2.6	<0.05
	250 µg/ml	117.4 ± 3.3	111.6 ± 0.8	119.7 ± 2.9	
	500 µg/ml	118.5 ± 2.1	116.2 ± 2.6	120.8 ± 3.5	
BA-Husk	**1 mg/ml**	118.9 ± 4.9	118.1 ± 1.2	119.8 ± 4.8	<0.05
	22.5 mg/ml	138.7 ± 2.3	125.2 ± 6.4	122.8 ± 5.7	
	45 mg/ml	135.0 ± 2.7	127.6 ± 7.8	127.3 ± 6.5	
WP-Husk	**1 mg/ml**	118.2 ± 3.4	118.8 ± 2.6	125.4 ± 0.9	<0.05
	22.5 mg/ml	128.5 ± 0.9	123.3 ± 2.4	125.9 ± 0.6	
	45 mg/ml	128.1 ± 9.2	130.3 ± 2.1	129.5 ± 2.6	
Arecoline	**1 µg/ml**	109.8 ± 2.5	116.6 ± 6.4	105.8 ± 5.7	<0.05
	10 µg/ml	107.4 ± 4.2	106.5 ± 5.1	106.9 ± 5.3	
	100 µg/ml	105.9 ± 2.2	115.5 ± 3.8	97.3 ± 5.9	

Tested using Tukey comparison to see significant differences between different concentration and control in each day of incubation.
The significantly different with control value marked with different colours

Significantly higher number of cell proliferation
Significantly lower number of cell proliferation
No significant different

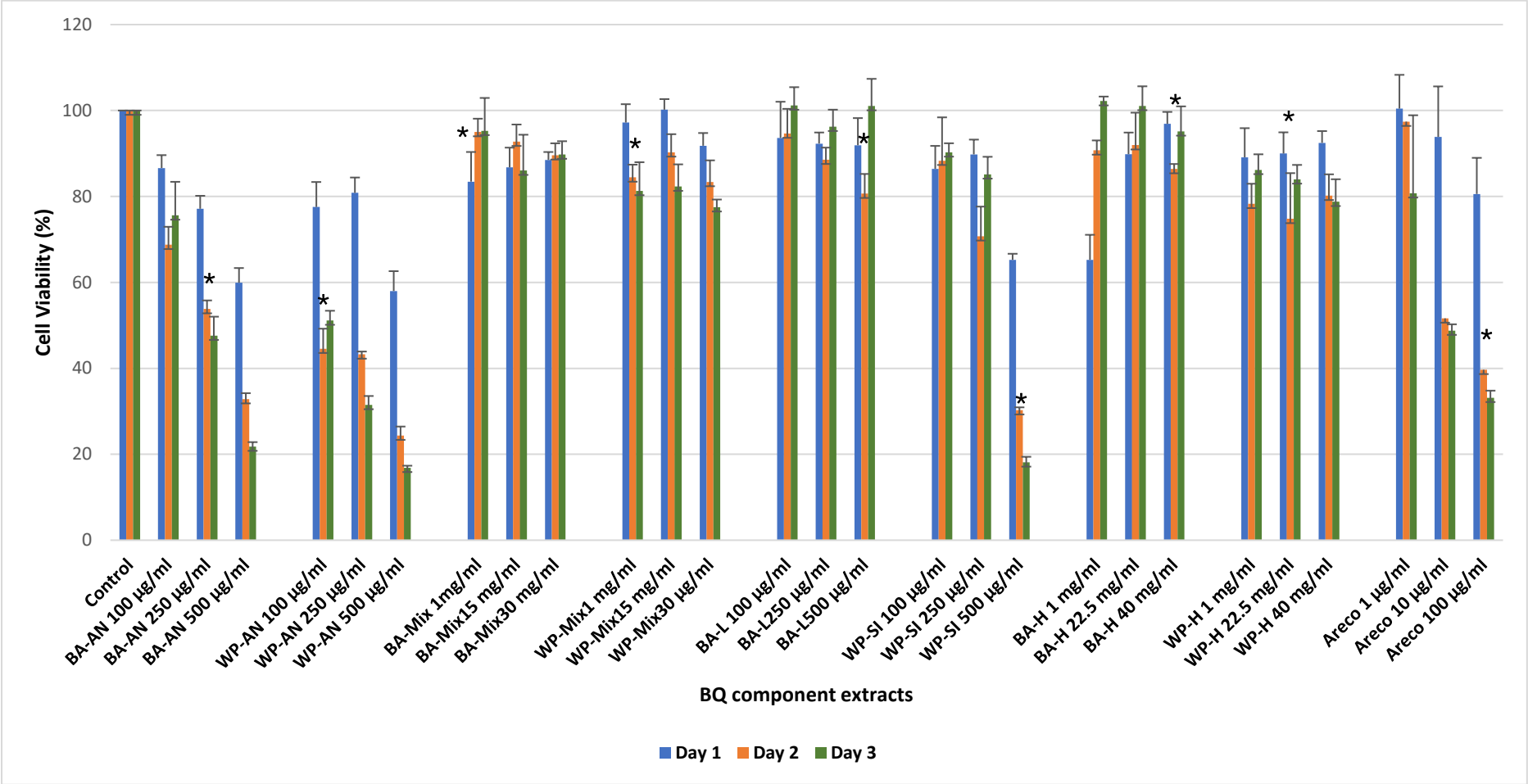


Figure 7. 1 Cell viability of BQ components extracts from BA and WP regions, and Arecoline on OKF6

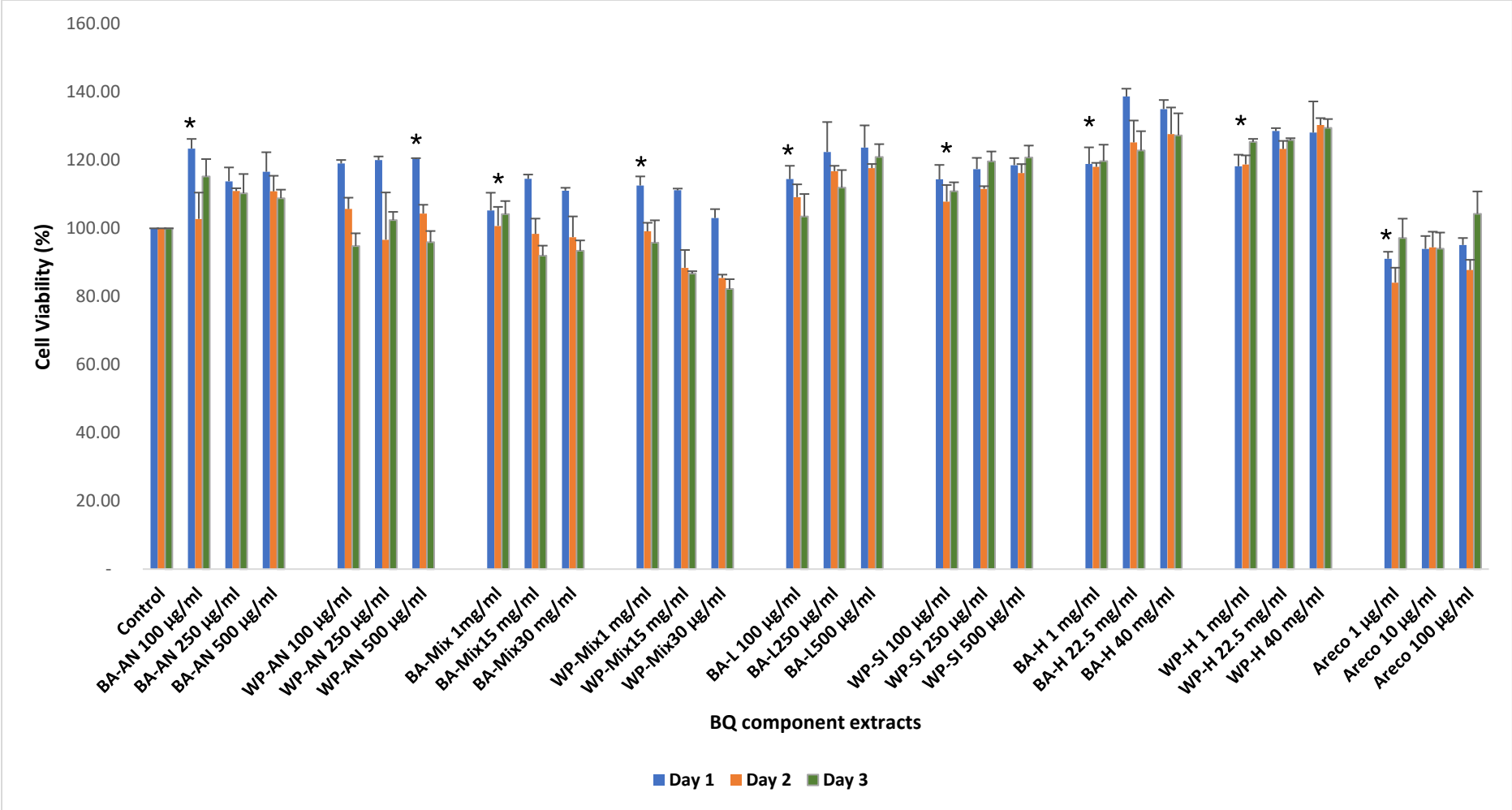


Figure 7. 2 Cell viability of BQ components extracts from BA and WP regions, and Arecoline on MMF-1

7.3.3 Morphological alteration of OKF-6 following exposure to BQ components from BA and WP regions

Morphological alterations in cultured OKF-6 following exposure to BQ components were observed. Untreated normal OKF-6 showed ovoid, rounded or polygonal appearances with well-defined intercellular spaces (**Figure 7.3A**). Exposure of OKF-6 to 500 μg/ml ANE from BA for 1 day induced loss of intercellular space and cell shrinkage (**Figure 7.3B**). Exposure of OKF-6 to 500 μg/ml of ANE from WP for 1 day showed substantial loss of intercellular spaces, cells were rounded and shrunk, the architecture of nuclei and nucleoli was not clearly demarcated, and some debris and dead cells were present, also intercellular vacuolization is evident (**Figure 7.3C**). Thus, both WP-AN and BA-AN caused morphological change consistent with cellular degeneration and death (**Figure 7.3**)

Cells exposed to BA-BQ Mixture extract (30 mg/ml) for 1 day showed polygonal appearance, intercellular spaces were sharply well-defined (**Figure 7.4A**). Exposure of OKF-6 to 30 mg/ml of BQ Mixture extract from WP over 1 day of incubation showed well-defined intercellular spaces, no loss intercellular spaces, cells were polygonal (**Figure 7.4B).** Keratinocytes exposed to 500 μg/ml of BA-Leaf extract for 1 day displayed both polygonal and elongated appearance, some dead cells were also present (**Figure 7.4C**). Exposure to 500 μg/ml of SI extract for 1 day induced loss of intercellular spaces, architectural changes including fibrastoid and shell-like appearance, and dead cells (**Figure 7.4D**). Exposure to BA-Husk extract (45 mg/ml) showing polygonal and rounded cell appearance, intercellular spaces were intact, some dead cells also present, and cell's architecture changed (**Figure 7.4E**). Exposure of OKF-6 to 45 mg/ml of WP-Husk extract for 1 day showed well-defined enlargement of intercellular spaces. Cytologically, oral keratinocytes were polygonal with evidence of shell-like appearance, vacuolization was evident (**Figure 7.4F**).

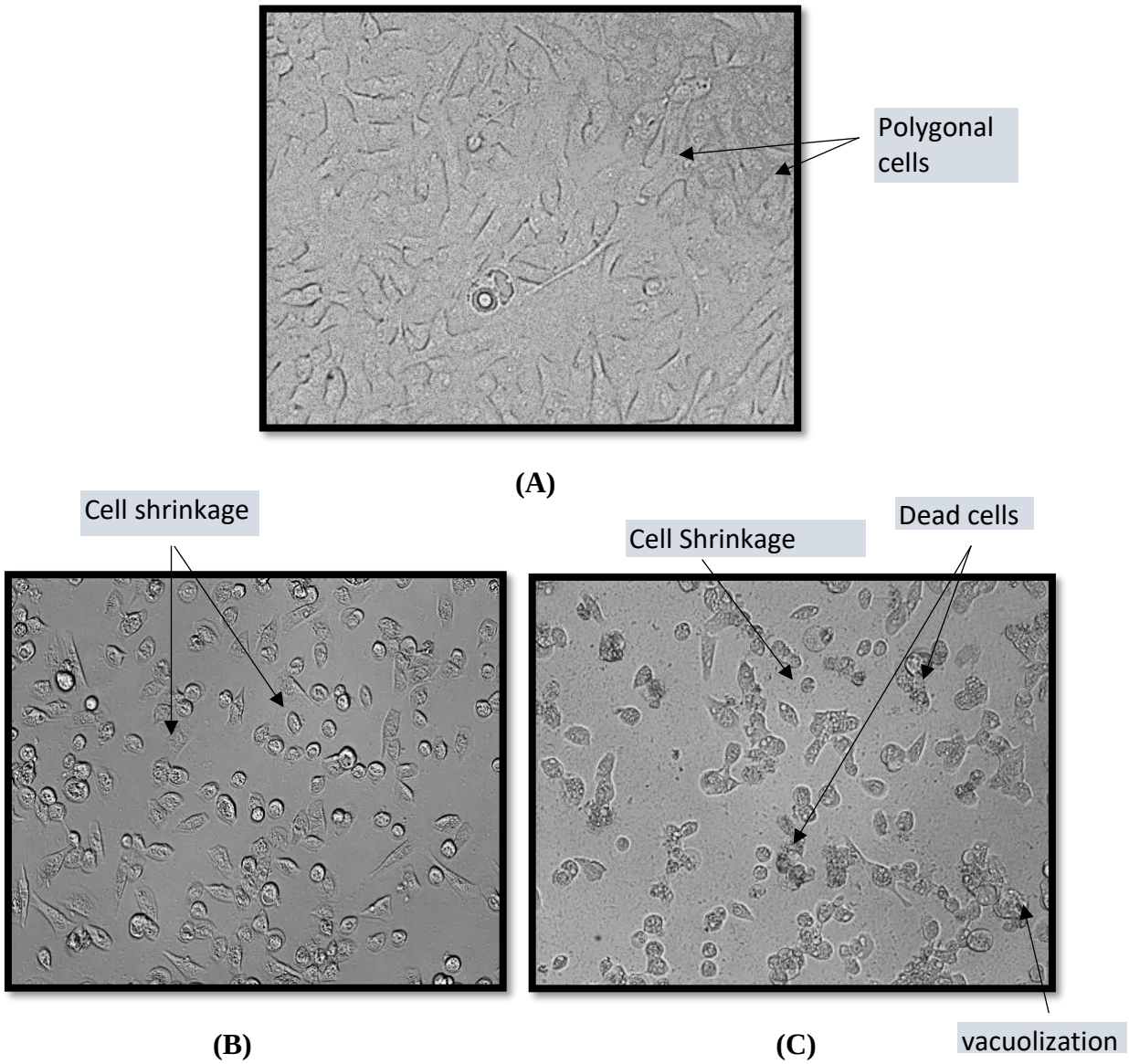


Figure 7. 3 OKF-6 morphology after 1-day growth in DMEM culture medium (Figure 7.3A), with addition of 500 µg/ml ANE from BA (Figure 7.3B) or WP (Figure 7.3C)

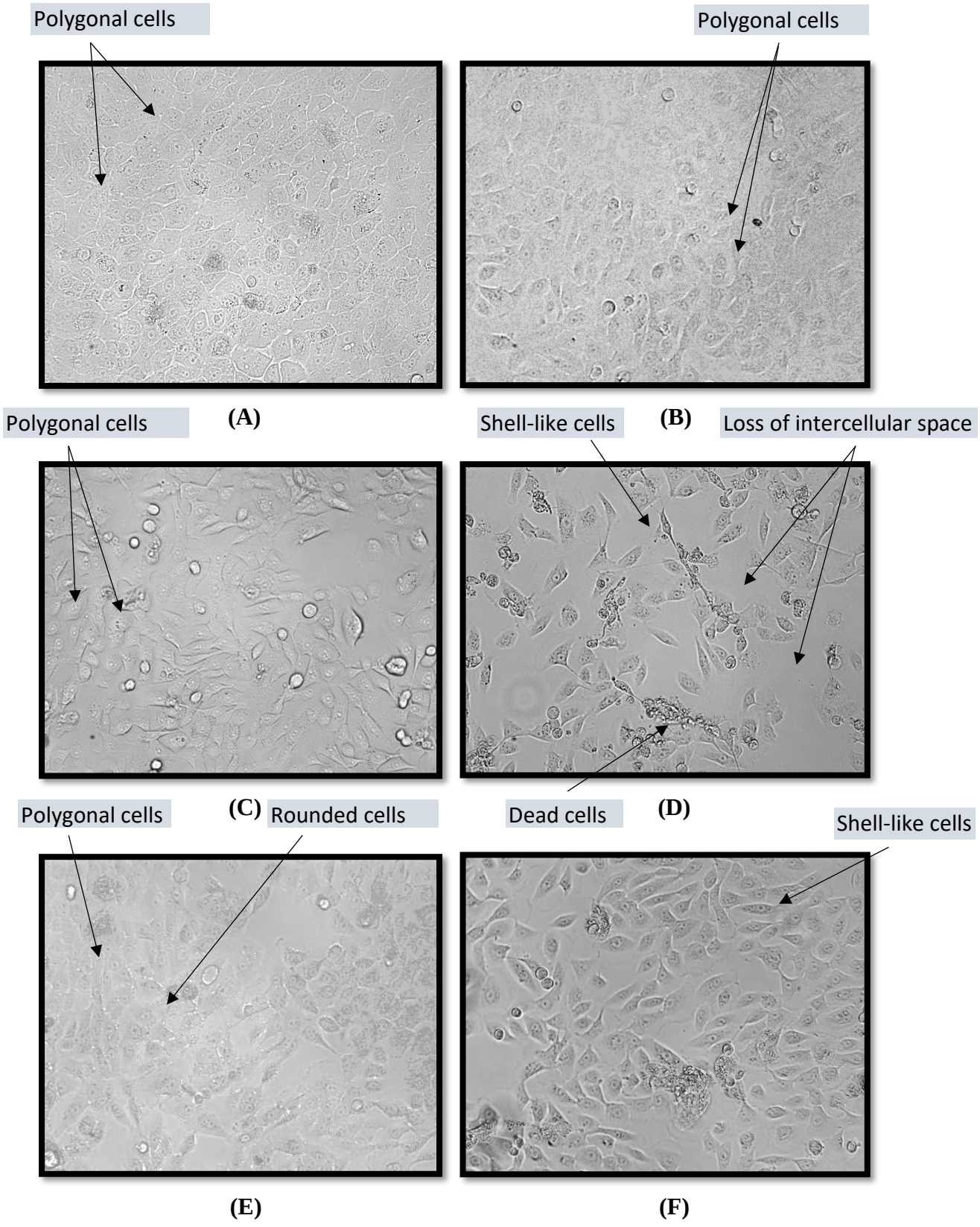


Figure 7. 4 Comparative morphological alteration of OKF-6 after 1-day incubation with DMEM culture media. 30mg/ml BQ Mixture from BA (Figure 7.4A) or WP (Figure 7.4B): 500 µg/ml of leaf extract from BA (Figure 7.4C) or WP (Figure 7.4D): 45mg/ml of husk extract from BA (Figure 7.4E) or WP (Figure 7.4F)

7.3.4 Morphological alteration of MMF-1 following exposure to BQ components from BA and WP regions

Morphological alteration of oral primary fibroblast exposed by BQ components was evident and various changes of cell characteristic were observed. The normal untreated MMF-1 (control) showed spindle shape with well-defined intercellular spaces (**Figure 7.5A**). MMF-1 was exposed to 500 μg/ml of ANE from BA for 1 day and showed stellate form, and unclear intercellular spaces (**Figure 7.5B**). The morphological change of MMF-1 exposed to 500 μg/ml of WP-ANE for 1 day showed stellate form with debris and dead cells were present, less of intercellular spaces observed (**Figure 7.5C**).

Exposure of MMF-1 to 30 mg/ml of BA-BQ Mixture for 1 day showed cell architectural changes (**Figure 7.6A**). Exposure of MMF-1 to 30 mg/ml of WP-BQ Mixture for 1 day showed stellate cells form and intercellular spaces (**Figure 7.6B**). Exposure of MMF-1 to 500 μg/ml BA-Leaf extract for 1 day showed epithelioid formation (**Figure 7.6C**). Exposure of MMF-1 to 500 μg/ml SI extract for 1 day showed epithelioid cell form, no intercellular spaces loss observed (**Figure 7.6D**). Exposure of MMF-1 to 45 mg/ml of husk extract from BA over 3 days of incubation showed epithelioid form with well-defined intercellular spaces (**Figure 7.6E**). Exposure of MMF-1 to 45 mg/ml of husk extract from WP showed cell architecture change into epithelioid form and no loss of intercellular spaces (**Figure 7.6F**)

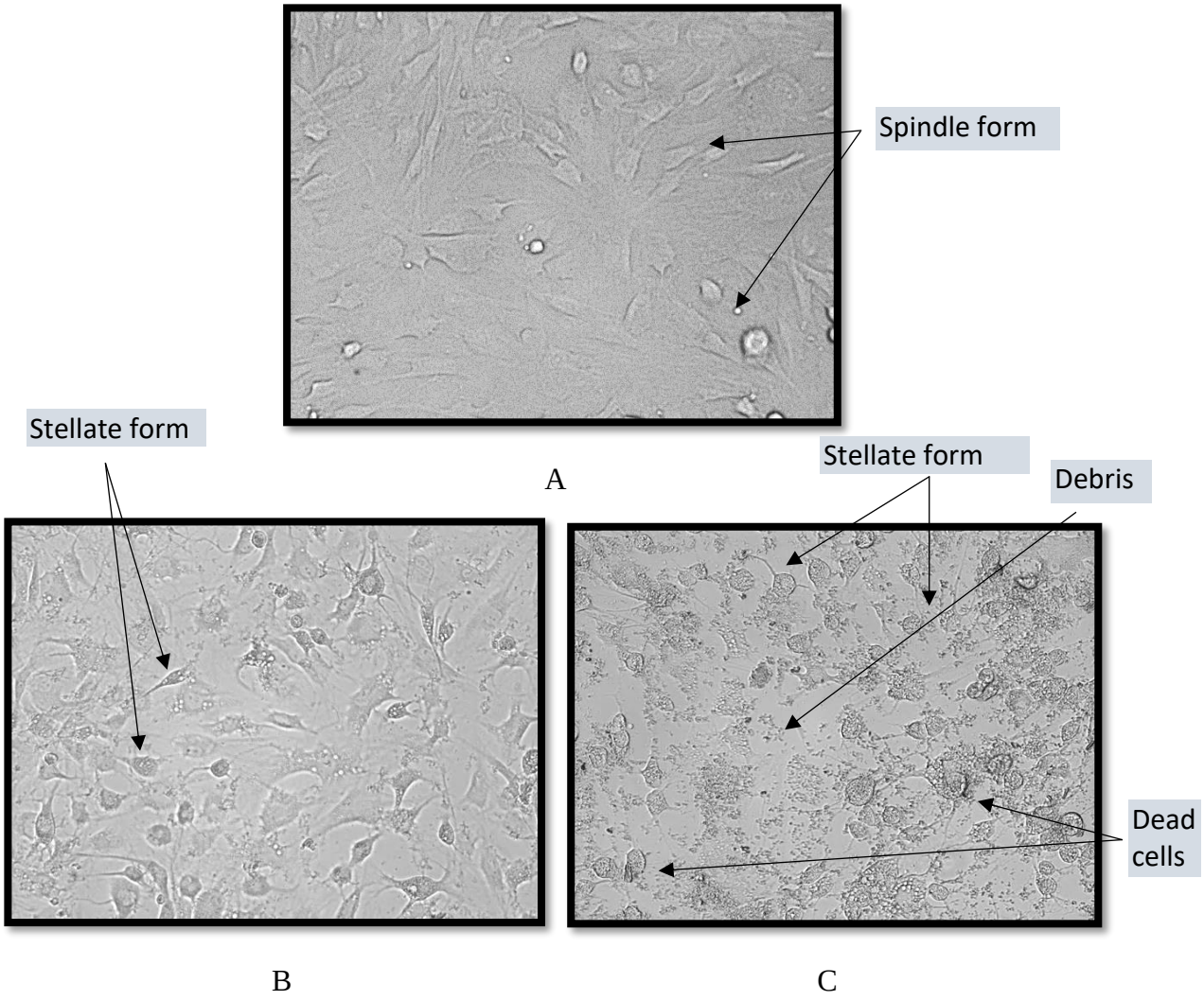


Figure 7. 5 MMF-1 morphology after 1-day growth in DMEM culture medium (Figure 7.5A), with addition of 500 µg/ml ANE from BA (Figure 7.5B) or WP (Figure 7.5C)

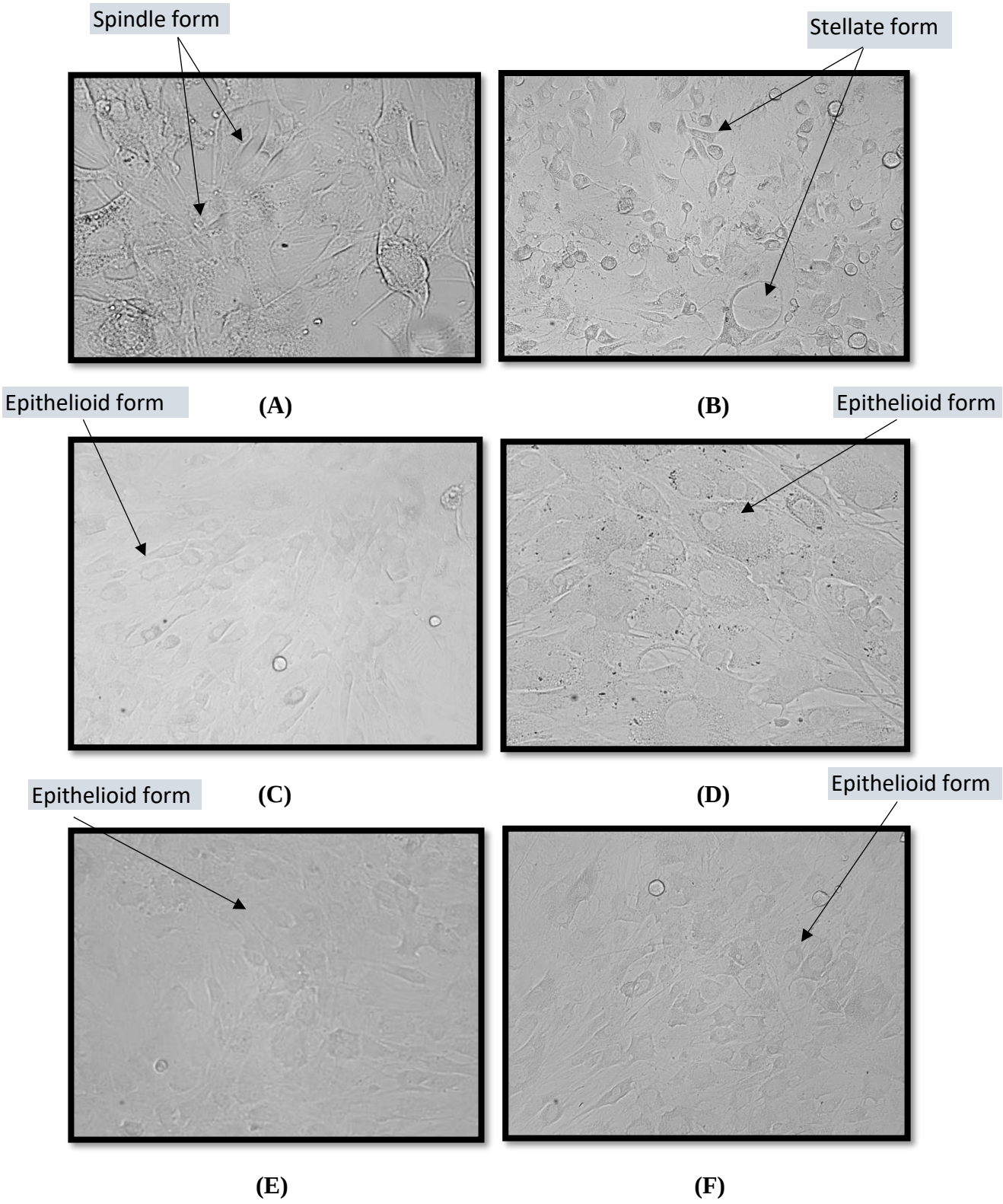


Figure 7. 6 Comparative morphological alteration of MMF-1 after 1-day incubation with DMEM culture media. 30mg/ml BQ Mixture from BA (Figure 7.6A) or WP (Figure 7.6B): 500 µg/ml of leaf extract from BA (Figure 7.6C) or WP (Figure 7.6D): 45mg/ml of husk extract from BA (Figure 7.6E) or WP (Figure 7.6F)

7.4. Discussion

In the present study, we show that BQ ingredients not only induce distinct functional and morphological effects in oral mucosal cells but, also, that these effects are mostly time, dose, and cell type dependent. Cell viability as measured by FDA assay was performed to investigate the basic pathobiological effect of BQ components on OKF-6 and MMF-1 cell lines. Whilst individual ingredients of BQ were cytotoxic at the concentration used, whole BQ mixtures induced cell proliferation and reduced cell viability only at much higher levels. Surprisingly, the same chemical components induced transient cell proliferation in oral fibroblasts.

7.4.1 Cytotoxicity of BA-AN compared with WP-AN on OKF-6

The different cytotoxicity seen between BA-AN and WP-AN may be due to the different maturity of AN. BA-AN is a ripe mature AN, while WP-AN is an unripe type. Our previous study (Chapter 5), that was consistent with previous studies, reported that unripe AN contains more polyphenols as an antimutagenic rather than ripe AN (155). WP-AN had antioxidants activities (TPC, FRAP, DPPH scavenging activities) almost 2-fold higher than BA-AN, and contains more polyphenols quantification as shown in **Table 5.1** and **5.2**. However, the excessive amount of phenolics could lead to cytotoxic instead of protective effects (332). Furthermore, we also showed that arecoline quantification by HPLC was higher in WP-AN compared to BA-AN. 250 mg/ml ANE from WP, contain 6.92±1.64 μg/ml, while 250 mg/ml ANE from BA contains 6.10±0.99 μg/ml. Arecoline is believed to be the major alkaloid identified in ANE and has been shown to exhibit cytotoxicity on epithelial cells in cancer cells line (378, 257, 253). Previous studies have shown that a concentration of 100 μg/ml of an aqueous ANE contains 10 μg/ml of arecoline (390, 254). A further study reported that ANE exhibited cytotoxicity to cultured gingival keratinocytes (C3H10T/12) at concentrations

higher than 320 mg/ mL, as shown by a decrease in colony numbers (251). These previously reported cytotoxic effects of AN in these studies are consistent with our current results. The variation of IC50 of ANE may have occurred due to different methods of extraction used, maturity of AN, and cytotoxicity assays used by researchers (155, 391, 254).

7.4.2 Cytotoxicity of BA-BQ Mixture compared with WP-BQ Mixture on OKF-6

Unlike ANE, no cytotoxic effect, as measured by 50% decreased cell viability, was induced by BQ Mixture from both regions at any concentration used over 3 days of incubation (Table 7.1). This can be due to the fact that BQ Mixture not only contains AN but also leaf or SI, as well as slaked lime which could counterbalance the effect of arecoline. Consistently, our previous phytochemical study showed that arecoline was not detected in betel leaf and SI samples. This finding is in line with other studies showing that betel leaf and SI mostly contains polyphenols without any alkaloids (341, 155, 307). Polyphenols contained in the leaf and SI might act as chemo-preventive compounds to oral cancer (392, 155). In particular, hydroxychavicol could reduce oral cancer risk in people who chew BQ with tobacco (217). Although slaked lime in the BQ Mixture could decrease the concentration of phenolics by extreme changes of pH as well as cause direct damage (177), long-term lime exposure to oral mucosa would be necessary to produce oral lesions. For example, previous study reported that only long-term exposure of Chinese hamster cheek pouch to slaked lime (calcium hydroxide) was able to create hyperplastic lesions in these animals. Further studies showed that repeated topical application of lime also could led to oral leukoplakia (393, 250). These data suggest that the small quantities of slaked lime in BQ would be able to induce cellular changes of the oral mucosa only if given repeatedly and for long term. In the present study, cells were exposed to slaked lime contained in BQ in a short-term over 3 days-time and in the presence of other

potentially chemo-preventive compounds such as betel leaf or SI. This might explain why BQ Mixture showed no cytotoxic effect on OKF-6 over 3 days.

7.4.3 Cytotoxicity of BA-Leaf compared with WP-SI on OKF-6

People living in BA region tend to chew BQ by wrapping all ingredients in leaf of *piper betle*, while in contrast people living in WP region pick a part of SI *piper betle* to be chewed together with other BQ components (320). The results of the current study show that BA-Leaf resulted in growth stimulation of OKF-6. Our previous phytochemical study identified a number of polyphenols in betel leaf and SI. Previous studies of betel leaf indicated that of hydroxy chavicol (69.46%), 4-chromanol (24%) and eugenol (4.86%), possess antioxidant, anti-inflammatory, anti-platelet and antithrombotic, antibacterial and antifungal activities (371). In contrast, WP-SI showed cytotoxic effect at 500 μg/ml after incubation for 2 days. Even though WP-SI contains a range of polyphenols that could be protective to the cell (250), it also contains other toxic phytochemicals that could overcome the effect of polyphenols. Our previous study using GC-MS identified safrole in high amount in WP-SI sample (Chapter 6) and several studies have shown safrole as a possible human carcinogen (155, 307). High concentration (15 mg/ml) of safrole has been found in SI (6). Consequently, chewing BQ containing SI may expose the oral mucosa to safrole and, consistently, this compound has been shown to be present in concentrations of about 420 μM in saliva during BQ chewing (7). Safrole is also classified as a hepatocarcinogen in mice and rats (352). A previous study reported that betel SI extract at concentration 100–400 mg/ml is cytotoxic on cultured gingival keratinocytes (378). These studies support the view that SI confers cytotoxic properties to BQ from WP.

7.4.4 Cytotoxicity of BA-Husk compared with WP-Husk on OKF-6

The husk extract from both regions did not exhibit cytotoxic effects on OKF-6 at any concentration used over the 3 days of incubation, although these did reduce cell viability significantly to a different extent. Our previous study on arecoline quantification using HPLC detected low arecoline concentrations in husk from most regions, where WP-Husk had the highest arecoline amount and BA-Husk did not contain detectable arecoline. Arecoline's role in cell inhibition may be reflected in WP-Husk-induced inhibition OKF-6 growth by dose and time fashion, while BA-Husk showed very little inhibition of cell growth. Both husks contain several polyphenols, such as sinapic acid and 5-(hydroxymethyl) furfural, which could slow the detrimental effect of arecoline on OKF-6. The cytotoxic effect of husk containing arecoline could provide additional information to some countries such as Guam, China, and Taiwan that include husk with BQ chewing habit (155). Furthermore, proper husk waste management need to be considered to avoid ongoing environmental pollution.

7.4.5 Cytotoxicity of BA-AN compared with WP-AN on MMF-1

The increase of cell viability compared to control indicated that BA-AN and WP-AN induced cell proliferation of oral primary fibroblasts over 3 days of incubation. Harvey et al also recorded increased human fibroblast proliferation with areca nut and showed cytotoxicity at 100 μg/ml in 5 days of incubation (256, 254). This result is consistent with our study that BA-AN and WP-AN showed increased number of oral fibroblasts. In our experiments, cell proliferation was transient and occurred primarily within the first 24 hours of incubation to then decrease steadily during the following 2 days.

7.4.6 Cytotoxicity of BA-BQ Mixture compared with WP-BQ Mixture on MMF-1

AN as the major ingredient of BQ Mixture is most likely to induce cell proliferation via the action of areca alkaloids, especially arecoline (256, 254). The increase of cell viability in oral fibroblasts incubated with BA and WP-BQ Mixture was seen on 1 day of incubation (**Table 7.2**). However, the number of viable cells started to decline within 2 days, in particular in WP-AN samples. The Mixture of BQ ingredients allows interaction between alkaloids (370, 256) and polyphenols (250) possessed by nut and leaf/SI. The polyphenols in leaf/SI and short-term exposure of slaked lime to cells could lead to delayed cell inhibition (177) that is consistent with the results of the current study.

7.4.7 Cytotoxicity of BA-Leaf compared with WP-SI on MMF-1

BA-Leaf and WP-SI did not exhibit cell cytotoxicity after 3 days of incubation with MMF-1. In contrast, cell proliferation occurred up to 3 days of incubation at all concentration used. This cell proliferation might be due to fact that leaf/SI contain polyphenols. Our phytochemistry study identified polyphenols in leaf and SI, such as sinapic acid and 5-(hydroxymethyl) furfural. A previous study that reported areca nut carcinogenicity was suppressed by the betel leaf when injected to mice (394). This areca alkaloids-antagonistic action by betel leaf has been attributed to the antioxidative nature of phenolics, particularly of appreciable quantities of hydroxychavicol. Antioxidative property of betel leaf has been reported to be much more either butylated hydroxyanisole or butylated hydroxytoluene (395, 396).

7.4.8 Cytotoxicity of BA-Husk compared with WP-Husk on MMF-1

BA-Husk and WP-Husk did not exhibit cytotoxic effect on MMF-1 up until 3 days of incubation. The appreciable numbers of identified polyphenols and small amount of arecoline in both husks might slow the inhibition of cell growth and rather more likely to stimulate cell growth as shown by the increased cells number compared to control.

7.4.9 The comparison of cytotoxic effects of arecoline on OKF-6 and MMF-1

Arecoline exhibited cytotoxicity on OKF-6 at 100 μg/ml after incubation for 2 days. Arecoline supressed growth of OKF-6 at 1 μg/ml, 10 μg/ml and 100 μg/ml respectively by 80.75%, 48.79%, and 33.16% after incubation for 3 days. However, arecoline induced cell proliferation on MMF-1 at all concentrations used over 3 days of incubation. Several researchers reported that arecoline supressed oral keratinocyte cell vitality at different ranges of concentration (378, 257, 250, 253, 251). In contrast, there are well-documented reports of proliferation and collagen synthesis in human buccal mucosal fibroblasts induced by arecoline and arecaidine (256). Our findings are consistent with the pathogenesis of OSMF, in which arecoline has a proliferative effect on fibroblast (397, 322, 398) and also has an anti-proliferative and/or cytotoxic effects on epithelial, which may have further implications on the oral mucosa and vascular function, respectively (322, 399). The effect of arecoline on fibroblasts, keratinocytes and endothelial cells contribute to the pathogenesis of OSMF (322, 399).

7.4.10 Morphological alteration of OKF-6 and MMF-1 after exposure to BQ components

Gross morphological changes were present in both OKF-6 and MMF-1 following BQ exposure. The morphological alteration differed widely and were dependent upon individual components and cell line tested.

It was observed that ANE exposure to OKF-6 led to cell shrinkage and death as well as intercellular space loss. Jeng et al (378) reported morphological alterations after exposure to AN in the change of cell characterization, form, and size, consistent with our current study. The exposure of leaf, BQ Mixture, and husk extracts to OKF-6 caused less to no intercellular space's loss.

When comparing exposure to AN from BA and WP region by the same dose and incubation time to OKF-6, WP-AN was associated with the presence of more dead cells and a higher loss of intercellular spaces. WP-Husk extract led to higher loss of intercellular spaces rather than BA-Husk extract and this is more likely due to higher arecoline concentration carried by WP-AN.

The normal spindle form of MMF-1 was altered to stellate form following exposure to BA-AN and WP-AN. However, the loss of intercellular spaces was more prominent in MMF-1 exposed by WP-AN compared to BA-AN. The oral primary fibroblast morphology alteration most likely occurred due to areca arecoline in the ANE , as also reported by Abhisek et al (400).

7.5 Conclusion

Our present study showed that BA-AN and WP-ANE had cytotoxic effects on OKF-6 over 3 days of incubation. SI extracts were also cytotoxic to OKF-6, but to a lesser extent. In contrast, the extract of leaf, Mixture BQ and husk did not exhibit cytotoxic effect up until 3 days of incubation. The exposure of BQ components to MMF-1 showed no cytotoxicity at any concentrations used. BA-AN and WP-AN led to some MMF-1 proliferation after 1 day of incubation with subsequent inhibition of growth of MMF-1 on 2 days of incubation in a dose and time dependent manners.

Arecoline is thought to be the key component in the pathogenesis of OSMF. In our study this compound exhibited cytotoxicity to OKF-6, with an IC50 reached at 100 μg/ml as early as 2 days after incubation. Different findings were seen when MMF-1 was exposed to arecoline; specifically, arecoline induced MMF-1 proliferation at all concentration used over 3 days of incubation.

Morphological changes following exposure to BQ components to OKF-6 and MMF-1 were observed. Alteration involved a range of cellular features, such as cell architecture, form, size, loss of cell demarcation and formation of intercellular spaces.

The highest arecoline concentration in the previous study (Chapter 5) was observed in AN from WP, followed by AN from BA region. However, in the form of mixed BQ, WP-BQ Mixture showed the highest arecoline level, while BA-BQ Mixture had the lowest one. Both WP-AN and BA-AN contained the high value of total phenolic content (TPC) and antioxidant activities (Chapter 5). These findings of phytochemicals are consistent with the observed cytotoxic effect on both cultured fibroblast and keratinocytes.

Taken together, these results suggest that unripe areca nut from WP, SI and husk might contribute to the development of OSMF. It may well be postulated that the consumption of ripe areca nuts, without the use of either stem inflorescence of *piper betle* or areca husk has potential

to decrease OSMF development and ultimately diminish the morbidity and mortality associated with this horrific disease.

Chapter 8: General Discussion and Future Directions

Oral cancer (OC) is ranked as the sixth most common cancer worldwide and accounts for 3% of cancer globally (1). The disease represents a group of conditions with a range of sites and varied aetiology (1). The incidence of OC annually is estimated to be about 275,000 however, its incidence is increasing (3-6). More than half of OC incidence globally occurs in Asia where an estimated 168,850 new OC cases have arisen in this region alone. Of these, approximately 11% were from South East Asia (SEA) countries such as Indonesia (7, 6).

OC is considered as a public health problem that carries significant morbidity and mortality (21). Presently, Indonesia has insufficient data of epidemiology studies in relation with OPMD or OC prevalence and their risk factors. It is also important to understand the knowledge of GDPs in recognizing OC risk factors, early symptoms of OC, as well as in performing COE. Without a comprehensive understanding of these factors, efforts to prevent, detect and manage this disease are likely to be ineffective in terms of outcomes and use of resources (22). The present study consists of both epidemiology studies and laboratory studies. The overall aims of the epidemiology studies were to understand the prevalence and risk factors of OC and OPMD among Indonesians as well as to assess Indonesian GDP's knowledge in risk factors for OC and in performing COE. The aims of the laboratory studies were to identify antioxidants activities and phytochemical components of Indonesian BQ using HPLC, LCMS, and GC-MS, as well as to know the pathobiological response of Indonesian BQ components on oral keratinocyte (OKF-6) and oral fibroblast (MMF-1).

8.1 Hypothesis 1 and 2, aims 1 and 2

The first hypothesis was that Indonesian GDPs have inadequate knowledge of the risk factors for oral cancer and are inadequate in performing COE. The second hypothesis was that there will be a significant improvement in Indonesian GDPs' knowledge of the risk factors for oral cancer as well as in performing COE after being given a one-day training for oral cancer. The first aim was to determine Indonesian GDPs' knowledge, awareness, and confidence in oral cancer screening. The second aim was to assess the impact of a one-day training on OC on Indonesian GDPs' knowledge of the risk factors for oral cancer as well as in performing COE.

The present study showed that 92.7% GDPs (164 out of 177) claimed to have insufficient training on OC in their dental training. All GDPs from WP region (N=20) responded to have had insufficient training, and only 18.18% (8 out of 44) GDPs from BA who felt to receive a sufficient OC training at dental school. These results should become a 'strong alarm' that needs to be addressed. Curriculum improvement on OC training should be a national wide learning objective for all dental partitioners.

Almost three-quarters (72.4%) of GDPs were shown to have sufficient knowledge in recognising risk factors, one of the most important aspects in preventing OC. Surprisingly, 14 (7.9%) out of 177 GDPs did not know any risk factors of OC, and 16 only reported smoking as a risk factor.

According to GDPs response to post-training questionnaire, the OC training significantly increased GDPs knowledge of indicating OC risk factors**.** The number of GDPs who had sufficient knowledge of risk factor increased from 66.7% (before training) to 88.6% after training. No GDPs answered with either blank or do not know for risk factor after training. However, 10.4% (15 out of 177) GDPs remained with insufficient answers. This may be due

to a lack of correlation between habits and OC development in the whole community which can only be changed with government by developing national OC awareness.

In recognising types of OPMD as early sign of OC, 28.8% GDPs did not know any type of OPMD. Before training for OC, only 21.5% GDPs had sufficient knowledge in recognising type of OPMD. This increased to 80.56% after training. GDPs should always remain alert of early sign and symptom of OC by properly recognising and distinguishing OPMD that appear in the oral cavity (294, 91).

GDPs are encouraged to perform an 8 step COE and be able to identify any pathologic changes in oral mucosa. This complete examination should always be part of GDP's routine examination in daily practice. The present study showed that gingival examination was the most frequent examination step undertaken by GDPs (90.9%), followed by a buccal examination (82,5%), labial (76.3%) and lips (76.3%) examination. However, bimanual and floor of the mouth (FOM) examination were the least frequent examination performed by GDPs (22%), while tongue examination was only performed by 54.2% of GDPs. Thus, most GDPs skipped bimanual and floor of the mouth examination and more half of them neglecting tongue examination. This is a worrisome finding as those two areas are high risk area where OPMD could potentially develop into carcinoma (102, 295, 101).

Measuring GDPs' confidence in performing an 8 step COE is essential so as to assess out why GDPs tend to do incomplete examination. Previous to the training, the most avoided steps by GDPs was in performing bimanual and FOM examination (84%). This number drastically changed after given OC training, GDPs' responded to both questions with 90% feeling confident and 6% very confident in performing bimanual and FOM examination. The drastic increased number of GDPs whose confidence in performing COE steps after training conducted is reassuring, as they a critical part of an OC prevention program.

Taken together these results, indicate that the method of a one-day training for oral cancer improves GDPs' knowledge of risk factors as well as in performing COE. This method can be adopted to develop an oral cancer national training in Indonesia.

8.2 Hypothesis 3, and aims 3 and 4

The third hypothesis was that Indonesia has a high prevalence of OPMD or OC that are strongly associated with known risk factors. The aims of the study were to assess the prevalence type, site and regional variation of OPMD or OC in Indonesian. A further aim was to assess the most significant risk factors for OPMD or OC in Indonesia.

The present study showed that the prevalence of OPMD in Indonesia was surprisingly high (14.3%). The most common affected site of OPMD was buccal mucosa (71.2%). Previous studies showed that approximately 70% of leukoplakias are found on buccal mucosa, lip vermilion, and gingiva, and yet lesions on buccal mucosa rarely show dysplastic or malignant change compared to lesions seen on the floor of the mouth, lip vermilion and gingivae (102). Six (4 %) respondents had OPMD that was found in the floor of the mouth (FOM) together with other sites. In the present study, we found lateral tongue and floor of the mouth sites were the second and third most common sites for OPMD. Two respondents with a large single ulcer on lateral border of the tongue were diagnosed with oral squamous cell carcinoma (OSCC). Our results suggest that the risk of developing OC from OPMD is not negligible in the Indonesian population and requires urgent management.

A strong association was observed between some risk factors and OPMD. The highest number of risk factor was smoking cigarettes (14.5%), followed by betel quid chewing (12.6%) and alcohol consumption (3.9%). Thus, in addition to smoking and BQ chewing, alcohol consumption is a significant risk factor for OPMD in Indonesia, in a univariate analysis.

Sixty (44.1%) out of the 139 respondents who had an OPMD were living in WP and the other 55.9% of OPMD were spread diversely amongst other 4 regions. The highest OPMD occurred in people from WP where there is a strong culture in BQ chewing passed down through many generations as well as drinking traditional made alcohol beverage. This could explain why the OPMDs were found with high prevalence in West Papua with conversely,

people who live in JKT had the lowest risk of developing OPMD, with a better understanding about OC risk factors, good social economic status and healthy nutrition. Previous studies have also reported that high SES results in a decreased risk of having OC (302, 303).

The various ingredients of BQ are also suspected to differentially influence OPMD development. Most BQ are composed of areca nut, slaked lime and betel leaf or betel SI, and some additional spices and tobacco. In the present study, only people living in WP consumed BQ containing betel SI (BQ-SI) and they are Melanesian in ethnicity. Taiwanese and Melanesian commonly chose betel SI (flower or pods part) rather than betel leaf to be chewed together with other BQ components (155). The logistic regression analysis showed that betel quid containing SI (BQ-SI) chewers had 4.2 times higher risk of having OPMD compared to betel quid containing betel leaf (BQ-L) chewers. Both betel leaf and betel SI alone have been shown to contain extensive polyphenolic as antimutagenic compounds (155, 307, 262). We have also shown that none of betel leaf chewers had OPMD in the oral cavity. Safrole is the major phenolic in betel SI and may act as a carcinogen (307). Further research is needed to assess whether BQ-SI or the combination with different slaked lime causes a higher risk of OPMD. The addition of tobacco to BQ-L chewing could increase the risk (OR=10.2) compared to consuming BQ-L without tobacco. Several studies have shown that tobacco could indeed increase the frequency of onset of OSMF and accelerate the OPMD development into cancerous lesions (165, 142). One study in Pakistan found that adding tobacco to BQ increased the severity of clinical grading of OSMF in a statistically significant manner (157).

The occurrence of OPMD was not significantly associated with the different type of cigarettes consumed. However, when comparing non-smokers to individuals smoking different types of cigarettes, non-smokers respondents had 3.8 and 5.3-times lower risk of having OPMD compared to kretek and filter cigarette smokers, respectively. Kretek is Indonesian traditional cigarette which characterised by the use of a significant amount of clove, the sun-dried flower

buds of the clove tree (*Syzygium aromaticum, syn. Eugenia aromaticum*), added to the tobacco, while filter cigarettes are imported cigarette that do not contain clove but only tobaccos with lower tar and nicotine level compared to kretek (308).

Alcohol drinking was the most causative factor of OPMD that was found in the present study. The respondents who consumed alcohol had 12.2-fold risk of OPMD compared to non-alcohol drinkers. Studies have reported alcohol strongly correlated with OC (15, 102, 295, 103) and the risk of OC increased with greater alcohol intake, and considered no safe limit intake to cancerous change (313). All types of alcohol drinking brands could cause OPMD to oral mucosal. Arak putih (tuak Dayak) is one of Indonesian traditional homemade alcohol beverage, which contains alcohol typically near 27-34% (314). The alcohol is fermented from rice (314). In the present study, most of arak putih consumers were from Suku Dayak, Pontianak, WK region. On the other hand, the beer brand consumed in this study is Saguer, this beverage is also traditionally made and the level of alcohol could possibly reach 70% or more (314). Most respondents who consumed beer (Saguer) were from WP region. Saguer itself originally comes from North Sulawesi's culture and adapted by people from surrounding islands, including WP. Saguer is made from fermentation of various palms to make sugar (314).

The respondents who had knowledge of OC risk factors had a significantly lower rate of OPMD (OR=0.2) compared to those who do not have knowledge with 82.2% admitting that GDPs did not give any information about OC. Inadequate knowledge of OC is a common obstacle for OC awareness and early screening programs (315, 316, 18).

Respondents who confidently and properly brushed their teeth (OR=0.6) were at a lower risk of OPMD. Additionally, the respondents who brush their teeth twice a day could lower the risk of OPMD compared to those who brush their teeth once a day. A study in Iran reported that the good behaviour in properly maintaining oral health significantly correlated with the behaviour in avoiding oral disease risk factors, such as tobacco use and poor diet

nutrition with those who carefully watched their oral health status mostly aware of their oral health in general (293).

We have shown that Papua (Papua Island) and Dayak (WK) ethnicities have 23.6 and 21.3-fold higher risk than Aceh ethnic (BA). Javanese, Balinese and East Nusa Tenggaranese (ENT) groups were slightly more likely to have OPMD than Aceh ethnic, whereas Sulawesi and Maluku ethnic shows 7.01 times of higher risk to Aceh ethnic. There is growing evidence of intra-country ethnic differences; such that, a significantly higher number of deaths from OC have been recorded in men from the Indian subcontinent in the UK, than in the indigenous UK population, and OC appears to be most prevalent in areas with a high Asian population (7, 317).

In the current study, we measured SES by the location of COE examination, occupation, health insurance, and whether it was easy or not to travel to the dental health facilities. Some studies reported that OC risk is associated with low SES significantly related to lifestyle risk factors (318, 302, 303). Respondents who were screened at urban areas had a lowered risk (OR=0.43) of OPMD or OC compared to those screened at rural areas. This may be due to people who living in a rural area may commonly have a lack of hygiene, poor nutrition, bad health facilities, and poor living conditions that may attribute to causation of OC by complex interactions (302).

The low and unstable income received by non-government employees, such as fishermen, traders, and farm workers are reason for poor living conditions and difficulty in buying good nutrition food. Thus, non-government employees had a 3.3-fold higher rate of OPMD compared to government employee respondents. The un-employed respondents were insignificantly associated with OPMD, and most of the unemployed respondents (Total n=479, Female =422, Male =57) were home-stay mothers who depend on their husband's income. This

predominant home-stay mother is un-employed but may not necessary mean they had no family income.

The respondents' income also could be assessed by whether they were covered by government insurance or purchased the insurance by themselves. The respondents who had non-government insurance had a lower risk (OR=0.33) of OPMD compared to those who had government insurance. The respondents who had non-government insurance could be considered economically capable as they can purchase insurance or were covered by their working company.

In the current study analysis showed no significant difference whether it was easy or not to reach dental health facilities with the occurrence of OPMD. The ability to access dental health facilities is also a factor thought to be associated with SES. Most low-income population who live in remote areas had difficulty in accessing dental health facilities, that may impact on OC development (302, 303).

The demographic difference of OC screening can also contribute to SES measurement in this survey. People living in WP and WK regions were found to have the highest risk of OPMD among all respondents and these are considered very remote areas. WP and WK region were difficulty to be reached from capital city of Jakarta. Limited small domestic flights are available, and another long-distance trip by boat or car is required to reach the area. Thus, the socioeconomic status would greatly different compared to WJ, JKT and BA region.

In the current study, alcohol drinking was found to be not significant compared to all significant risk factors to cause OPMD or OC. Thus, here are multiple factors influencing the risk for OPMD or OC. When all these factors were analysed collectively in a multiple logistic regression analysis, interestingly, alcohol drinking was found to be not significantly related to the presence of OPMD or OC. The most significant risk factors for OPMD or OC in the present study were smoking, betel quid chewing, and ethnicity. A large number of the 973 enrolled

were smokers (14.5%) and BQ chewers (12.6%). Ethnicity itself has also previously been reported to associate with OPMD or OC (7, 317), and in the current study we observed the same with ethnicity significantly related to OPMD or OC.

The future direction of our study is to urge Indonesian government for primary prevention of OC programs in Indonesia and conduct a national survey. The risk of developing OC from OPMD is not negligible in the Indonesian population and should be addressed seriously. The primary prevention of OC program aimed to decrease the mortality and morbidity rate of this disease among Indonesian.

8.3 Hypothesis 4, and aims 5 and 6

The fourth hypothesis was that each Indonesian BQ component from the four different regions has different carcinogenic and antimutagenic constituents identifiable using HPLC-MS or LC-MS. The aim was to identify and quantify carcinogenic and antimutagenic constituents in Indonesian BQs from the four different regions using HPLC-MS or LC-MS. A further aim of this study was to determine the total phenolic content and antioxidant activities using the FRAP and DPPH assays in Indonesian BQ components.

BQ compounds have distinct chemical features that are mixture, and region-specific. Interestingly, the alkaloid and phenolic content as well as antioxidant activity of individual ingredients changes significantly when these are combined into a BQ mixture. We observed that BQ compounds from WP were distinctive from other regions, and that AN and BQ mixture from this region contained the highest TPC, antioxidant activities, polyphenols and arecoline content, anthoxanthins, and stilbenes which may help explain the high prevalence of OSMF and oral cancer in that region. Further research is required to better elucidate the association between the specifically identified arecoline and phenolics with the development of OSMF in these regions of Indonesia.

Arecoline was detected in all ANs and mixture samples and was significantly correlated with catechin and epicatechin, and significantly negatively correlated with *p*-hydroxybenzoic acid. Phenolic acids and flavanols content in betel quid mixture were low, likely due to the addition of lime, which also led to lower antioxidant activity and radical scavenging capacity in the mixture. In the current study, alkaloid and flavonoid (catechin and tannin) components were simultaneously identified in AN. The alkaloids are considered to be the most potent component causing OSMF, while flavonoids have a synergistic role (321). Flavonoids could decrease collagenase, stabilize the collagen fibrils to resist degradation by collagenase (137).

Catechin and epicatechin are the main phenolic compounds detected in BQ, while WP had the highest concentrations in nut samples. The high amount of catechin and epicatechin could change the effect of antioxidant into pro-oxidant leading to a raise in the potential of developing OSMF. It has been suggested that regular and frequent polyphenol intake may be helpful to protect against oral cancer (332). However, polyphenols can also act as pro-oxidant at high concentrations under certain conditions (327, 328). In the present study, we found the polyphenols catechin and epicatechin in high amounts, especially in WP-AN sample.

The ripeness of AN is directly related to the amount of both polyphenols and arecoline. The unripe AN contained a higher concentration of polyphenols and arecoline compared to ripe AN, suggesting that consuming the ripe AN could lower the potential of developing OSMF. The immature AN from WK and WP were interestingly less affected in losing arecoline after added slaked lime, potentially due to chemical and biological process in the mixture component (339). This could indicate that phenolics and arecoline have a synergic protection from pH change that preserve the amount of arecoline i.e. the more phenolics in AN, the less arecoline damage that occurs by slaked lime in BQ mixture.

Other polyphenols identified were ferulic acid and resveratrol that have been previously reported in AN (348, 235). However, in the present study ferulic acid was not detected in any nut samples and resveratrol was only detected in nuts from WP. Further, polyphenols such as quercetin and kaempferol were also reported in AN (348, 235). These compounds were detected in the current study and interestingly observed in leaf and husk samples as well. Previous studies on BQ phenolic compounds mainly reported phenolic compounds in the nuts (218, 349, 348, 235, 207, 208), but a few studies reported these compounds in husk, leaf and SI. Therefore, the phenolics data of husk, leaf and SI reported in the current study explain the antioxidant capacity of these samples and provided insights for future research investigating antioxidants in BQ.

8.4 Hypothesis 5, aims 7 and 8

The fifth hypothesis was that GC-MS can be used as an alternative method to identify carcinogenic and antimutagenic constituents in Indonesian BQ components. The aim of this study was to assess if GC-MS can be used as an alternative method to identify and quantify carcinogenic and antimutagenic constituents in Indonesian BQ components. A further aim was to identify and quantify other alkaloids and polyphenols in Indonesian BQ components that previously could not be identified using HPLC-MS or LC-MS.

This is the first study to use GC-MS to profile volatile compounds in different Indonesian BQ components. We have successfully established a method using GC-MS to analyse 3 types of alkaloids in BQ components, however, guvacine could not be analysed using this method. Extremely long tailing was observed for guvacine with unstable peaks, therefore, it could not be identified and quantified. This unstable peak was likely due to the high melting point of guvacine (306-309^{o}C), which exceeded the highest analysis temperature used in the current method (300^{o}C). Thus, this method is not suitable for the identification and quantification of guvacine.

In our previous study, the undetected polyphenol using LC-MS was safrole, while in the present study we demonstrated that safrole was detected in betel SI, betel leaf and areca husk samples using GC-MS. A very high concentration of safrole was found in WP-SI (761.6 mg/g DM), being more than 700-fold higher concentration of safrole compared to other samples. Safrole was not detected in BA-AN, WK-AN, and NS-AN. This finding suggests that adding betel SI and husk into BQ compound could raise the risk of having OSMF and oral cancer even more as safrole is a possible potential human carcinogen (341, 307).

Eugenol was only found in the betel leaf and SI samples, and WP-SI contained the highest eugenol concentration compared to eugenol found in BA-Leaf, NS-Leaf, and WK-Leaf. While it was undetected in areca nuts samples. The benefit of eugenol for human health has

extensively studied for cancer prevention, antimicrobial properties and as an antioxidant (375-377).

Acid, aldehyde, alcohol, and ester were mostly found in the husk group, especially what has been indicated at highest level in NS-Husk, such as 2,3-Butanediol, glycerine, L-Lactic acid Other detected compounds were mainly fatty acids and esters, likely contributing to the fragrance of betel quid. The WP-Husk contained hexadecanoic acid, Linoleic acid ethyl ester and Methyl nicotinate at the highest concentration compared to other samples.

Thus, GC-MS is an extremely good alternative method for identifying carcinogenic and mutagenic constituents. This method demonstrated very clearly the very high level of the polyphenol such as safrole present in WP sample, 700-fold greater than other samples. Future analyses of the carcinogenic constituents of various combinations of betel chewing components must therefore include the use GC-MS as part of the analyses.

8.5 Hypothesis 6, aim 9

The sixth hypothesis was that BQ components from WP and BA have different cytotoxic effects on oral keratinocyte and oral fibroblast. The aim of this study was to determine whether BQ components from WP have different cytotoxic effect on oral keratinocytes and oral fibroblasts compared to BQ components from BA.

BA-AN and WP-AN extracts had cytotoxic effects on OKF-6 over 3 days of incubation. Arecoline is believed as the major alkaloid identified in areca nut shown to have carcinogenic potential inhibiting epithelial cell growth (378, 257, 253). Previous studies have shown this at a concentration of 100 μg/ml arecoline as well as an aqueous areca nut extract (ANE) contains 10 μg/ml of arecoline (390, 254). A further study reported that ANE exhibited cytotoxicity to cultured gingival keratinocytes (C3H10T/12) cells at concentrations higher than 320 mg/ mL as shown by a decrease in colony numbers (251). These previously reported that cytotoxic effects of areca nut in these studies are consistent with our current result.

Betel SI extracts were also cytotoxic to OKF-6, but to a lesser extent compared with areca nut. Even though WP-SI contains a range of polyphenols that are chemo-protective to the cell (250), it also contained safrole as a strong toxic phytochemical. Safrole was identified using GC-MS in high concentration in WP-SI sample (chapter 6) and several studies have shown safrole as a possible human carcinogenic polyphenols (155, 307). A previous study also has been shown a high concentration (15 mg/ml) of safrole in SI (6). Consequently, chewing BQ containing SI may attribute to exposure of safrole and it has been shown that there is 420 μM of safrole in saliva during chewing (7). Safrole is classified as a hepatocarcinogen in mice and rats (352). Further, a previous study reported that betel SI extract at concentration 100–400 mg/ml has cytotoxicity effect on cultured gingival keratinocytes (378).

In contrast to betel SI, the extract of betel leaf, mixture BQ and husk did not exhibit cytotoxic effect over 3 days of incubation. Betel leaf has shown to have no alkaloids, and it

contains polyphenols that are chemo-protective to cells. BQ mixture compose not only areca nut but also betel leaf/betel SI and small amount of slaked lime. The mixture allows betel leaf/betel SI to slow the cell growth inhibition that possibly caused by AN and slaked lime. Areca husk itself has shown to contain an appreciable number of polyphenols and small amounts of arecoline. Thus, this may explain why betel leaf, mixture BQ and husk did not show cytotoxic effect at any concentration over 3 days of incubation.

No cytotoxicity effect was observed following the exposure of BQ components to MMF-1 at any concentrations used. BA-AN and WP-AN resulted in some MMF-1 proliferation after 1 day of incubation with subsequent inhibition of growth of MMF-1 in a dose and time dependent manners. Previous studies have also shown that areca arecoline increased proliferation of human fibroblast as well as showed cytotoxicity effect at 100 μg/ml in 5 days of incubation (256, 254).

In our present study arecoline exhibited cytotoxicity to OKF-6, with an IC50 reached at 100 μg/ml as early as 2 days after incubation. Different findings were seen when MMF-1 was exposed to arecoline; specifically, arecoline induced MMF-1 proliferation at all concentrations used over 3 days of incubation. Our findings are consistent with the pathogenesis of OSMF, and it has previously been reported that arecoline has a proliferative effect on fibroblast (397, 322, 398) as well as an anti-proliferative and cytotoxic effect on endothelial cells, which may have a further effect on vascular function impairment (322, 399). Both the effect of arecoline on fibroblast and endothelial cells contribute to the pathogenesis of OSMF (322, 399).

Morphological changes following exposure to BQ components to OKF-6 and MMF-1 were observed. Alteration involved a range of cellular features, such as cell architecture, form, size, loss of cell demarcation and formation of intercellular spaces.

We conclude that unripe areca nut from WP, SI and husk highly contribute to the development of OSMF. We suggest that consuming BQ containing ripe areca nuts, without the use of either stem inflorescence of *piper betle* or areca husk has the potential to decrease OSMF development and ultimately diminish the morbidity and mortality associated with this horrific disease

Bibliography

1. Brocklehurst, P., Kujan, O., O'Malley, L. A., Ogden, G., Shepherd, S., *et al.* Screening programmes for the early detection and prevention of oral cancer. Cochrane Database of Systematic Reviews.(2013)

2. Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., *et al.* Global cancer statistics 2018: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 68(6):394-424.(2018)

3. Patton, L. L. The effectiveness of community-based visual screening and utility of adjunctive diagnostic aids in the early detection of oral cancer. Oral Oncology. 39(7):708-723.(2003)

4. Pindborg, J. J., Mehta, F. S., Gupta, P. C., Daftary, D. K. Prevalence of oral submucous fibrosis among 50,915 indian villagers. Br J Cancer 22:646–654.(1968)

5. Psoter, W. J., Morse, D. E., Sánchez-Ayendez, M., Vélez Vega, C. M., Aguilar, M. L., *et al.* Increasing opportunistic oral cancer screening examinations: Findings from focus groups with general dentists in puerto rico. Journal of Cancer Education. 30(2):277-283.(2014)

6. Warnakulasuriya., S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncology. 45(4-5):309-316.(2009)

7. Cheong, S. C., Vatanasapt, P., Yi-Hsin, Y., Zain, R. B., Kerr, A. R., *et al.* Oral cancer in south east asia. Transl. Res. Oral Oncol. 2.(2017)

8. IARC. Cancer incidence in five continents ci5, vol ix.: Lyon, IARCPress; 2010 [

9. Ferlay J, Soerjomataram I, Dikshit R, al., e. Cancer incidence and mortality worldwide: Sources, methods and major patterns in globocan 2012. Int J Cancer. 136:E359-386.(2015)

10. Vatanasapt P, Suwanrungruang K, Kamsa-Ards, al., e. Epidemiology of oral and pharyngeal cancers in khon kaen, thailand: A high incidence in females. APJCP. 12:2505-2508.(2011)

11. Alwan A, M. D., Riley LM, et al. . Monitoring and surveillance of chronic non-communicable diseases: Progress and capacity in high-burden countries. Lancet. 376:1861-1868.(2010)

12. Ng CJ, T. C., Abdullah N, et al. . Relationships between cancer pattern, country income and geographical region in asia. BMC Cancer. 15:613.(2015)

13. Ansara DL, A. F., Kishor S, et al. Tobacco use by men and women in 49 countries with demographic health surveys. Maryland: Icf international. ICF International.65.(2013)

14. Eriksen M, M. J., Schluger NW, et al. . The tobacco atlas, 5th ed. Georgia: American Cancer Society,.(2015)

15. BDA. Opprotunistic oral cancer screening. A management strategy for dental practice. 6.(2000)

16. Frenandez GL, S. R., Lence AJJ, Rodriguez SA, Maxwell PD. . An evaluation of the oral cancer control program in cuba. Epidemiology. 6:428-431.(1995)

17. Rogers SN, V. S., Lowe D. Reasons for delayed presentation in oral and oropharyngeal cancer: The patients perspective. Brit J Oral Max Surg. (49):349-353.(2011)

18. Paudyal, P., Flohr, F. D., Llewellyn, C. D. A systematic review of patient acceptance of screening for oral cancer outside of dental care settings. Oral Oncology. 50(10):956-962.(2014)

19. Warnakulasuriya KA, H. C., Scarrott DM, et al. An alarming lack of public awareness towards oral cancer. Br Dent J 187:319-322.(1999)

20. Mignogna MD, F. S., Lo Russo L, Ruoppo E, Lo Muzio L. Oral and pharyngeal cancer: Lack of prevention and early detection by health care providers. Eur J Cancer Prev. 10:381-383.(2001)

21. Warnakulasuriya KAAS, N. B. Reproducibility of an oral cancer and precancer detection program using a primary health care model in sri lanka. Cancer Detect Prev 15:331-334.(1991)

22. Ford, P. J., Farah, C. S. Early detection and diagnosis of oral cancer: Strategies for improvement. J Cancer Policy. 1(1-2):e2-e7.(2013)

23. Kujan, O., Glenny, A. M., Duxbury, A. J., Thakker, N., Sloan, P., *et al.* Screening programmes for the early detection and prevention of oral cancer. The cochrane database of systematic reviews.(2003)

24. Mignogna MD, F. S., Lo Russo L, Ruoppo E, Lo Muzio L. Costs and effectiveness in the care of patients with oral and pharyngeal cancer: Analysis of a paradox. Eur J Cancer Prev. 11:205-208.(2001)

25. Rethman MP, C. W., Cohen EE, Epstein J, Evans CA, Flaitz CM, et al. Evidence-based clinical recommendations regarding screening for oral squamous cell carcinomas. Journal of the American Dental Association. 141:509-520.(2010)

26. Giovannacci, I., Vescovi, P., Manfredi, M., Meleti, M. Non-invasive visual tools for diagnosis of oral cancer and dysplasia: A systematic review. Medicina oral, patologia oral y cirugia bucal. 21(3):e305-315.(2016)

27. Mehrotra, R., Gupta, D. K. Exciting new advances in oral cancer diagnosis: Avenues to early detection. Head & neck oncology. 3:33.(2011)

28. Moles DR, Downer MC, PM, S. Meta-analysisofmeasuresofperformance reported in oral cancer and precancer screening studies. British dental journal. 6:340-344.(2002)

29. Lingen, M. W., Kalmar, J. R., Karrison, T., Speight, P. M. Critical evaluation of diagnostic aids for the detection of oral cancer. Oral Oncol. 44(1):10-22.(2008)

30. HC., S. Cytological screening for early mouth cancer. Cancer 15:1119-1124.(1962)

31. Jr., S. S. Early diagnosis of oral cancer. Cancer 62(8 Suppl):1796–1799.(1988)

32. Shugars DC, P. L. Detecting, diagnosing, and preventing oral cancer. Nurse Pract. 105:109-110.(1997)

33. Downer MC., Evans AW., Hughes Hallet CM., J., ullien JA, Speight PM., *et al.* Evaluation of screening for oral cancer and precancer in a company headquarters. Community Dent Oral Epidemiol. 23(2):84-88.(1995)

34. Jullien JA., Downer MC., Zakrzewska JM., PM, S. Evaluation of a screening test for the early detection of oral cancer and precancer. Community Dent Health. 12(1):3-7.(1995)

35. Downer MC., Moles DR., Palmer S., PM, S. A systematic review of test performance in screening for oral cancer and precancer. Oral Oncol. 40(3):264-273.(2004)

36. Ramadas K., Sankaranarayanan R., Jacob BJ., Thomas G., Somanathan T., *et al.* Interim results from a cluster randomized controlled oral cancer screening trial in kerala. Oral Oncol. 39(6):580-588.(2003)

37. Sankaranarayanan R., Mathew B., Jacob BJ., Thomas G., Somanathan T., *et al.* Early findings from a community-based, cluster-randomized, controlled oral cancer screening trial in kerala, india. The trivandrum oral cancer screening study group. Cancer. 88(3):664-673.(2000)

38. Mignogna MD., S, F. Oral cancer screening: 5 minutes to save a life. Lancet. 365(9475):1905-1906.(2005)

39. Kujan OGA., Duxbury AJ., Thakker N., P, S. Screening programmes for the early detection and prevention of oral cancer. Cochrane Database Syst Rev. 4:CD004150.(2003)

40. Sankaranarayanan R., Ramadas K., Thomas G., Muwonge R., Thara S., *et al.* Effect of screening on oral cancer mortality in kerala, india: A cluster-randomised controlled trial. Lancet. 365(9475):1927-1933.(2006)

41. British Columbia Oral Cancer Prevention Program, B. C. C. A., College of Dental Surgeons of British, C. Guideline for the early detection of oral cancer in british columbia 2008. Journal. 74(3):245.(2008)

42. D., E. Brush biopsy 'saves lives'. J Am Dent Assoc. 133(6):688, 690, 692.(2002)

43. T, F. S. The oral brush biopsy: Separating fact from fiction. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 96 (6):654-655.(2003)

44. Fedele, S. Diagnostic aids in the screening of oral cancer. Head & neck oncology. 1:5.(2009)

45. <s-2014-transepithelial brush biopsy - oral cdx.Pdf>.

46. Omar, E. Current concepts and future of noninvasive procedures for diagnosing oral squamous cell carcinoma - a systematic review. Head & Face Medicine. 11(1).(2015)

47. Omar, E. Future imaging alternatives: The clinical non-invasive modalities in diagnosis of oral squamous cell carcinoma (oscc). The open dentistry journal. 9:311-318.(2015)

48. JJ., S. Improving detection of precancerous and cancerous oral lesions. Computer-assisted analysis of the oral brush biopsy. Us collaborative oralcdx study group. J Am Dent Assoc. 130:1445-1457.(1999)

49. Farah, C. S., McCullough, M. J. Oral cancer awareness for the general practitioner: New approaches to patient care. Australian Dental Journal. 53(1):2-10.(2008)

50. Poate TW., Buchanan JA., Hodgson TA., al., e. Hodgson ta, et al. An audit of the efficacy of the oral brush biopsy technique in a specialist oral medicine unit. Oral Oncol 40:829-834.(2004)

51. Remmerbach, T. W., Meyer-Ebrecht, D., Aach, T., Würflinger, T., Bell, A. A., *et al.* Toward a multimodal cell analysis of brush biopsies for the early detection of oral cancer. Cancer Cytopathology. 117(3):228-235.(2009)

52. Fedele, S. Diagnostic aids in the screening of oral cancer. Head & Neck Oncology. 1(1).(2009)

53. Böcking, A., Sproll, C., Stöcklein, N., Naujoks, C., Depprich, R., *et al.* Role of brush biopsy and DNA cytometry for prevention, diagnosis, therapy, and followup care of oral cancer. Journal of Oncology. 2011:1-7.(2011)

54. Pallagatti, S., Sheikh, S., Aggarwal, A., Gupta, D., Singh, R., *et al.* Toluidine blue staining as an adjunctive tool for early diagnosis of dysplastic changes in the oral mucosa. Journal of clinical and experimental dentistry. 5(4):e187-191.(2013)

55. Ujaoney, S., Motwani, M. B., Degwekar, S., Wadhwan, V., Zade, P., *et al.* Evaluation of chemiluminescence, toluidine blue and histopathology for detection of high risk oral precancerous lesions: A cross-sectional study. BMC Clin Pathol. 12:6.(2012)

56. Vashisht, N., Ravikiran, A., Samatha, Y., Rao, P. C., Naik, R., *et al.* Chemiluminescence and toluidine blue as diagnostic tools for detecting early stages of oral cancer: An invivo study. J Clin Diagn Res. 8(4):ZC35-38.(2014)

57. Epstein, J. B., Guneri, P. The adjunctive role of toluidine blue in detection of oral premalignant and malignant lesions. Current opinion in otolaryngology & head and neck surgery. 17(2):79-87.(2009)

58. A., M. Final evaluation of tolonium chloride rinse for screening of high-risk patients with asymptomatic squamous carcinoma. J Am Dent Assoc. 106(3):319–323.(1983)

59. Barrellier P., Babin E., Louis MY., A, M.-G. The use of toluidine blue in the diagnosis of neoplastic lesions of the oral cavity. Rev Stomatol Chir Maxillofac. 94(1):51-54.(1993)

60. Warnakulasuriya KA., NW, J. Sensitivity and specificity of orascan (r) toluidine blue mouthrinse in the detection of oral cancer and precancer. J Oral Pathol Med. 25(3):97-10.(1996)

61. Onofre MA., Sposto MR., CM, N. Reliability of toluidine blue application in the detection of oral epithelial dysplasia and in situ and invasive squamous cell carcinomas. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 91(5):535-540.(2001)

62. Seoane Leston, J., Diz Dios, P. Diagnostic clinical aids in oral cancer. Oral Oncol. 46(6):418-422.(2010)

63. Sambandham, T., Masthan, K. M., Kumar, M. S., Jha, A. The application of vizilite in oral cancer. J Clin Diagn Res. 7(1):185-186.(2013)

64. Drezek, R., Guillaud, M., Collier, T., Boiko, I., Malpica, A., *et al.* Light scattering from cervical cells throughout neoplastic progression: Influence of nuclear morphology, DNA content, and chromatin texture. Journal of biomedical optics. 8(1):7-16.(2003)

65. Nagi, R., Reddy-Kantharaj, Y. B., Rakesh, N., Janardhan-Reddy, S., Sahu, S. Efficacy of light based detection systems for early detection of oral cancer and oral potentially malignant disorders: Systematic review. Medicina Oral Patología Oral y Cirugia Bucal.0-0.(2016)

66. Mehrotra, R., Singh, M., Thomas, S., Nair, P., Pandya, S., *et al.* A cross-sectional study evaluating chemiluminescence and autofluorescence in the detection of clinically innocuous precancerous and cancerous oral lesions. Journal of the American Dental Association (1939). 141(2):151-156.(2010)

67. Ram S., CH, S. Chemiluminescence as a diagnostic aid in the detection of oral cancer and potentially malignant epithelial lesions. Int J Oral Maxillofac Surg 34:521-527.(2005)

68. Rajmohan M., Rao UK., Joshua E., Rajasekaran ST., R, K. Assessment of oral mucosa in normal, precancer and cancer us- ing chemiluminescent illumination, toluidine

blue supravital stain- ing and oral exfoliative cytology. J Oral Maxillofac Pathol. 16:325-329.(2012)

69. Ujaoney S., Motwani MB., Degwekar S., al, e. Evaluation of chemiluminescence, toluidine blue and histopathology for detection of high risk oral precancerous lesions: A crosssectional study. BMC Clin Pathol. 12:6.(2012)

70. Farah CS., MJ, M. A pilot case control study on the efficacy of acetic acid wash and chemiluminescent illumination (vizilite trade mark) in the visualisation of oral mucosal white lesions. Oral Oncol. 43:820-824.(2007)

71. Farah, C. S., McCullough, M. J. Oral cancer awareness for the general practitioner: New approaches to patient care. Australian dental journal. 53(1):2-10; quiz 99.(2008)

72. McCullough, M. J., Prasad, G., Farah, C. S. Oral mucosal malignancy and potentially malignant lesions: An update on the epidemiology, risk factors, diagnosis and management. Australian dental journal. 55 Suppl 1:61-65.(2010)

73. Farah, C. S., McIntosh, L., Georgiou, A., McCullough, M. J. Efficacy of tissue autofluorescence imaging (velscope) in the visualization of oral mucosal lesions. Head & neck. 34(6):856-862.(2012)

74. Bhatia, N., Matias, M. A., Farah, C. S. Assessment of a decision making protocol to improve the efficacy of velscope in general dental practice: A prospective evaluation. Oral Oncol. 50(10):1012-1019.(2014)

75. Farah, C. S., McIntosh, L., Georgiou, A., McCullough, M. J. Efficacy of tissue autofluorescence imaging (velscope) in the visualization of oral mucosal lesions. Head & Neck. 34(6):856-862.(2012)

76. McNamara, K. K., Martin, B. D., Evans, E. W., Kalmar, J. R. The role of direct visual fluorescent examination (velscope) in routine screening for potentially malignant oral

mucosal lesions. Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology. 114(5):636-643.(2012)

77. Awan, K. H., Morgan, P. R., Warnakulasuriya, S. Evaluation of an autofluorescence based imaging system (velscope) in the detection of oral potentially malignant disorders and benign keratoses. Oral Oncol. 47(4):274-277.(2011)

78. Awan, K., Yang, Y., Morgan, P., Warnakulasuriya, S. Utility of toluidine blue as a diagnostic adjunct in the detection of potentially malignant disorders of the oral cavity--a clinical and histological assessment. Oral diseases. 18(8):728-733.(2012)

79. ID, M. The diagnostic uses of saliva. J Oral Pathol Med. 19(3):119-125.(1990)

80. JV Jokerst, J. M. Programmable nano-bio-chips: Multifunctional clinical tools for use at the point-of-care. Nanomed. 5(1):143-155.(2010)

81. Zimmermann BG, P. N., Wong DT. Genomic targets in saliva. Ann N Y Acad Sci. 1098:184–191

82. Floriano PN., Christodoulides N., Miller CS., Ebersole JL., Spertus J., *et al.* Use of saliva-based nano-biochip tests for acute myocardial infarction at the point of care: A feasibility study. Clin Chem. 55(8):1530-1538.(2009)

83. Malati, T. Tumour markers: An overview. Indian J Clin Biochem. 22(2):17-31.(2007)

84. Hasselmann D., Rappl G., Tilgen W., U, R. Extracellular tyrosinase mrna within apoptotic bodies is protected from degradation in human serum. Clin Chem. 47:1488-1489.(2001)

85. Ratajczak J, Wysoczynski M., Hayek F., Janowska-Wieczorek A., MZ, R. Membrane-derived microvesicles: Important and underappreciated mediators of cell-to-cell communication. Leukemia. 20:1487-1495.(2006)

86. García JM., García V, Peña C., Domínguez G., Silva J., *et al.* Extracellular plasma rna from colon cancer patients is confined in a vesicle-like structure and is mrna-enriched. 14.1424-1432.(2008)

87. Simpson RJ., Jensen SS., JW, L. Proteomic profiling of exosomes: Current perspectives. Proteomics. 8:4083-4099.(2008)

88. Skog J., Würdinger T., van Rijn S., Meijer DH., Gainche L., *et al.* Glioblastoma microvesicles transport rna and proteins that promote tumour growth and provide diagnostic biomarkers. . Nat Cell Biol. 10:1470-1476.(2008)

89. Wong, D. Salivary diagnostics powered by nanotechnologies, proteomics and genomics. JADA. 137:313-321.(2008)

90. Speight PM., PR, M. The natural history and pathology of oral cancer and precancer. 10 Suppl. 1:31-41.(1993)

91. Jamileh Bigom Taheri1, Z. N., Somayyeh Azimi3, Masomeh Mehdipour1, Romina Behrovan4, Kosar Rezaei Far1*. Knowledge of oral precancerous lesions considering years since graduation among dentists in the capital city of iran: A pathway to early oral cancer diagnosis and referral? Asian pacific journal of cancer prevention 19:2103-2108.(2018)

92. Joseph, B. K., Sundaram, D. B., Sharma, P. Oral cancer awareness among dentists in kuwait. Med Princ Pract. 21(2):164-170.(2012)

93. Gómez I, W. S., Varela-Centelles PI, López-Jornet P, Suárez-Cunqueiro M, Diz-Dios P, et al. . Is early diagnosis of oral cancer a feasible objective? Who is to blame for diagnostic delay? Oral Dis. 16:333-342.(2010)

94. Varela-Centelles, P., Insua, A., Seoane-Romero, J. M., Warnakulasuriya, S., Rapidis, A., *et al.* Available web-based teaching resources for health care professionals on screening for oral cancer. Medicina Oral Patología Oral y Cirugia Bucal.e144-e149.(2015)

95. Morse, D. E., Vélez Vega, C. M., Psoter, W. J., Vélez, H., Buxó, C. J., *et al.* Perspectives of san juan healthcare practitioners on the detection deficit in oral premalignant and early cancers in puerto rico: A qualitative research study. BMC Public Health. 11(1).(2011)

96. Maksimovskaya LN., Abramova MY., AA, E. Implementation of the national program of cancer screening of oral precancerous and oncological diseases in the russian federation. Stomatologiia (Mosk). 98(4):44-47.(2019)

97. Maybury, C., Horowitz, A. M., Goodman, H. S. Outcomes of oral cancer early detection and prevention statewide model in maryland. Journal of Public Health Dentistry. 72:S34-S38.(2012)

98. S, W. Living with oral cancer: Epidemiology with particular reference to prevalence and life-style changes that influence survival. Oral Oncol. 46:407-410.(2010)

99. Warnakulasuriya S., Kovacevic T., Madden P, al., e. Factors predicting malignant transformation in oral potentially malig- nant disorders among patients accrued over a 10-year period in south east england. J Oral Pathol Med 40:677-683.(2011)

100. El-Naggar AK., Chan JKC., Grandis JR., Takata T., PJ, S. Who classification of head and neck tumours (iarc who classification of tumours). 4th edn. World Health Organization, 2017.(2017)

101. Warnakulasuriya, S., Johnson, N. W., Van Der Waal, I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. Journal of Oral Pathology & Medicine. 36(10):575-580.(2007)

102. Mortazavi, H., Baharvand, M., Mehdipour, M. Oral potentially malignant disorders: An overview of more than 20 entities. J Dent Res Dent Clin Dent Prospects. 8(1):6-14.(2014)

103. Yardimci, G., Kutlubay, Z., Engin, B., Tuzun, Y. Precancerous lesions of oral mucosa. World journal of clinical cases. 2(12):866-872.(2014)

104. E., S. Die idiopathischen schleimhautplaques der mundhöhle (leukoplakia buccalis). Arch Dermat Syph. 9:570-611.(1877)

105. George A, B., SS, Varghese SS, Thomas J, Gopakumar D, Mani V. Potentially malignant disorders of oral cavity. OMPJ. 2:95-100.(2011)

106. Axéll T., Pindborg JJ., Smith CJ., I, v. d. W. Oral white lesions with special reference to precancerous and tobacco-related lesions: Conclu- sions of an international symposium held in uppsala, sweden may18–21 1994. An international collaborative group on oral white lesions. J Oral Pathol Med 25:49-45.(1994)

107. Waal, I. v. d. Potentially malignant disorders of the oral and oropharyngeal mucosa; terminology, classification and present concepts of management. Oral Oncol. 45(4-5):317-323.(2009)

108. Bánóczy J, G. Z., Dombi C. Tobacco use and oral leukoplakia. J Dent Educ. 65:322-327.(2001)

109. RO, G. Pathology of malignant and premalignant oral epithelial lesions. Otolaryngol Clin North Am. 39:249-275.(2006)

110. Petti, S. Pooled estimate of world leukoplakia prevalence: A sys- tematic review. Oral Oncol 39:77-80.(2003)

111. Soares, A. B., Perschbacher, K., Perez-Ordonez, B. Oral potentially malignant disorders. Diagnostic Histopathology. 24(5):161-165.(2018)

112. McCullough, M. J., Prasad, G., Farah, C. S. Oral mucosal malignancy and potentially malignant lesions: An update on the epidemiology, risk factors, diagnosis and management. Aust Dent J. 55:61-65.(2010)

113. Napier SS, S. P. Natural history of potentially malignant oral lesions and conditions: An overview of the literature. J Oral Pathol Med. 37:1-10.(2008)

114. John G. Batsakisa, P. S., Adel K. El-Naggar. Proliferative verrucous leukoplakia and its related lesions. 35:354-359.(1999)

115. Capella, D. L., Gonçalves, J. M., Abrantes, A. A. A., Grando, L. J., Daniel, F. I. Proliferative verrucous leukoplakia: Diagnosis, management and current advances. Brazilian Journal of Otorhinolaryngology. 83(5):585-593.(2017)

116. Silverman S., M, G. Proliferative verrucous leukoplakia: A follow-up study of 54 cases. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 84:154-157.(1997)

117. Hansen LS., Olson JA., S, S. Proliferative verrucous leuko- plakia. A long-term study of thirty patients. Oral Surg Oral Med Oral Pathol. 60:285-298.(1985)

118. Bagan J., Jiménez Y., Murillo J., Poveda R., Díaz JM., *et al.* Epstein-barr virus in oral proliferative verrucous leuko- plakia and squamous cell carcinoma: A preliminary study. Med Oral Patol Oral Cir Bucal. 13:110-113.(2008)

119. Maymone, M. B. C., Greer, R. O., Kesecker, J., Sahitya, P. C., Burdine, L. K., *et al.* Premalignant and malignant oral mucosal lesions: Clinical and pathological findings. J Am Acad Dermatol. 81(1):59-71.(2019)

120. Holmstrup, P. White lesions oral leukoplakia, a premalignant lesion. London: Henry Stewart Talks,; 2017. Available from: https://ezp.lib.unimelb.edu.au/login?url=https://hstalks.com/bs/3546/ Connect to online video (University of Melbourne only)

121. Kumar, A., Cascarini, L., McCaul, J. A., Kerawala, C. J., Coombes, D., *et al.* How should we manage oral leukoplakia? British Journal of Oral and Maxillofacial Surgery. 51(5):377-383.(2013)

122. Tradati N, G. R., Calabrese L, et al. . Oral leukoplakias: To treat or not? Oral Oncol. 33:317-321.(1997)

123. Chandu A, S. A. The use of co2 laser in the treatment of oral white patches: Outcomes and factors affecting recurrence. Int J Oral Maxillofac Surg. 34:396-400.(2005)

124. van der Hem PS, N. J., van der Wal JE, Roodenburg JL. The results of co2 laser surgery in patients with oral leukoplakia: A 25 year follow up. Oral Oncol 41:31-37.(2005)

125. Chiesa F, T. N., Grigolato R, et al. Randomized trial of fenretinide (4-hpr) to prevent recurrences, new localizations and carcinomas in patients operated on for oral leukoplakia: Long-term results. Int J Cancer. 115:625-629.(2005)

126. Mulshine JL, A. J., Greer RO, et al. Randomized, double-blind, placebo- controlled phase iib trial of the cyclooxygenase inhibitor ketorolac as an oral rinse in oropharyngeal leukoplakia. Clin Cancer Res 10:1565-1573.(2004)

127. Gaballah K, H. A., Curiel D, Hallden G, Harrison P, Partridge M. . Lysis of dysplastic but not normal oral keratinocytes and tissue-engineered epithelia with conditionally replicating adenoviruses. Cancer Res. 67:7284-7294.(2007)

128. Rudin CM, C. E., Papadimitrakopoulou VA, et al. An attenuated adenovirus, onyx-015, as mouthwash therapy for premalignant oral dysplasia. J Clin Oncol. 21:4546-4552.(2003)

129. Kvaal SI, W. T. Photodynamic treatment of oral lesions. J Environ Pathol Toxicol Oncol. 26:127-133.(2007)

130. Axe´ ll T, H. P., Kramer IRH, Pindborg JJ, Shear M. International seminar on oral leukoplakia and associ- ated lesions related to tobacco habits. Community Dent Oral Epidemiol. 12:145-154.(1984)

131. Reichart PA, P. H. Oral erythroplakia--a review. Oral Oncol. 41:551-561.(2005)

132. Edwards PC, K. R. Oral lichen planus: Clinical presentation and management. Journal (Canadian Dental Association). 66:494-499.(2002)

133. Neville BW., Damm DD., Allen CR., JE, B. Oral and maxillofacial pathology. 2nd ed. Philadelphia: WB Saunders; 2002.2002l

134. Neville BW., TA, D. Oral cancer and precancerous lesions. CA Cancer J Clin. 52:195-215.(2002)

135. Shafer WG., CA, W. Erythroplakia of the oral cavity. Cancer. 36:1021-1028.(1975)

136. I, v. d. W. Potentially malignant disorders of the oral and oropharyngeal mucosa; present concepts of management. Oral Oncol 46:423-425.(2010)

137. Arakeri, G., Brennan, P. A. Oral submucous fibrosis: An overview of the aetiology, pathogenesis, classification, and principles of management. Br J Oral Maxillofac Surg. 51(7):587-593.(2013)

138. Dhariwal R, R. J., Pattanayak SM, Swain N. . Oral submucous fibrosis: A report of two pediatric cases and a brief review. J Indian Soc Pedod Prev Dent. 30:85-88.(2012)

139. Gupta VK, M. S., Patil R, Tripathi A. Oral submucous fibrosis at pediatric age, now time to think: Series of two cases. 34:107-110.(2013)

140. Mundra RK, G. S., Gupta Y. Oral submucous fibrosis in paediatric age group. Indian J Otolaryngol Head Neck Surg. 51:60-62.(1999)

141. Guo F, Jian XC., Zhou SH., Li N., Hu YJ., *et al.* A retrospective study of oral squamous cell carcinomas originated from oral submucous fibrosis. Zhonghua Kou Qiang Yi Xue Za Zhi. 46:494-497.(2011)

142. Wollina, U., Verma, S. B., Ali, F. M., Patil, K. Oral submucous fibrosis: An update. Clin Cosmet Investig Dermatol. 8:193-204.(2015)

143. Auluck A, R. M., Zhang L, Sumanth KN. . Oral submucous fibrosis, a clinically benign but potentially malignant disease: Report of 3 cases and review of the literature. J Can Dent Assoc 74:735-740.(2008)

144. Angadi PV, R. S. Areca nut in pathogenesis of oral submucous fibrosis: Revisited. Oral Maxillofac Surg 15:1-9.(2011)

145. R, R. Oral submucous fibrosis: Etiology, pathogenesis, and future research. Bull World Health Organ. 72:985-996.(1994)

146. R, R. Oral submucous fibrosis. J Oral Maxillofac Pathol. 7:1-4.(2003)

147. Wollina, U., Verma, S. B., Faridi, M., Patil, K. Oral submucous fibrosis: An update. Clinical, Cosmetic and Investigational Dermatology.(2015)

148. Haider SM, M. A., Fikree FF, Rahbar MH. Clinical and functional staging of oral submucous fibrosis. Br J Oral Maxillofac Surg. 38 (1):12-15.(2000)

149. Alam S, A. I., Giri KY, Gokkulakrishnan S, Natu SS, Faisal M, Agarwal A, Sharma H. Efficacy of aloe vera gel as an adjuvant treatment of oral submucous fibrosis. Oral Surg Oral Med Oral Pathol Oral Radiol 116:717-724.(2013)

150. Bhadage CJ, U. H., Shah K, Välimaa H. Vasodilator isoxsuprine alleviates symptoms of oral submucous fibrosis. Clin Oral Investig. 17:1375-1382.(2013)

151. Mehrotra R, S. H., Gupta SC, Singh M, Jain S. Pentoxifyl-line therapy in the management of oral submucous fibrosis. Asian Pac J Cancer Prev. 12:971-974.(2011)

152. Sharma AK, G. R., Gupta HP, Singh AK. Haemodynamic effects of pan masala in healthy volunteers. J Assoc Physicians India. 48 (4):400-401.(2000)

153. Jacob BJ, S. K., Thomas G, et al. Betel quid without tobacco as a risk factor for oral precancers. Oral Oncol. 40(7):697–704.(2004)

154. Gupta, P. C., Warnakulasuriya, S. Global epidemiology of areca nut usage. Addict Biol. 7(1):77-83.(2002)

155. IARC. Monographs on the evaluation of carcinogenic risks to humans. Betel-quid and areca-nut chewing and some areca-nut-derived nitrosamines. 85 ed: Lyon, IARC Press; 2004.2004l

156. Williams, S., Malik, A., Chowdhury, S., Chauhan, S. Sociocultural aspects of areca nut use. Addict Biol. 7(1):147-154.(2002)

157. Hosein, M., Mohiuddin, S., Fatima, N. Association between grading of oral submucous fibrosis with frequency and consumption of areca nut and its derivatives in a wide age group: A multi-centric cross sectional study from karachi, pakistan. Journal of cancer prevention. 20(3):216-222.(2015)

158. Lee, C. H., Ko, Y. C., Huang, H. L., Chao, Y. Y., Tsai, C. C., *et al.* The precancer risk of betel quid chewing, tobacco use and alcohol consumption in oral leukoplakia and oral submucous fibrosis in southern taiwan. British journal of cancer. 88(3):366-372.(2003)

159. Zain, R. B., Ikeda, N., Razak, I.A., Axéll, T., Majid, Z.A., Gupta, P.C. Yaacob, M. A national epidemiological survey of oral mucosal lesions in malaysia. Community dent. Oral epidemiol. Community dent. oral
Epidemiol. 25,:377–383.(1997)

160. Fotlona, W. Hubungan status kesehatan periodontal dengan kebiasaan menyirih pada mahasiswa etnis papua di manado.
Https://ejournal.Unsrat.Ac.Id/index.Php/egigi/article/view/3156. 2018(November 15th).(2013)

161. Budhy, T. I., Soenarto, S.D., Yaacob, H.B. & Ngeow, W.C. . Changing incidence of oral and maxillofacial tumours in east java, indonesia,part 2: Malignant tumours. J. oral maxillofac. Surg. 39:460–464.(2001)

162. Hazare VK, G. R., Gupta PC. Oral submucous fibrosis, areca nut and pan masala use: A case-control study. Natl Med J India. 11(6):299.(1998)

163. Gupta PC, S. P., Bhonsle RB, Pawar VS, Mehta HC. Oral submucous fibrosis in india: A new epidemic? Natl Med J India. 11(3):113–116.(1998)

164. Shah N, S. P. Role of chewing and smoking habits in the etiology of oral submucous fibrosis (osf): A case-control study. J Oral Pathol Med. 27(10)(475–479).(1998)

165. Babu S, B. R., Kumar PU, et al. A comparative clinico-pathological study of oral submucous fibrosis in habitual chewers of pan masala and betelquid. . J Toxicol Clin Toxicol. 34(3):317–322:317–322.(1996)

166. Bathi RJ, R. R., Mutalik S. Gst null genotype and antioxidants: Risk indicators for oral pre-cancer and cancer. Indian journal of dental research : official publication of Indian Society for Dental Research. 20(3):298–230.(2009)

167. Nair U, B. H., Nair J. Alert for an epidemic of oral cancer due to use of the betel quid substitutes gutkha and pan masala: A review of agents and causative mechanisms. Mutagenesis. 19(4):251–262.(2004)

168. Misra SP, M. V., Dwivedi M, Gupta SC. Oesophageal subepithelial fibrosis: An extension of oral submucosal fibrosis. Postgrad Med J. 74(878):733–736.(1998)

169. SR, A. Oral submucous fibrosis: Case report and review of diagnosis and treatment. J Oral Maxillofac Surg 66:2386–2389.(2008)

170. Tilakaratne WM, K. M., Saku T, Peters TJ, Warnakulasuriya S. Oral submucous fibrosis: Review on aetiology and pathogenesis. 42:561-568.(2006)

171. Utsunomiya H, T. W., Oshiro K, et al. Extracellular matrix remodeling in oral submucous fibrosis: Its stage-specific modes revealed by immunohistochemistry and in situ hybridization. J Oral Pathol Med. 34:498–507.(2005)

172. Rajalalitha P, V. S. Molecular pathogenesis of oral submucous fibrosis—a collagen metabolic disorder. J Oral Pathol Med. 34:321-328.(2005)

173. Ma RH, T. C., Shieh TY. Increased lysyl oxidase activity in fibroblasts cultured from oral submucous fibrosis associated with betel nut chewing in taiwan. J Oral Pathol Med. 24:407-412.(1995)

174. Trivedy CR, W. K., Peter TJ, Senkus R, Hazarey VK. Raised tissue copper levels in oral submucous fibrosis. J Oral Pathol Med 29:241–248.(2000)

175. Khanna JN, A. N. Oral submucous fibrosis: A new concept in surgical management. Report of 100 cases. Int J Oral Maxillofac Surg. 24:433-439.(1995)

176. Sarode, S. C., Mahuli, A., Sarode, G. S., Mahuli, S. Why only areca nut chewing cannot cause oral submucous fibrosis? Med Hypotheses. 81(1):47-49.(2013)

177. Steven J. Thomas, R. M. Slaked lime and betel nut cancer in papua new guinea. Lancet. 340:577-578.(1992)

178. Chiang CP, H. R., Chen TH. High incidence of autoantibodies in taiwanese patients with oral submucous fibrosis. J Oral Pathol Med. 31:402-409.(2002)

179. R, R. Oral submucous fibrosis. J Oral Maxillofac Pathol 7:1-4.(2003)

180. Dufresne RG, C. M. Actinic cheilitis. A treatment review. Dermatol Surg. 23:15-21.(1997)

181. Martins-Filho PR, D. S. L., Piva MR. The prevalence of actinic cheilitis in farmers in a semi-arid northeastern region of brazil. Int J Dermatol. 50:1109-1114.(2011)

182. Vieira RA, M. E., Marques ME, Marques SA. Actinic cheilitis and squamous cell carcinoma of the lip: Clinical, his- topathological and immunogenetic aspects. An Bras Dermatol. 87:105-114.(2012)

183. de Santana Sarmento DJ, d. C. M. M., Queiroz LM, Godoy GP, da Silveira EJ. Actinic cheilitis: Clinicopathologic profile and association with degree of dysplasia. Int J Dermatol. 53:466-472.(2014)

184. Epstein JB, W. L., Gorsky M, Zhang L. Oral lichen planus: Progress in understanding its malignant potential and implications for clinical management. Oral Surg Oral Med Oral Pathol Oral Radiol. 96:32-37.(2003)

185. Fornatora M, J. A., Kerpel S, Freedman P. Human papillomavirus-associated oral epithelial dysplasia (koilocytic dysplasia). An entity of unknown biologic potential. . Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 82:47-56.(1996)

186. van der Meij EH, S. K.-P., van der Wall I. The possible malignant character of oral lichen planus and oral lichenoid lesions: A prospective study. Oral Surg Oral Med Oral Pathol 96:164-171.(2003)

187. S, S. Oral lichen planus: A potentially premalignant lesion. J Oral Maxillofac Surg. 58:1286-1288.(2000)

188. Thornhill MH, S. V., Xu XJ, et al. The role of histopathological characteristics in distinguishing amal- gam-associated oral lichenoid reactions and oral lichen planus. J Oral Pathol Med. 35:233-240.(2006)

189. Sazwi, N. N., Nalina, T., Abdul Rahim, Z. H. Antioxidant and cytoprotective activities of piper betle, areca catechu, uncaria gambir and betel quid with and without calcium hydroxide. BMC Complement .Altern. Med. 13:351.(2013)

190. Fathilah AR, S. R., Norhanom AW, Adenan MI. Antiproliferative activity of aqueous extract of piper betle l. And psidium guajava l. On kb and hela cell lines. J Med Plants Res. 4:987-990.(2010)

191. Nalina T, R. Z. The crude aqueous extract of piper betle l. And its antibacterial effect towards streptococcus mutans. Am J Biotechnol Biochem. 3:10-15.(2007)

192. Arambewela LSR, A. L., Ratnasooriya WD. Antidiabetic activities of aqueous and ethanolic extracts of piper betle leaves in rats. J Ethnopharmacol. 102:239-245.(2005)

193. Hajare R, D. V., Shewale A, Patil V. Evaluation of antihistaminic activity of piper betel leaf in guinea pig. Afr J Pharm Pharmacol. 5:113-117.(2011)

194. Bhandare AM, K. A., Vyawahare NS, Hadambar AA, Thorve VS. Potential analgesic, anti-inflammatory and antioxidant activities of hydroalcoholic extract of areca catechu l. Nut. Food Chem Toxicol. 48:3412–3417.(210)

195. Azeez S, A. S., Adiga S, Rao N, Rao N, Udupa LA. Wound healing profile of areca catechu extracts on different wound models in wistar rats. Kuwait Med J. 39:48-52.(2007)

196. Dar A, K. S. Behavioral and biochemical studies of dichloromethane fraction from the areca catechu nut. Pharmacol Biochem Behav. 65:1-6.(2000)

197. Vermani K, G. S. Herbal medicines for sexually transmitted diseases and aids. J Ethnopharmacol. 80:49-66.(2002)

198. Dasgupta N, D. B. Antioxidant activity of piper betle l. Leaf extract in vitro. Food Chem. 88:219-222.(2004)

199. Li CC, L. E. Antiradical capacity and reducing power of different extraction method of areca catechu seed. Afr J Biotechnol. 9:7831-7836.(2010)

200. Lim YY, L. T., Tee JJ. Antioxidant properties of several tropical fruits: A comparative study. . Food Chem. 103:1003-1008.(2007)

201. Agoramoorthy G, C. F., Venkatesalu V, Kuo DH, Shea PC. Evaluation of antioxidant polyphenols from selected mangrove plants of india. Asian J Chem. 20:1311–1322.(2008)

202. Devasagayam TPA, T. J., Boloor KK. Review: Free radicals and antioxidants in human health: Current status and future prospects. J Assoc Physician India. 52:794-804.(2004)

203. Anani K, H. J., De-Souza C, Akpagana K, Tower GHN, Arnason JT, Gbeassor M. Investigation of medicinal plants of togo for antiviral and antimicrobial activities. J Pharm Biol 38:40-45.(2000)

204. Craciunescu O, C. D., Gaspar A, Toma L, Utoiu E, Moldovan L. Evaluation of antioxidant and cytoprotective activities of arnica montana l. And artemisia absinthium l. Ethanolic extracts. Chem Cent 6:97.(2012)

205. Kondo S, Y. H., Miwa N. Cytoprotective effect of fruit extracts associated with antioxidant activity against ultraviolet rays. Food Chem 104:1272–1276.(2007)

206. Saeed N, K. M., Shabbir M. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts torilis leptophylla l. BMC Complem Altern M. 12:221.(2012)

207. Zhang, W. M., Huang, W. Y., Chen, W. X., Han, L., Zhang, H. D. Optimization of extraction conditions of areca seed polyphenols and evaluation of their antioxidant activities. Molecules. 19(10):16416-16427.(2014)

208. Zhang, X., Wu , J., Han , Z., Mei , W. L., Dai, H. F. Antioxidant and cytotoxic phenolic compounds of areca nut(areca catechu). Chem Res Chin University 26:161-184.(2010)

209. O. Folin, W. D. A colorimetric estimation of phenol and phenol and derivatives in urine. J. Biol. Chem. 22:305-308.(1915)

210. Singleton, V. L., Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents,. Am. J. Enol. Vitic. 16:144-158.(1965)

211. V. Singleton, R. O., R.M. Lamuela-Raventos. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteau reagent. Methods Enzymol. 299:152–178.(1999)

212. BENZIE IFF, JJ, S. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of

total antioxidant power and ascorbic acid concentration by iris f. F. Benzie and j. J. Strain. Methods in Enzymology. 299:15-27.(1996)

213. Gadow , A., Joubert, E., Hansmann, C. F. J. Comparison of the antioxidant activity of aspalathin with that of other plant phenols of rooibos tea (aspalathus linearis), α-tocopherol, bht, and bha. J Agric Food Chem. 45:632.(1997)

214. Elleuch, H., Mihoubi, W., Mihoubi, M., Ketata, E., Gargouri, A., *et al.* Potential antioxidant activity of morita-baylis-hillman adducts. Bioorganic Chemistry. 78:24-28.(2018)

215. D. Villaño, M. S. F. n.-P. n., M.L. Moyá, A.M. Troncoso, M.C. García- Parrilla. Radical scavenging ability of polyphenolic compounds towards dpph free radical. Talanta. 71(1):230-235.(2007)

216. N. Liang, D. D. K. Antioxidant property of coffee components: Assessment of methods that define mechanisms of action. Molecules. 19(11):19180-19208.(2014)

217. Amonkar, A. J., Padma, P. R., Bhide, S. V. Protective effect of hydroxychavicol, a phenolic component of betel leaf, against the tobacco-specific carcinogens. Mutat Res. 210:249-253.(1989)

218. Chavan, Y. V., Singhal, R. S. Separation of polyphenols and arecoline from areca nut (areca catechu l.) by solvent extraction, its antioxidant activity, and identification of polyphenols. J Sci Food Agric. 93(10):2580-2589.(2013)

219. Cox, S., Piatkov, I., Vickers, E. R., Ma, G. High-performance liquid chromatographic determination of arecoline in human saliva. J Chromatogr. 1032(1-2):93-95.(2004)

220. Jain, V., Garg, A., Parascandola, M., Chaturvedi, P., Khariwala, S. S., *et al.* Analysis of alkaloids in areca nut-containing products by liquid chromatography-tandem mass spectrometry. J Agric Food Chem. 65(9):1977-1983.(2017)

221. Li, L., Luo, Z., Liu, Y., Wang, H., Liu, A., *et al.* Screening and identification of the metabolites in rat plasma and urine after oral administration of areca catechu l. Nut extract by

ultra-high-pressure liquid chromatography coupled with linear ion trap-orbitrap tandem mass spectrometry. Molecules. 22(6).(2017)

222. Lu Y, T. L., He Y, Lu Y, Liang X, et al Development and optimization of a rp-hplc method to quantify midazolam in rat plasma after transdermal administration: Validation and application in pharmacokinetic study. Pharm Anal Acta. 6:329.(2015)

223. Tyagi A, S. N., Mittal K, Mashru R, Bhardwaj T, et al. Hptlc-densitometric and rp-hplc method development and validation for determination of salbutamol sulphate, bromhexine hydrochloride and etofylline in tablet dosage forms. Pharm Anal Acta. 6:350.(2015)

224. Nardulli P, C. E., Perrone MG, Ferraiuolo S, Laforgia MR A combined hplc and lc-ms approach for evaluating drug stability in elastomeric devices: A challenge for the sustainability in pharmacoeconomics. J Pharmacovigilance. 3:57.(2015)

225. Singh A, T. S., Sand NK. Active ingredient estimation of clopyralid formulation by reversed phase hplc. J Chromatogr Sep Tech. 6:257.(2014)

226. Lovekesh Mehta, J. S. Rp-hplc method development and validation for the determination of bupropion hydrochloride in a solid dosage form. Research and Reviews: Journal of Pharmaceutical Analysis 2.(2013)

227. Sassi A, H. A., Kallel M, Jaidane M, Saguem S. Hplc method for quantification of halofuginone in human ureter: Ex-vivo application. J Chromatogr Sep Tech. 6:255.(2014)

228. Ezhilarasi K, S. V., Ramachandran G, Umapathy D, Rajaram R, et al. A simple and specific method for estimation of lipoic acid in human plasma by high performance liquid chromatography. J Chromatogr Sep Tech. 5:245.(2014)

229. H, S. Role of metastable and spore hydration to sterilize spores by nitrogen gas plasma exposure and dpa analysis by hplc and uv. Pharmaceut Reg Affairs. 3:125.(2014)

230. Divya, T. High performance liquid chromatography in analysis. RRJPPS. 3(2):73-75

231. Mustafa S, H. W., Khaliq S, Azizuddin, Khan RA An improved high performance liquid chromatographic method for tryptophan analysis in rat brain administrated by seaweed. J Anal Bioanal Tech. 5:188.(2014)

232. Caglar S, A. A. A validated high performance liquid chromatography method for the determination of saxagliptin and metformin in bulk, a stability indicating study. J Anal Bioanal Tech. 12:10.(2014)

233. Shah I, B. J., Barton SJ, Naughton DP A novel method for determination of fenofibric acid in human plasma using hplc-uv: Application to a pharmacokinetic study of new formulations. J Anal Bioanal Tech. S12:009.(2014)

234. Paranthaman R, K. S. A reversed-phase high- performance liquid chromatography (rp-hplc) determination of pesticide residues in tender coconut water (elaneer/nariyal pani). J Chromatograph Separat Techniq 4:208.(2013)

235. Yang, W. Q., Wang, H.C., Wang, W.J., Wang, Y., Zhang, X.Q., Ye, W.C. Chemical constituents from the fruits of areca catechu. J Chin Med Materials ([in Chinese]). 35:400-402.(2012)

236. Gross, J. H. Mass spectrometry, second ed. Heidelberg.(2011)

237. Kruve, A., Rebane, R., Kipper, K., Oldekop, M. L., Evard, H., *et al.* Tutorial review on validation of liquid chromatography-mass spectrometry methods: Part i. Anal Chim Acta. 870:29-44.(2015)

238. B.W. Brooks, D. B. H. Human pharmaceuticals in the environment. Current and future perspectives2012.2012l

239. M. Jemal, Y. Q. X. Lc–ms development strategies for quantitative bioanalysis. Curr. Drug Metab. 7:491–502.(2006)

240. Koek, M. M., Jellema, R. H., van der Greef, J., Tas, A. C., & Hanke- meier, T. Quantitative metabolomics based on gas chro- matography mass spectrometry: Status and perspectives. Metabolomics. 7:307-328.(2011)

241. Mastrangelo, A., Ferrarini, A., Rey-Stolle, F., García, A., & Barbas, C. From sample treatment to biomarker discovery: A tutorial for untargeted metabolomics based on gc-(ei)-q-ms. Analytica Chimica Acta. 900:21-35.(2015)

242. Villas-Bôas, S. G., Mas, S., Akesson, M., Smedsgaard, J., & Nielsen, J. Mass spectrometry in metabolome analysis. Mass Spectrometry Reviews. 24:613-646.(2005)

243. Tsugawa, H., Tsujimoto, Y., Arita, M., Bamba, T., Fukusaki, E., *et al.* Gc/ms based metabolomics: Development ofa data mining system for metabolite identification by using soft independent modeling of class analogy (simca). BMC Bioinformatics. 12:131-131.(2011)

244. Beale, D. J., Pinu, F. R., Kouremenos, K. A., Poojary, M. M., Narayana, V. K., *et al.* Review of recent developments in gc-ms approaches to metabolomics-based research. Metabolomics. 14(11):152.(2018)

245. Martens, L., Chambers, M., Sturm, M., Kessner, D., Levander, F., *et al.* Mzml—a community standard for mass spectrometry data. Molecular & Cell Proteomics. 10:R110.000133.(2011)

246. Adusumilli, R., & Mallick, P. Data conversion with pro- teowizard msconvert. Methods Mol Biol. 1550:339-368.(2017)

247. Madhumita, M., Guha, P., & Nag, A. Extraction of betel leaves (piper betle l.) essential oil and its bio-actives identification: Process optimization, gc-ms analysis and anti-microbial activity. Industrial Crops and Products. 138.(2019)

248. Meisam Omidi, A. F., Masomeh Farahani, Zahra Akbari, Saleheh Shahmoradi, FatemehYazdian, Mohammadreza Tahriri, Keyvan Moharamzadeh, Lobat Tayebi, Daryoosh

Vashaee. 7 - characterization of biomaterials. Biomaterials for Oral and Dental Tissue Engineering.97-115.(2017)

249. Kearsley, t. T. a. J. Betel quid and oral cancer: A review. Oral Oncol, Eur J Cancer. 298 No.4:251-255.(1992)

250. Soumitra Sen, G. T., Archana Sharma Betel cytotoxicity. Journal of Ethnopharmacology.217 - 247.(1989)

251. Yeh, C. Y., Chen, H. M., Chang, M. C., Kok, S. H., Lee, J. J., *et al.* Cytotoxicity and transformation of c3h10t1/2 cells induced by areca nut components. Journal of the Formosan Medical Association = Taiwan yi zhi. 115(2):108-112.(2016)

252. IARC. Monographs on the evaluation of carcinogenic risk of chemicals to humans 37, iarc, lyon.(1985)

253. Tseng, S.-K., Chang, M.-C., Hsu, M.-L., Su, C.-Y., Chi, L.-Y., *et al.* Arecoline inhibits endothelial cell growth and migration and the attachment to mononuclear cells. Journal of Dental Sciences. 9(3):258-264.(2014)

254. Van Wyk CW, O. A., De Miranda CM, Van Der Bijl P, Grobler-Rabie AF. Observation on the effect of areca nut extracts on oral fibroblast proliferation. J Oral Pathol Med. 23:145-148.(1994)

255. Elias ST, Diniz J, Rubens S.S., Almeida, Alvarenga N, *et al.* Cytotoxic effect of tobacco extract on human oral squamous cell carcinoma cell-line. Oral oncology. 46:869-873.(2010)

256. Harvey W, S. A., S. Mechji A,S. J. P. Caniff J.P Stimulation of human buccal mucosa fibrobalst in vitro by betel nut alkaloids

. Archs oral Bid. 31:45-49.(1986)

257. Lee, P. H., Chang, M. C., Chang, W. H., Wang, T. M., Wang, Y. J., *et al.* Prolonged exposure to arecoline arrested human kb epithelial cell growth: Regulatory mechanisms of cell cycle and apoptosis. Toxicology. 220(2-3):81-89.(2006)

258. Mao, L. Steps to further reduce oral cancer burden for marylanders. J Public Health Dent. 72 Suppl 1:S39-40.(2012)

259. Warnakulasuriya, S., Johnson, N. W., van der Waal, I. Nomenclature and classification of potentially malignant disorders of the oral mucosa. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 36(10):575-580.(2007)

260. Riset kesehatan dasar 2010. p. http://labdata.litbang.depkes.go.id/riset-badan-litbangkes/menu-riskesnas/menu-riskesdas/148-rkd-2010. 2016 (February 1st)

261. Penyajian pokok-pokok hasil riset dasar kesehatan 2013. http://kesga.kemkes.go.id/images/pedoman/Data%20Riskesdas%202013.pdf. 2016 (January 25th).

262. Wang C.K., Su H.Y., Lii C.K. Chemical composition and toxicity of taiwanese betel quid extract. Food Chem Toxicol. 37:135-144.(1999)

263. Kammerer, D., Claus,A., Carle, R., Schieber, A. Polyphenol screening of pomace from red and white grape varieties (vitis vinifera l.) by hplc-dad. J Agric Food Chem. 52:4360-4367.(2004)

264. Singleton, V., & Rossi, JR, J. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. J. Enol. Viticult. 16:144-158.(1965)

265. Granato, D., Santos, J. S., Maciel, L. G., Nunes, D. S. Chemical perspective and criticism on selected analytical methods used to estimate the total content of phenolic compounds in food matrices. TrAC Trends in Analytical Chemistry. 80:266-279.(2016)

266. T. Margraf, A. R. K., N.D. Rosso, D. Granato. Comparison between folin-ciocalteu and prussian blue assays to estimate the total phenolic content of juices and teas using 96-well microplates. J. Food Sci. 80:2397–2403.(2015)

267. Medina, M. B. Simple and rapid method for the analysis of phenolic compounds in beverages and grains. J. Agric. Food Chem. 59:1565–1571.(2011)

268. Benzie, I., & Strain, J. The ferric reducing ability of plasma (frap) as a measure of ‘‘antioxidant power’’: The frap assay. Analytical Biochemistry. 239(292): 70-76.(1996)

269. Chavan, Y., & Singhal, R. . Separation of polyphenols and arecoline from areca nut (areca catechu l.) by solvent extraction, its antioxidant activity, and identification of polyphenols. Journal of the science of food and agriculture. 93:2580–2589.(2013)

270. Berkov, S., Bastida, J., Viladomat, F., Codina, C. Development and validation of a gc–ms method for rapid determination of galanthamine in leucojum aestivum and narcissus ssp.: A metabolomic approach. Talanta. 83(5):1455-1465.(2011)

271. Stich H.F, S.S, T. Promoting activity of betel quid ingredients and their inhibition by retinol. Cancer letter. 45:71-77.(1989)

272. Gonzales-Reyes, S., Guzman-Beltran, S., Medine-Campos, O. N., Pedraza-Chaverri, J. Curcumin pretreatment induces nrf2 and an antioxidant response and prevents hemin-induced toxicity in primary cultures of cerebellar granule neurons of rats. Oxid Med Cell Longev.(2013)

273. Prime, S. S., Nixon, S. V., Crane, I. J., STONE, A., , Matthews, J. B., Maitland, N. J., *et al.* The behaviour of human oral squamous cell carcinoma in cell culture. J Pathol. 160:259-269.(1990)

274. Mortazavi, H. B., M. Mehdipour, M. Oral potentially malignant disorders: An overview of more than 20 entities. J Dent Res Dent Clin Dent Prospect. 8(1):6-14.(2014)

275. Sciubba, J. J. Oral cancer. The importance of early diagnosis and treatment. Am J Clin Dermatol. 2(4):239-251.(2001)

276. Petti, S. Pooled estimate of world leukoplakia prevalence: A systematic review. Oral Oncol. 39(8):770-780.(2003)

277. J. Ferlay, H.-R. S., F. Bray, D. Forman, C. Mathers, and D. M. Parkin. Estimates of worldwide burden of cancer in 2008 globocan 2008. International Journal of Cancer. 127(12):2893-2917.(2010)

278. Sharma, N., Om, H. Usage of probabilistic and general regression neural network for early detection and prevention of oral cancer. The Scientific World Journal. 2015:1-11.(2015)

279. A. Jemal, R. S., J. Xu, and E. Ward. Cancer statistics, 2010. CA: A Cancer Journal for Clinicians. 60. no.5:277-300.(2010)

280. R. Sankaranarayanan, E. M., R. Swaminathan, J. Ferlay, and S. Whelan. Head and neck cancer: A global perspective on epidemiology and prognosis. Anticancer Research. 18, no. 6:4779–4786.(1998)

281. Crossman, T., Warburton, F., Richards, M. A., Smith, H., Ramirez, A., *et al*. Role of general practice in the diagnosis of oral cancer. Br J Oral Maxillofac Surg. 54(2):208-212.(2016)

282. Gigliotti, J., Madathil, S., Makhoul, N. Delays in oral cavity cancer. Int J Oral Maxillofac Surg. 48(9):1131-1137.(2019)

283. R. Sankaranarayanan a, L. F. G., J. Lence Anta , P. Pisani a, A. Rodriguez Salva Visual inspection in oral cancer screening in cuba: A case-control study. Oral Oncology 38:131-138.(2001)

284. Yellowitz, J. A., Goodman, H. S. Assessing physicians' and dentists' oral cancer knowledge, opinions and practices. J Am Dent Assoc. 126(1):53-60.(1995)

285. Lutfan Lazuardia, G. Y. S., Ika Candradewib Åsa Holmnerc. Automatic platelets counter for supporting dengue case detection in primary health care in indonesia. MEDINFO 2013.585-588.(2013)

286. Rahardjo, A., Maharani, D. A. A review of indonesia's dental health - past, present and future. International Journal of Clinical Preventive Dentistry. 10(3):121-126.(2014)

287. Warnakulasuriya S, F. N., Diz P, Seoane J, Rapidis A. . An appraisal of oral cancer and pre-cancer screening programmes in europe: A systematic review. J Oral Pathol Med.(2014)

288. Soendoro, T. Report on result of national basic health research (riskesdas) 2013: Dental health. 1st ed. . Jakarta: The National Institute of Health Research and Development Ministry of Health Republic of Indonesia. 1st ed:110-119.(2013)

289. Irwin JY, T. T., Spallek H, Wali T, Kerr AR, Schleyer T. . English and spanish oral cancer information on the internet: A pilot surface quality and content evaluation of oral cancer web sites. J Public Health Dent. 71:106-116.(2011)

290. Rethman MP, C. W., Cohen EE, Epstein J, Evans CA, Flaitz CM, et al. . Evidence-based clinical recommendations regard- ing screening for oral squamous cell carcinomas. J Am Dent Assoc. 141:509-520.(2010)

291. Soendoro, T. Report on result of national basic health research (riskesdas) 2007. Dental Health. 1st ed. Jakarta: The National Institute of Health Research and Development Ministry of Health Republic of Indonesia.128-146.(2008)

292. ISMPI. Tentang ispmi. 2018.

293. Asgari, F., Majidi, A., Koohpayehzadeh, J., Etemad, K., Rafei, A. Oral hygiene status in a general population of iran, 2011: A key lifestyle marker in relation to common risk factors of non-communicable diseases. Int J Health Policy Manag. 4(6):343-352.(2015)

294. Azhar, N., Doss, J. G. Health-seeking behaviour and delayed presentation of oral cancer patients in a developing country: A qualitative study based on the self-regulatory model. Asian Pac J Cancer Prev. 19(10):2935-2941.(2018)

295. Sreedhar, G., Narayanappa Sumalatha, M., Shukla, D. An overview of the risk factors associated with multiple oral premalignant lesions with a case report of extensive field cancerization in a female patient. Biomedical Papers. 159(2):178-183.(2015)

296. Awan, K. Oral cancer: Early detection is crucial. Journal of international oral health : JIOH. 6(5):i-ii.(2014)

297. Parkin DM, W. S., Ferlay J, Teppo L, Thomas DB. Eds. Cancer incidence in five continents. Cancer incidence in five continents. Lyon: IARC Press.(2002)

298. Prakash C. Gupta , Jens J. Pindborg*, R. B. Bhonsle, P. R. Murti, Fali S. Mehta, *et al.* Intervention study for primary prevention of oral cancer among 36000 indian tobacco users. The Lancet. 31;1(8492):1235-1239.(1986)

299. Paulino, Y. C., Hurwitz, E. L., Warnakulasuriya, S., Gatewood, R. R., Pierson, K. D., *et al.* Screening for oral potentially malignant disorders among areca (betel) nut chewers in guam and saipan. BMC oral health. 14:151.(2014)

300. Nagao, T., Warnakulasuriya, S., Ikeda, N., Fukano, H., Fujiwara, K., *et al.* Oral cancer screening as an integral part of general health screening in tokoname city, japan. J Med Screen. 7(4):203-208.(2000)

301. Lim, K., Moles, D. R., Downer, M. C., Speight, P. M. Opportunistic screening for oral cancer and precancer in general dental practice: Results of a demonstration study. British dental journal. 194(9):497-502; discussion 493.(2003)

302. Conway, D. I., Petticrew, M., Marlborough, H., Berthiller, J., Hashibe, M., *et al.* Socioeconomic inequalities and oral cancer risk: A systematic review and meta-analysis of case-control studies. International Journal of Cancer. 122(12):2811-2819.(2008)

303. Warnakulasuriya, S. Significant oral cancer risk associated with low socioeconomic status. Evid Based Dent. 10(1):4-5.(2009)

304. A Anis Najiha, Nadiah, W. A. W. Alkohol (arak dan etanol) dalam makanan halal Jurnal Intelek. 9(1):40-51.(2014)

305. Wang, M., Xiao, C., Ni, P., Yu, J. J., Wang, X. W., *et al.* Correlation of betel quid with oral cancer from 1998 to 2017: A study based on bibliometric analysis. Chinese medical journal. 131(16):1975-1982.(2018)

306. Ananta, A., Utami, D. R. W. W., Handayani, N. B. Statistics on ethnic diversity in the land of papua, indonesia. Asia & the Pacific Policy Studies. 3(3):458-474.(2016)

307. IARC. Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Some naturally occurring substances (1976)

308. Roemer, E., Dempsey, R., Schorp, M. K. Toxicological assessment of kretek cigarettes. Regulatory Toxicology and Pharmacology. 70:S2-S14.(2014)

309. Guidotti, T. L. Critique of available studies on the toxicology of kretek smoke and its constituents by routes of entry involving the respiratory tract. . Arch. Toxicol. 63:7-12.(1989)

310. Piade, J. J., Roemer, E., Dempsey, R., Hornig, G., Deger Evans, A., *et al.* Toxicological assessment of kretek cigarettes: Part 2: Kretek and american-blended cigarettes, smoke chemistry and in vitro toxicity. Regul Toxicol Pharmacol. 70 Suppl 1:S15-25.(2014)

311. Piade, J. J., Roemer, E., Dempsey, R., Weiler, H., Meurrens, K., *et al.* Toxicological assessment of kretek cigarettes part 3: Kretek and american-blended cigarettes, inhalation toxicity. Regul Toxicol Pharmacol. 70 Suppl 1:S26-40.(2014)

312. Roemer, E., Dempsey, R., Lawless-Pyne, J., Lukman, S., Evans, A. D., *et al.* Toxicological assessment of kretek cigarettes part 4: Mechanistic investigations, smoke chemistry and in vitro toxicity. Regul Toxicol Pharmacol. 70 Suppl 1:S41-53.(2014)

313. Ogden, G. R. Alcohol and mouth cancer. British dental journal. 225(9):880-883.(2018)

314. Paramita, T. Saguer, arak khas minahasa yang masih laris manis. 2017.

315. Humphris GM, F. E. The immediate effect on knowledge, attitudes and intentions in primary care attenders of a patient information leaflet: A randomized control trial replication and extension. . Brit Dent J. 194:683-688.(2003)

316. Humphris GM, I. R., Field EA. Randomised trial of the psychological effect of information about oral cancer in primary care settings. . Oral Oncol. 37:548-552.(2001)

317. Scully, C., Bedi, R. Ethnicity and oral cancer. The Lancet Oncology. 1(1):37-42.(2000)

318. Auluck, A., Walker, B. B., Hislop, G., Lear, S. A., Schuurman, N., *et al.* Socio-economic deprivation: A significant determinant affecting stage of oral cancer diagnosis and survival. BMC Cancer. 16:569.(2016)

319. Cheong, S., Vatanasapt, P., Yang, Y.-H., Zain, R., Kerr, A., & Johnson, N. Oral cancer in south east asia: Current status and future directions. Translational Research in Oral Oncology. 1-9.(2017)

320. Sari, E. F., McCullough, M., Cirillo, N. Oral pre-malignant and malignant lesion detection among indonesian ; the prevalence and risk factors. Head & Neck. 39 E58.(2017)

321. Angadi, P. V., Rao, S. S. Areca nut in pathogenesis of oral submucous fibrosis: Revisited. Oral and Maxillofac Surg. 15(1):1-9.(2010)

322. Arakeri, G., Rai, K. K., Hunasgi, S., Merkx, M. A. W., Gao, S., *et al.* Oral submucous fibrosis: An update on current theories of pathogenesis. J Oral Pathol Med. 46(6):406-412.(2017)

323. Franke, A. A., Lai, J. F., Kawamoto, C. T., Pokhrel, P., Herzog, T. A. University of hawai'i cancer center connection: Areca (betel) nut consumption: An underappreciated cause of cancer. Hawai'i journal of medicine & public health : a journal of Asia Pacific Medicine & Public Health. 73(12):400-403.(2014)

324. Reichart, P. A. Toothpastes containing betel nut (areca catechu l.) from england of the nineteenth century. Journal of the history of medicine and allied sciences. 39(1):65-68.(1984)

325. Maisuthisakul, P., Suttajit, M., Pongsawatmanit R. Assessment of phenolic content and free radical-scavenging capacity of some thai indigenous plants. Food Chem.1409-1428.(2007)

326. Antonio J. León-González, C. A., Valérie B. Schini-Kerth. Pro-oxidant activity of polyphenols and its implication on cancer chemoprevention and chemotherapy. Biochem Pharmacol. 371-380(98).(2015)

327. Park, E. J., Pezzuto J.M. Flavonoids in prevention, anticancer agents. Med. Chem. 12:836-851.(2012)

328. Prochazkova D, Bousova I, N, W. Antioxidant and prooxidant properties of flavonoids. Fitoterapia. 82:513-523.(2011)

329. Elbling, L., Weiss , R. M., T, eufelhofer, O., Uhl, M., Knasmueller, S., *et al.* Green tea extract and (-)-epigallocatechin-3-gallate, the major tea catechin, exert oxidant but lack antioxidant activities. FASEB J. 19:807–809.(20015)

330. Galati, G., Lin, A., Sultan, A. M., O'Brien, P. J. Cellular and in vivo hepatotoxicitycaused by green tea phenolic acids and catechins. Free Radical Biol. Med. 570-580.(2006)

331. Lambert, J. D., Kennett, M. J., Sang, S., Reuhl, K. R., Ju, J., *et al.* Hepatotoxicity of high oral dose (−)-epigallocatechin-3-gallate in mice. Food Chem.Toxicol. 48:409–416.(2010)

332. Petti, S., Scully, C. Polyphenols, oral health and disease: A review. J Dent. 37(6):413-423.(2009)

333. Tapiero H, T. K., Ba GN, Mathe G. Polyphenols: Do they play a role in prevention of hu- man pathologies? Biomed Pharmacother. 56:200-220.(2002)

334. Sharan, R. N., Choudhury, Y. Betel nut and susceptibility to cancer. In: Environmental factors, genes, and the development of human cancers. New York: Springer; 2010. 401-428 p.2010l

335. Gupta, A. K., Tulsyan, S., Thakur, N., Sharma, V., Sinha, D. N., *et al.* Chemistry, metabolism and pharmacology of carcinogenic alkaloids present in areca nut and factors affecting their concentration. Regul Toxicol Pharmacol. 110:104548.(2020)

336. Senica, M., Stampar, F., Veberic, R., Mikulic-Petkovsek, M. The higher the better? Differences in phenolics and cyanogenic glycosides in sambucus nigra leaves, flowers and berries from different altitudes. J Sci Food Agric. 97(8):2623-2632.(2017)

337. Bryła, M., Ksieniewicz-Woźniak, E., Waśkiewicz, A., Podolska, G., Szymczyk, K. Stability of ergot alkaloids during the process of baking rye bread. Lwt. 110:269-274.(2019)

338. Jiang, Z.-M., Wang, L.-J., Gao, Z., Zhuang, B., Yin, Q., *et al.* Green and efficient extraction of different types of bioactive alkaloids using deep eutectic solvents. Microchem J. 145:345-353.(2019)

339. Kukula-Koch, W. A., Widelski, J. Alkaloids. Pharmacognosy. 2018(December 11th).(2017)

340. Raghavan, V., Baruah, H.K Arecanut: India’s popular masticatory — history, chemistry and utilization. . Econom. Bot. 12:315–325.(1958)

341. Hwang, L. S., Wang, C. K., Sheu, M. J., Kao, L. S., . Phenolic compounds of piper betle flower as flavoring and neuronal activity modulating agents. In: Ho, c.T., lee, c.Y. & huang, m.T., eds, phenolic compounds in food and their effects on health, vol. 1, analysis, occurrence and chemistry. Food and their Effects on Health.(1992)

342. Hodnick, W. F., Milosavljevic, E. B., Nelson, J. H., Pardini, R. S. Electrochemistry of flavonoids. Relationships between redox potentials, inhibition of mitochondrial respiration, and production of oxygen radicals by flavonoids. Biochem. Pharmacol. 37:2607–2611.(1988)

343. Choi, S. I., Jeong, C. S., Cho, S. Y., Lee , Y. S. Mechanism of apoptosis induced by apigenin in hepg2 human hepatoma cells: Involvement of reactive oxygen species generated by nadph oxidase. Arch. Pharm. Res. 30:1328–1335.(2007)

344. Mazzanti, G., Menniti-Ippolito, F., Moro, P. A., Cassetti, F., Raschetti, R., *et al.* Hepatotoxicity from green tea: A review of the literature and two unpublished cases. Eur. J. Clin. Pharmaco. 65:331–341.(2009)

345. Stevens, T., Qadri, A., Zein, N. N. Two patients with acute liver injury associated with use of the herbal weight-loss supplement hydroxycut. Ann. Intern. Med. 142:477-478.(2005)

346. Belobrov, S., Seers, C., Reynolds, E., Cirillo, N., McCullough, M. J. Functional and molecular effects of a green tea constituent on oral cancer cells. JOP. 48(7):604-610.(2019)

347. Bravo, L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance

. Lead Review Article. 317-333.(1998)

348. Peng, W., Liu, Y. J., Wu, N., Sun, T., He, X. Y., *et al.* Areca catechu l. (arecaceae): A review of its traditional uses, botany, phytochemistry, pharmacology and toxicology. J Ethnopharmacol. 164:340-356.(2015)

349. Gilani, A. H., Ghayur, M. N., Saify, Z. S., Ahmed, S. P., Choudhary, M. I., *et al.* Presence of cholinomimetic and acetylcholinesterase inhibitory constituents in betel nut. Life Sci. 75(20):2377-2389.(2004)

350. Benegal, V., Rajkumar, R., Muralidharan, K. Does areca nut use lead to dependence? Drug alcohol depend. https://doi.org/10.1016/j.drugalcdep.2008.03.016.114-121.(2008)

351. Soman, S., Chacko, A. S., Prasad, V. S., Anju, P., Surya, B. S., *et al.* Self-assembly of oleylamine modified nano-fibrillated cellulose from areca husk fibers into giant vesicles. Carbohydr Polym. 182:69-74.(2018)

352. Chiu-Lan Chen , Chin-Wen Chi , Kuo-Wei Chang , Liu, T.-Y. Safrole-like DNA adducts in oral tissue from oral cancer patients with a betel quid chewing history. carcinogenesis. 20 no.12 2331–2334.(1999)

353. Chung, Y., Chen, C., Wu, C., Chan, S., Chi, C., *et al.* Safrole-DNA adduct in hepatocellular carcinoma associated with betel quid chewing. Toxicology Letters. 183(1-3):21-27.(2008)

354. Tsou, H.-H., Ko, H.-T., Chen, C.-T., Wang, T.-W., Lee, C.-H., *et al.* Betel quid containing safrole enhances metabolic activation of tobacco specific 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (nnk). Environmental Pollution. 251:13-21.(2019)

355. M. Kreh, R. M., L. Witte. Capillary gas chromatography-mass spectrometry of amaryllidaceae alkaloids. Capillary gas chromatography-mass spectrometry of amaryllidaceae alkaloids. Phytochemistryp. 773.(1995)

356. S. Berkov, J. B., B. Sidjimova, F. Viladomat, C. Codina. Phytochemical differentiation of galanthus nivalis and galanthus elwesii : A case study. Biochem. Syst. Ecol. 36:638.(2008)

357. Luo, J., Brotchie, J., Pang, M., Marriott, P. J., Howell, K., *et al.* Free terpene evolution during the berry maturation of five vitis vinifera l. Cultivars. Food Chemistry.125101.(2019)

358. Dave, B. J. T., A. H.; Adhvaryu, S. G. In vitro genotoxic effects of areca nut extract and arecoline. J. Cancer Res. Clin. Oncol. 118:283−288.(1992)

359. Eng, J. H. C., M. C.; Hahn, L. J. Role of areca nut in betel quid-associated chemical carcinogenesis: Current awareness and future perspectives. Oral Oncol. 37:477−492.(2001)

360. Huang, J. L. M., M. J. High-performance liquid chromatographic determination of the alkaloids in betel nut. J. Chromatogr. A. 475:447−450.(1989)

361. Nair, J. O., H.; Friesen, M.; Croisy, A.; Bhide, S. V.; Bartsch, H. Tobacco-specific and betel nut-specific n-nitroso compounds: Occurrence in saliva and urine of betel quid chewers and formation in vitro by nitrosation of betel quid. Carcinogenesis. 6:295−303.(1985)

362. Prokopczyk, B. R., A.; Bertinato, P.; Brunnemann, K. D.; Hoffmann, D. 3-(methylnitrosamino)propionitrile: Occurrence in saliva of betel quid chewers, carcinogenicity, and DNA methylation in f344 rats. Cancer Res. 47:467−471.(1987)

363. undqvist, K. L., Y.; Nair, J.; Bartsch, H.; Arvidson, K.; Grafstrom, R. C. Cytotoxic and genotoxic effects of areca nut-related compounds in cultured human buccal epithelial cells. Cancer Res. 49:5294−5298.(1989)

364. Cox, S., Piatkov, I., Vickers, E. R., & Ma, G. High-performance liquid chromatographic determination of arecoline in human saliva. J Chromatogr. 1032(1-2):93-95.(2004)

365. Jantarat, C., Sirathanarun, P., SONGSRM, W., Srinornate, W., & Daengprom, S. A simple and rapid hplc technique for determination of arecoline in areca nut (areca catechu l.) extrac. Walailak Journal of Science and Technology (WJST). 10(1):57-66.(2013)

366. Giri, S., Idle, J. R., Chen, C., Zabriskie, T. M., Krausz, K. W., *et al.* A metabolomic approach to the metabolism of the areca nut alkaloids arecoline and arecaidine in the mouse. Chemical research in toxicology. 19(6):818-827.(2006)

367. Hameed, I. H., Jasim, H., Kareem, M. A., & Hussein, A. O. Alkaloid constitution of nerium oleander using gas chromatography-mass spectroscopy (gc-ms). Journal of Medicinal Plants Research. 9(9):326-3334.(2015)

368. Jasim, H., Hussein, A. O., Hameed, I. H., & Kareem, M. A. Characterization of alkaloid constitution and evaluation of antimicrobial activity of solanum nigrum using gas chromatography mass spectrometry (gc-ms). Journal of Pharmacognosy and Phytotherapy. 7(4):56-74.(2015)

369. Wang, C.-K. L., W.-H.; Peng, C.-H. Contents of phenolics and alkaloids in areca catechu linn. During maturation. J. Agric. Food Chem. 45:1185−1188.(1997)

370. Franke, A. A., Biggs, L., Yew, J. Y., Lai, J. F. Areca alkaloids measured from buccal cells using dart-ms serve as accurate biomarkers for areca nut chewing. Drug Test Anal.(2019)

371. L, M., Krishna, A., Reddy, J., Nirmala, G. S. Optimization studies on extraction of phytocomponents from betel leaves. Resource-Efficient Technologies. 3(4):385-393.(2017)

372. Iqbal, J., Abbasi, B. A., Mahmood, T., Kanwal, S., Ali, B., *et al.* Plant-derived anticancer agents: A green anticancer approach. Asian Pacific Journal of Tropical Biomedicine. 7(12):1129-1150.(2017)

373. Chen, J.-J., Lin, Y.-H., Day, S.-H., Hwang, T.-L., Chen, I.-S. New benzenoids and anti-inflammatory constituents from zanthoxylum nitidum. Food Chemistry. 125(2):282-287.(2011)

374. Preethi R, P. P. Biosynthesis and bioactivity of silver nanobioconjugates from grape (vitis vinifera) seeds and its active component resveratrol. Int J Pharma Sci Res. 7(10):4253.(2016)

375. Bonilla, J., Poloni, T., Lourenço, R. V., Sobral, P. J. A. Antioxidant potential of eugenol and ginger essential oils with gelatin/chitosan films. Food Bioscience. 23:107-114.(2018)

376. Chatterjee, D., Bhattacharjee, P. Comparative evaluation of the antioxidant efficacy of encapsulated and un-encapsulated eugenol-rich clove extracts in soybean oil: Shelf life and frying stability of soybean oil. Journal of Food Engineering. 117:545–550.(2013)

377. Woranuch, S., Yoksan, R. Eugenol-loaded chitosan nanoparticles: Ii. Application in bio-based plastics for active packaging. Carbohydrate Polymers. 96:586–592.(2013)

378. Jeng JH, H. L., Lin BR, Hsieh CC, Chan CP, Chang MC. Effects of areca nut, inflorescence piper betle extracts and arecoline on cytotoxicity, total and unscheduled DNA synthesis in cultured gingival keratinocytes. Oral Pathol Med 28:64-71.(1999)

379. Kiyama, R. Estrogenic terpenes and terpenoids: Pathways, functions and applications. European Journal of Pharmacology. 815:405-415.(2017)

380. Türkez, H., ., Çelik, K., ., Toğar, B. Effects of copaene, a tricyclic sesquiterpene, on human lymphocytes cells in vitro. Cytothecnology. 66, Issue 4(4):597-603.(2013)

381. Vinholes, J., Gonçalves, P., Martel, F., Coimbra, M. A., Rocha, S. Assessment of the antioxidant and antiproliferative effects of sesquiterpenic compounds in in vitro caco-2 cell models. Food Chem. 156:204-211.(2014)

382. Crowell, P. L., Gould, M. N. Chemoprevention and therapy of cancer by d-limonene. Crit. Rev.Oncog. 5:1-22.(1994)

383. Mauro, M., Catanzaro, I., Naselli, F., Sciandrello, G., Caradonna, F. Abnormal mitotic spindle assembly and cytokinesis induced by d-limonene in cultured mammalian cells. Mutagenesis. 28:631–635.(2013)

384. Chaudhary, S. C., Siddiqui, M. S., Athar, M., Alam, M. S. D-limonene modulates inflammation, oxidative stress and ras-erk pathway to inhibit murine skin tumorigenesis. Hum. Exp. Toxicol. 31:798-811.(2012)

385. Vigushin, D. M., Poon, G. K., Boddy, A., English, J., Halbert, G. W., *et al.* Research campaign phase i/ii clinical trials committee. Phase i and pharmacokinetic study of d-limonene in patients with advanced cancer. Cancer Chemother. Pharmacol. 42:111-117.(1998)

386. Zhang, Z., Guo, S., Liu, X., Gao, X. Synergistic antitumor effect of α-pinene and β-pinene with paclitaxel against non-small-cell lung carcinoma (nsclc). Drug Res. 65:214–218.(2015)

387. Saravanan, R., Rajendra Prasad N., KV., P. Effect of piper betle leaf extract on alcoholic toxicity in the rat brain. J Med Food. 6:261-265.(2003)

388. Begam K.M.F., Ravichandran, P., Manimekalai, V. Phytochemical analysis of some selected varieties of piper betle l. International Journal of Current Pharmaceutical Research. 10(2):89-93.(2018)

389. Sripradha, S. Betel leaf-the green gold. Pharm.Sci.&Res. 6(1):36-37.(2014)

390. KK;, W., RN., S. Aqueous extract of betel-nut in north-i-.Ast india induces DNA-stiand breaks and enhanc- es rate of cell proliferation in vitro. . J Cancer Rex Ctin Oncol. 114:579 578.(1988)

391. MN, A. Fate of betel nut chemical constituents following nut treatment prior lo chewing anj its relation to oral precancerous and cancerous lesions. Dent. J Malays. 10:33-37.(1988)

392. Baldasquin-Caceres, B., Gomez-Garcia, F. J., Lopez-Jornet, P., Castillo-Sanchez, J., Vicente-Ortega, V. Chemopreventive potential of phenolic compounds in oral carcinogenesis. Arch Oral Biol. 59(10):1101-1107.(2014)

393. Dunham, L. J., Muir, C.S. and Hamner, J.E. . Epithelial atypia in hamster cheek pouches treated repeatedly with calcium hydroxide. British journal of cancer. 20:586-593.(1966)

394. Shivapurkar, N. M., Ranadive, S.N., Gothoskar, S.V., Bhide, S.V. and Ranadive, K.J. . Tumorigenic effect of aqueous and polyphenolic fractions of betel nut in swiss strain mice. Indian Journal of Experimental Biology 18:1159-1161.(1980)

395. Patek R.S., Rajorhia, G. S. Antioxidative role of curry murray koenigi) and betel (piper betle) leaves in ghee. Journal of Food Science and Technology 16:158-160.(1991)

396. Sethi, S. C. A., J.S. . Stabilization of edible fats by spices. Part ii. A new antioxidant from betel leaf. Journal of Scientific and Industrial Research. 15B:34-36.(1956)

397. Angadi PV, R. S. Areca nut in pathogenesis of oral submucous fibrosis: Revisited. Oral Maxillofac Surg. 15:1-9.(2011)

398. Reichart PA, V. W. C., Becker J, Schuppan D. Distribution of procollagen type iii, collagen type iv and tenascin in oral submucous fibrosis. J Oral Pathol Med. 23:394-398.(1994)

399. Ekanayaka, R. P., Tilakaratne W.M. Oral submucous fibrosis: Review on mechanisms of pathogenesis and malignant transformation. J Carcinog Mutagen.S5:002.(2013)

400. Abhishek Banerjee, V.V Kamath, V. K., Kishore Bhatt. Fibroblastic phenotype in oral submucous fibrosis- a cell culture analysis Saudi J. Pathol.Microbiol. 2(3):36-47.(2017)

Appendices

Appendix 1. Plain language statement form (for GDPs)
Melbourne Dental School
Faculty of Medicine, Dentistry, and Health Science

Project: **Oral Pre-Malignant and Malignant Lesion Detection Among Ind**

A/Professor Nicola Cirillo (Responsible Researcher)
Tel: +61 39341 1473
Email: nicola.cirillo@unimelb.edu.au
Elizabeth Fitriana Sari [DDS, Oral Medicine Specialist] (student)
Email: esari@student.unimelb.edu.au

Introduction

Thank you for your interest in participating in this research project. The following few pages will provide you with further information about the project, so that you can decide if you would like to take part in this research.

Please take the time to read this information carefully. You may ask questions about anything you don't understand or want to know more about.

Your participation is voluntary. If you don't wish to take part, you don't have to. If you begin participating, you can also stop at any time.

What is this research about?

Based on previous research finding, most of the GDP and medical practitioners are still incapable to perform oral cancer early detection properly. Thus, this research purpose is to assess GDP's awareness, knowledge, and skill in performing oral cancer early detection prior to training. The GDP who consent to participate in this study will be enrolled to a one-day standardise oral cancer detection training.

What will I be asked to do?

Should you agree to participate after reading this document, you will be asked to indicate your consent by signing infromed consent form. You will than be asked to complete a questionnaire to assess your awareness and skill of performing oral cancer early detection. To complete the questionnaire it will take up to 10 minutes. You will than be having a one-day training of standardised education of oral pre-malignant and malignant lesion detection. You will subsequently be part of this study as co-researcher and involved in data collection.

A-post training questionnaire will be undertaken 6 to 12 weeks after one-day standardized training program. This will essentially the same as the pre-training questionnaire with several additional question directly related to training program. You will need to follow 3-days data collection in primary health care or selected village building.

What are the possible benefits?

By participating in this study, we believe that you will have better understanding and skill on how to perform oral cancer early detection adequately. As part of this study, you will get a free of charge of one-day training of standardised education on oral pre-malignant and malignant detection. In addition, you will have access to join oral cancer group consultation in social media (Whatsapp group and Facebook). This group will be beneficial for you to disscuss any of your oral diseases cases which might be found in your dental practice for a maximum period of 9 months after training.

What are the possible risks?

There will no any possible risk aside of providing extra time for attending one day training and another 3-5 days for collecting data as co-researcher.

Do I have to take part?

No. Participation is completely voluntary. There will not any force to participate this study, and you have the right to both reject and/or join. If you do consent, you also can withdrawl your data in the future without any reason. If you wish to withdraw, you can contact responsible researcher or Dr. Elizabeth Fitriana Sari (Phone: +61404732334, email: esari@studentunimelb.edu.au). If you decide to withdraw from the study, our research member will contact you and discuss with you.

Will I hear about the results of this project?

For the purpose of science and contribute to public, the outcome of this study will be published on some scientific journals, present in conferences as poster and/or oral presentation.

What will happen to information about me?

Every collected information and all aspects in this study will be strictly confidential. Only responsible researcher and Dr. Elizabeth Fitriana who have access to the data. We will keep all data securely for a minimum 5 years post publication. Both digital and non-digital data will be kept in the same secure storage or unit of my department. The digital data not intended to be kept will be disposed of by deleting the digital data in SurveyMonkey.com. The paper based data will also be disposed properly.

Where can I get further information?

If you would like to get more information about the project, please contact the researchers;

A/Professor Nicola Cirillo (Responsible Researcher)

Tel: +61 39341 1473, Email: nicola.cirillo@unimelb.edu.au

Elizabeth Fitriana Sari [DDS, Oral Medicine Specialist] (student)

Email: esari@student.unimelb.edu.au

Who can I contact if I have any concerns about the project?

This research project has been approved by the Human Research Ethics Committee of The University of Melbourne. If you have any concerns or complaints about the conduct of this research project, which you do not wish to discuss with the research team, you should contact the Manager, Human Research Ethics, Research Ethics and Integrity, University of Melbourne, VIC 3010. Tel: +61 3 8344 2073 or Email: HumanEthics-complaints@unimelb.edu.au. All complaints will be treated confidentially. In any correspondence please provide the name of the research team or the name or ethics ID number of the research project.

Appendix 2. Consent form (for GDPs)

Melbourne Dental School
Faculty of Medicine, Dentistry, and Health Science

Project: **Oral Pre-Malignant and Malignant Lesion Detection Among Indonesian**

A/Professor Nicola Cirillo (Responsible Researcher)
Tel: +61 39341 1473, Email: nicola.cirillo@unimelb.edu.au
Elizabeth Fitriana Sari [DDS, Oral Medicine Specialist] (student)
Email: esari@student.unimelb.edu.au

Name of Participant: _____

1. I consent to participate in this project, the details of which have been explained to me, and I have been provided with a written plain language statement to keep.
2. I understand that the purpose of this research is to investigate oral cancer cases among Indonesian
3. I understand that my participation in this project is for research purposes only.
4. I acknowledge that the possible effects of participating in this research project have been explained to my satisfaction.
5. In this project I will be required to indicate my consent by signing infromed consent form. I will than be asked to complete a questionnaire to assess my awareness and skill of performing oral cancer early detection. To complete the questionnaire will take up to 10 minutes. I will than be having a one-day training of standardised education of oral pre-malignant and malignant lesion detection. I will subsequently be part of this study as co-researcher and involved in data collection. A-post training questionnaire will be undertaken 6 to 12 weeks after one-day standardized training program. I will need to follow 3-days data collection in primary health care or selected village.
6. I understand that my participation is voluntary and that I am free to withdraw from this project anytime without explanation or prejudice and to withdraw any unprocessed data that I have provided.

7. I understand that the data from this research will be stored at the University of Melbourne and will be destroyed after 5 years post publication.
8. I have been informed that the confidentiality of the information I provide will be safeguarded subject to any legal requirements; my data will be password protected and accessible only by the named researchers.
9. I understand that given the small number of participants involved in the study, it may not be possible to guarantee my anonymity.
10. I understand that after I sign and return this consent form, it will be retained by the researcher.

Participant Signature: _____ **Date:** _____

Appendix 3. Pre-training questionnaire for GDPs

Personal Data

Name :

Qualification : GDP/ Spesialist_____ (Please circle, and fill in the blank if others)

Date of Birth :

Institution : Private clinic / Hospital/ Primary health care (Please circle)

Address :

Email :

Phone :

∗ Personal data will be treated confidentially

Please kindly answer the questions below. You may answer in Bahasa Indonesia or English.

1. Have you ever undertaken an oral cancer screening examination?

   a. Yes

   b. No

2. Do you always perform a complete oral mucosal examination to every patient?

   a. Yes

   b. No

3. Have you ever found patients with suspected oral malignant lesions?

   a. Yes, **often** (please roughly state the number of times each year_____)

   b. Yes, **seldom** (please roughly state the number of times each year_____)

   c. Never

4. Have you ever found patients with suspected oral pre-malignant lesions?

   a. Yes, often (please roughly state the number of it in a year_____)

   b. Yes, seldom (please roughly state the number of it in a year_____)

   c. Never

5. Do you regularly advise your patients about oral cancer risk factors?
    a. Yes
    b. No
6. Do you regularly encourage patients to reduce oral cancer risk factors?
    a. Yes
    b. No
7. Do you believe that you have sufficient knowledge concerning prevention of oral cancer?
    a. Yes
    b. No
8. If not routine, do you perform oral cancer screening in high-risk patients?
    a. Yes
    b. No
9. Do you believe you have sufficient training, knowledge, and technique to perform oral cancer detection?
    a. Yes
    b. No
10. Do you believe you have sufficient training, knowledge, and technique to detect oral premalignant lesions or early oral cancer lesions?
    a. Yes
    b. No
11. Do you believe that you have received sufficient training on oral cancer at Dental school?
    a. Yes
    b. No

12. Please indicate below what you believe as risk factors of oral cancer.
13. Please indicate below any pathologic changes in oral cavity associated with oral cancer.
14. Please indicate below any pathologic changes in oral cavity associated with oral malignant lesions.
15. Do you have the required equipment to perform an oral cancer screening?
    a. Bright white light (Yes/No)
    b. Dental Mirror (Yes/No)
    c. Gauze square (Yes/No)
16. Do you perform extra-oral examinations?
    a. Perform
    b. Do not perform
17. How confident are you in undertaking an extra-oral examination?
    a. Very confident
    b. Confident
    c. Not confident
    d. Very not confident
18. How confident are you in identifying pathologic changes on extra-oral?
    a. Very Confident
    b. Confident
    c. Not confident
    d. Very not confident
19. Do you perform lip examinations?
    a. Perform
    b. Do not perform

20. How confident are you in undertaking a lip examination?
    a. Very confident
    b. Confident
    c. Not confident
    d. Very not confident
21. How confident are you in identifying pathologic change on lips?
    a. Very Confident
    b. Confident
    c. Not confident
    d. Very not confident
22. Do you perform labial examinations?
    a. Perform
    b. Do not perform
23. How confident are you in undertaking a labial examination?
    a. Very confident
    b. Confident
    c. Not confident
    d. Very not confident
24. How confident are you identifying pathologic changes on labial mucosa?
    a. Very Confident
    b. Confident
    c. Not confident
    d. Very not confident
25. Do you perform buccal mucosa examinations?
    a. Perform

b. Do not perform

26. How confident are you in undertaking a buccal mucosa examination?

e. Very confident

a. Confident

b. Not confident

c. Very not confident

27. How confident are you in identifying pathologic changes on buccal mucosa?

a. Very Confident

b. Confident

c. Not confident

d. Very not confident

28. Do you perform gingival examinations?

a. Perform

b. Do not perform

29. How confident are you in undertaking a gingival examination?

a. Very confident

b. Confident

c. Not confident

d. Very not confident

30. How confident are you in identifying pathologic change on gingival?

a. Very Confident

b. Confident

c. Not confident

d. Very not confident

31. Do you perform a ventral tongue and floor of the mouth examinations?
    a. Perform
    b. Do not perform
32. How confident are you in undertaking a ventral tongue and floor of the mouth examination?
    a. Very confident
    b. Confident
    c. Not confident
    d. Very not confident
33. How confident are you in identifying pathologic changes on ventral tongue and floor of the mouth?
    a. Very Confident
    b. Confident
    c. Not confident
    d. Very not confident
34. Do you perform a bimanual palpation on floor of the mouth?
    a. Perform
    b. Do not perform
35. How confident are you in undertaking a bimanual palpation floor of the mouth examination?
    a. Very confident
    b. Confident
    c. Not confident
    d. Very not confident

36. How confident are in identifying pathologic changes on floor of the mouth?

    e. Very Confident

    a. Confident

    b. Not confident

    c. Very not confident

37. Do you believe you have the ability to perform an oral mucosal biopsy?

    a. Yes,

       If yes, have you performed an oral mucosal biopsy? Please circle the answer (Yes/No)

    b. No

38. Do you believe that you have the ability to perform Toluidine blue staining?

    a. Yes, where did you learn? please choose:

        - Dental school
        - Course
        - Others, please specify_____

    b. No

39. Do you believe that you have the ability to use the adjunct tool for oral pre-cancer/cancer detection such as Velscope?

    a. Yes, where did you learn? Please choose:

        - Dental school
        - Course
        - Others, please specify_____

    b. No

40. Have you done oral diseases detection utilizing Velscope or any other tools? Please circle

    a. Yes, please specify_____

    b. No

41. Will you refer your patients suspected to have oral malignant lesion?

    a. Yes

    If yes, where will you refer them to? Please circle the answer (to Oral Medicine Specialist/Oral Surgery/Oral Pathology/ else _____)

    b. No, as I have never suspected any of my patients to have had an oral malignant lesion.

42. Will you refer your suspected patients with oral pre-malignant lesions?

    a. Yes

    If yes, where will you refer them to? Please circle the answer (to Oral Medicine Specialist/Oral Surgery/Oral Pathology/ else _____)

    b. No, as I have never suspected any of my patients to have had an oral pre-malignant.

43. What will you prescribe at the first appointment when you suspect a patient has an oral malignant or pre-malignant lesion?

    a. Mouthwash

    b. Analgesic

    c. Antibiotic

    d. Antiviral

    e. Do not give any medication and only do referral

    f. Other, please specify_____

Appendix 4. Post-training questionnaire for GDPs

Personal Data

Name :

Qualification : GDP/ Specialist_____ (Please circle, and fill in the blank if others)

Date of Birth :

Institution : Private clinic / Hospital/ Primary health care (Please circle)

Address :

Email :

Phone :

∗ Personal data will be treated confidentially

Please kindly answer the questions below. You may answer in Bahasa Indonesia or English.

1. This training has provided me with adequate information and knowledge regarding oral malignant and pre-malignant (OPL) lesions.
    1. Strongly agree
    2. Agree
    3. Neither agree or disagree
    4. Disagree
    5. Strongly disagree
2. My awareness of oral malignant and OPL has increased significantly because of this training.
    1. Strongly agree
    2. Agree
    3. Neither agree or disagree
    4. Disagree
    5. Strongly disagree

3. This training has increased my confidence in detecting oral malignant lesions and OPL:
    1. Strongly agree
    2. Agree
    3. Neither agree nor disagree
    4. Disagree
    5. Strongly disagree
4. I will always perform a complete oral mucosal examination.
    1. Strongly agree
    2. Agree
    3. Neither agree nor disagree
    4. Disagree
    5. Strongly disagree
5. I will always routinely educate my patients about oral cancer and risk factors.
    1. Strongly agree
    2. Agree
    3. Neither agree nor disagree
    4. Disagree
    5. Strongly disagree
6. I believe that this training is important for GDP.
    1. Strongly agree
    2. Agree
    3. Neither agree nor disagree
    4. Disagree
    5. Strongly disagree

7. I believe that this training should be conducted regularly.
    1. Strongly agree
    2. Agree
    3. Neither agree or disagree
    4. Disagree
    5. Strongly disagree
8. Please indicate below some risk factors associated with oral cancer.

9. Please indicate below any pathologic changes in oral cavity associated with oral cancer

10. Please indicate below any pathologic changes in oral cavity associated with oral malignant lesions.

11. How confident are you in undertaking an extra-oral examination?
    a. Very confident
    b. Confident
    c. Not confident
    d. Very not confident
12. How confident are you in identifying pathologic changes on extra-oral?
    a. Very Confident
    b. Confident
    c. Not confident
    d. Very not confident

13. How confident are you in undertaking a lip examination?

    a. Very confident
    b. Confident
    c. Not confident
    d. Very not confident

14. How confident are you in identifying pathologic change on lips?

    a. Very Confident
    b. Confident
    c. Not confident
    d. Very not confident

15. How confident are you in undertaking a labial examination?

    a. Very confident
    b. Confident
    c. Not confident
    d. Very not confident

16. How confident are you in identifying pathologic changes on labial mucosa?

    a. Very Confident
    b. Confident
    c. Not confident
    d. Very not confident

17. How confident are you in undertaking a buccal mucosa examination?

    a. Very confident
    b. Confident
    c. Not confident
    d. Very not confident

18. How confident are you in identifying pathologic changes on buccal mucosa?
    a. Very Confident
    b. Confident
    c. Not confident
    d. Very not confident
19. How confident are you now in undertaking a gingival examination?
    a. Very confident
    b. Confident
    c. Not confident
    d. Very not confident
20. How confident are you in identifying pathologic change on gingival?
    a. Very Confident
    b. Confident
    c. Not confident
    d. Very not confident
21. How confident are you in undertaking a ventral tongue and floor of the mouth examinations?
    a. Very confident
    b. Confident
    c. Not confident
    d. Very not confident
22. How confident are you in identifying pathologic changes on ventral tongue and floor of the mouth?
    a. Very Confident
    b. Confident

c. Not confident

d. Very not confident

23. How confident are you in undertaking a bimanual palpation floor of the mouth examination?

    a. Very confident

    b. Confident

    c. Not confident

    d. Very not confident

24. How confident are you in identifying pathologic changes on floor of the mouth with bimanual palpation?

    a. Very Confident

    b. Confident

    c. Not confident

    d. Very not confident

Appendix 5. Questionnaire for respondents (patients)

Personal Data

Name :

Gender :

Date of birth :

Ethnicity :

Monthly income :

Marital status :

Address :

*Personal data will be treated confidentially

Please kindly answer all the questions below;

1. What is the main reason you have come to the GDP today?
    a. Pain
    b. Bleeding
    c. Hole in tooth
    d. Loss teeth
    e. Missing teeth
    f. Sore gums
    g. Other, please specify _____
2. How long have you had your chief complaint?
    a. 1 day
    b. less than a week
    c. between 1 and 4 weeks
    d. more than a month
    e. more than a year

3. When you have oral problems, where do you go?

   a. GDP

   b. medical doctor

   c. nurse

   d. others, please specify_____

4. Is it easy for you to travel to this dental health facility? Yes/No

5. Who will pay for your dental health treatment?

   a. you

   b. insurance (Government/others_____)

   c. other, please specify_____

6. Have you ever been diagnosed with oral cancer?

   a. No

   b. Yes, Specify the diagnosis_____

7. Has any of your extended family members ever been diagnosed with oral cancer?

   a. No

   b. Yes, Specify the diagnosis_____

8. Has any of your friends ever been diagnosed with oral cancer?

   a. No

   b. Yes, Specify the diagnosis_____

9. Have you ever heard of someone not known to you being diagnosed with oral cancer from others or from online information?

   a. No

   b. Yes, specify the diagnosis_____

10. Do you smoke?
    a. No
    b. Yes, please answer the questions below
        - How many cigarettes do you smoke daily? please circle
          (Less than 10/ between 10 and 20 / more than 20)
        - When did you start to smoke? please circle
          (in the last 5 years / 5-15 years ago/ more than 15 years ago)
        - What kind of smoke do you inhale? (Pease circle: Filtered / Kretek/ other, please specify_____)
11. Do you have a habit of chewing betel nuts?
    a. No
    b. Yes, please answer the questions below
        - how many times in a day do you chew? please circle (once a day, 2-5 times a day/ 6-10 times a day / more than 10 times a day)
        - When did you start this habit?
          (in the last 5 years/ 5-15 years ago / more than 15 years ago)
        - What are the ingredients you put in your betel nuts? Please circle
          (Betel leaf only / Betel leaf + areca nut + lime / Betel leaf + areca nut + lime + tobacco / others, please specify_____)
        - Do you prepare it by yourself? Please circle the answer: yes/no
        - Or do you buy it in a package? Please circle the answer: yes/no
12. Do you drink alcohol?
    a. No
    b. Yes, please answer below questions:

- how many times in a day do you drink? please circle (once a day, 2-5 times a day/ 6-10 times a day / more than 10 times a day)
- When did you start this habit? (in the last 5 years/ 5-15 years ago / more than 15 years ago)
- What kind of alcohol do you drink? please specify_____

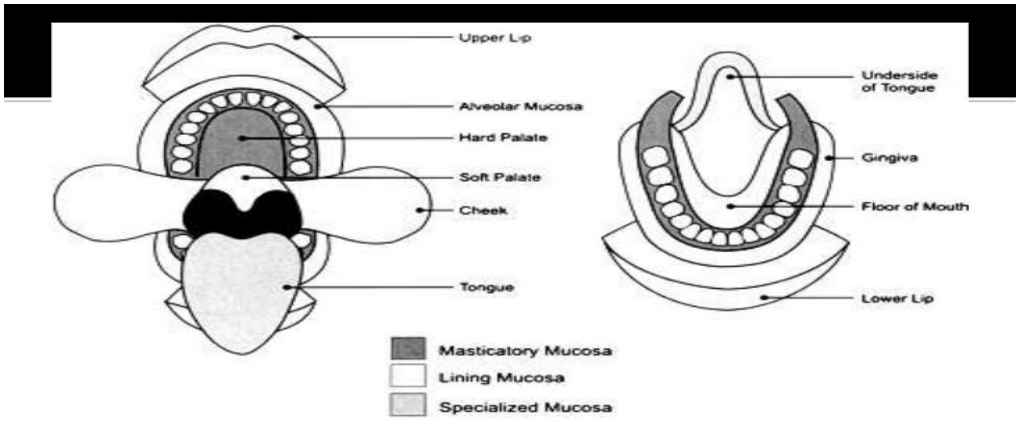
13. Do you think that there might be other causes of oral cancer apart from smoking, areca nut, and alcohol?
    a. No
    b. Yes, please choose (food/ viruses/ oral sex/ other_____)
14. Do you work mostly exposed by UV?
    a. No
    b. Yes, please answer below question;
        - How many hours roughly in a day?
        - How many days in a month do you work?
15. Do you brush your teeth on a daily basis?
    a. No
    b. Yes
16. How many times do you brush your teeth daily?
17. Do you know how to brush your teeth properly?
    a. No
    b. Yes, please answer the question below:

       Where did you get the information about tooth brushing? (family members/GDPs/teachers at school/ others, please specify_____)

18. How many times do you eat in a day? Please choose: (once a day/ twice a day/ three times a day/ more than three times a day)
19. Do you eat meat every day?
    a. No
    b. Yes
20. Do you eat vegetables every day?
    a. No
    b. Yes
21. Do you eat fruit every day?
    a. No
    b. Yes
22. Have you ever received any information about oral cancer from your GDP?
    a. No
    b. Yes
23. Have you ever received any information about oral cancer from the government?
    a. No
    b. Yes

Appendix 6. Examination form

Name	
Age	
Gender	
Doctor name	



EXTRA ORAL EXAMINATION

A. LYMPH NODES

Lymph nodes	**Enlargement (+/-)**	**pain (+/-)**	**fixed (+/-)**	**Note**
Left Submandibular				
Right Submandibular				
Submental				
Right Cervical				
Left Cervical				

B. **FACE**

Symmetric/asymmetric (Please give detail explanation)	**Other finding such as lump, enlargement, tumor, pain, numbness, unusual pigmentation, tenderness. Please give detail explanation about size, region, consistency**

C. **LIPS**

Cheilitis (+/-)	**Bleeding, Pain, numbness, crusted (+/-)**	**Melanosis pigmentation/other pigmentation (+/-)**	**Vermillion border**	**Other condition**

Please explain any finding in detail (size, region, colour, consistency, outline, etc)

INTRA ORAL EXAMINATION:

Lesions:

1. Leukoplakia
2. Eritroplakia
3. Eritroleukoplakia
4. OSMF
5. Single ulcer
6. Multiple ulcer
7. Lichen planus
8. Candidiasis
9. Melanosis
10. Bleeding
11. Others (explain)

Oral mucosa	Lesion	Site	Note
Labial superior			
Labial anterior			
Right buccal			
Left buccal			
Gingiva			
Hard palate			
Soft palate			
Dorsum tongue			
Right lateral tongue			
Left lateral tongue			
Ventral tongue			
Floor of the mouth			

Is this patient required follow up treatment? Yes / No (please circle)?

If Yes:

1. Give patient a clear instruction to withdraw the suspected risk factors for 2 weeks (smoke, drink alcohol, betel nut chewing)
2. Give patient instruction to be followed up after 2 weeks from initial screening to re-examined for further treatment such as biopsy. If they required to have biopsy, please refer to the given address of specialist for referral bellow;
    - Banda Aceh, Patients will be referred to Oral Medicine Department of Dental Hospital, Faculty of Dentistry of University of Syiah Kuala. Aceh. Coordinator: Srie Rejeki, drg, Sp.PM
    - Bandung, West java, Patients will be refered to Oral Medicine and Oral Surgery Department of Hasan Sadikin Hospital. Coordinator: Yanti, drg
    - Pontianak, West Kalimantan, Patients will be referred to Oral Surgery Department of RSUD Bethesda. Coordinator : Hanny Christina, drg, Sp.PM
    - West Papua, (waiting for confirmation) Coordinator: Rosalin, drg
    - Jakarta, Patients will be referred to Cipto Mangunkusumo Hospital. Coordinator: Syaukani, drg

Appendix 7. Plain language statement (for patients)

Melbourne Dental School
Faculty of Medicine, Dentistry, and Health Science

Project: **Oral Pre-Malignant and Malignant Lesion Detection Among Indonesian**

A/Professor Nicola Cirillo (Responsible Researcher)
Tel: +61 39341 1473, Email: nicola.cirillo@unimelb.edu.au
Elizabeth Fitriana Sari [DDS, Oral Medicine Specialist] (student)
Email: esari@student.unimelb.edu.au

Introduction

Thank you for your interest in participating in this research project. The following few pages will provide you with further information about the project, so that you can decide if you would like to take part in this research.

Please take the time to read this information carefully. You may ask questions about anything you don't understand or want to know more about.

Your participation is voluntary. If you don't wish to take part, you don't have to. If you begin participating, you can also stop at any time.

What is this research about?

It is predicted that Indonesian have a high number of oral cancer cases as Indonesian people have been exposed many years by oral cancer risk factors such as smoking cigarette, betel nut chewing, and alcohol drinking. However, the number of oral cancer cases among Indonesian people is still unknown. Thus, the purpose of the research project is to get factual number of oral cancer cases among Indonesians, to assess risk factors associated with oral cancer, and to promote awareness by detecting oral cancer early.

What will I be asked to do?

Should you agree to participate after reading this document, you will be asked to indicate your consent by signing infromed consent form. You will than be asked to complete a questionnaire to assess your awareness and risk factors related with oral cancer. To complete the questionnaire will take up to 10 minutes. The next step is, you will undergo a visual and palpation examination on your head, neck and mouth areas to see any abnormalities. For having a detail examination on entire tongue, we will use gauze to retract it gently. All examination steps will not cause any pain and it will only take 5-10 minutes to complete.

What are the possible benefits?

By participating this study, we believe that you will get better understanding about oral cancer and how to prevent it, and as part of this study you will have a free of charge examination for oral cancer detection. Furthermore, If by examination you are suspected to have cancer or pre-cancer condition, than we will reffer you to selected specialist in your region for futher treatments.

There will not any cost arise to take part as participant, either to your insurance or to your own payment. Additionally, our study will not cause any further physical complication, as it is only a simple oral cancer detection.

After completing questionnaire and examination, as our appreciation for your participation, you will be given tools to aid maintaining oral health such as toothbrush and dental paste.

What are the possible risks?

There are no possible risk arise aside of spending extra 15-20 minutes longer than you usually need for your dental treatment, as you need to fill in a questionnaire and undergo examination prior to your dental treatment by your GDP.

Do I have to take part?

No. Participation is completely voluntary. There will not any force to participate this study, and you have the right to both reject and/or join. If you do consent, you also can withdraw your data in the future without any reason. If you wish to withdraw, you can contact responsible researcher or Dr. Elizabeth Fitriana Sari (Phone: +61404732334, email: esari@studentunimelb.edu.au). If you decide to withdraw from the study, our research member will contact you and discuss with you.

Will I hear about the results of this project?

The individual result of this research will be given to you straight away after all research steps have been done completely (questionnaire and examination) together with detail information whether you have suspected lesion or not, and whether you will need further treatment or not.

For the purpose of science, the results of this study will be published on some journals or media and present the results in conferences as poster and/or oral presentation.

What will happen to information about me?

Every collected information and all aspects in this study will be strictly confidential. Only responsible researcher and Dr. Elizabeth Fitriana who have access to the data. We will keep all data securely for a minimum 5 years post publication. Both digital and non-digital data will be kept in the same secure storage or unit of my department. The digital data not intended to be kept will be disposed of by deleting the digital data in SurveyMonkey.com. The paper-based data will also be disposed properly.

Where can I get further information?

If you would like to get more information about the project, please contact the researchers;

A/Professor Nicola Cirillo (Responsible Researcher)

Tel: +61 39341 1473 Email: nicola.cirillo@unimelb.edu.au

Elizabeth Fitriana Sari [DDS, Oral Medicine Specialist] (student)

Email: esari@student.unimelb.edu.au

Who can I contact if I have any concerns about the project?

This research project has been approved by the Human Research Ethics Committee of The University of Melbourne. If you have any concerns or complaints about the conduct of this research project, which you do not wish to discuss with the research team, you should contact the Manager, Human Research Ethics, Research Ethics and Integrity, University of Melbourne, VIC 3010. Tel: +61 3 8344 2073 or Email: HumanEthics-complaints@unimelb.edu.au. All complaints will be treated confidentially. In any correspondence please provide the name of the research team or the name or ethics ID number of the research project

Appendix 8. Consent form (For Patient)

Melbourne Dental School
Department of Oral Medicine
Faculty of Medicine, Dentistry, and Health Science

Project: **Oral Pre-Malignant and Malignant Lesion Detection Among Indonesian**

A/Professor Nicola Cirillo (Responsible Researcher)
Tel: +61 39341 1473 Email: nicola.cirillo@unimelb.edu.au
Elizabeth Fitriana Sari [DDS, Oral Medicine Specialist] (student)
Email: esari@student.unimelb.edu.au

Name of Participant: _____

1. I consent to participate in this project, the details of which have been explained to me, and I have been provided with a written plain language statement to keep.
2. I understand that the purpose of this research is to investigate oral cancer cases among Indonesian
3. I understand that my participation in this project is for research purposes only.
4. I acknowledge that the possible effects of participating in this research project have been explained to my satisfaction.
5. In this project I will be required to indicate my consent by signing infromed consent form. I will than be asked to complete a questionnaire to assess my awareness and risk factors related with oral cancer. To complete the questionnaire will take up to 10 minutes. The next step is, I will undergo a visual and palpation examination on my head, neck and mouth areas to see any abnormality. All examination steps will not cause any pain and it will only take 5-10 minutes to complete.
6. I understand that my participation is voluntary and that I am free to withdraw from this project anytime without explanation or prejudice and to withdraw any unprocessed data that I have provided.

7. I understand that the data from this research will be stored at the University of Melbourne and will be destroyed after 5 years post publication.
8. I have been informed that the confidentiality of the information I provide will be safeguarded subject to any legal requirements; my data will be password protected and accessible only by the named researchers.
9. I understand that given the small number of participants involved in the study, it may not be possible to guarantee my anonymity.
10. I understand that after I sign and return this consent form, it will be retained by the researcher.

Participant Signature: _____ **Date:** _____

Appendix 9. Ethics approval from Indonesia

KEMENTERIAN RISET, TEKNOLOGI DAN PENDIDIKAN TINGGI
UNIVERSITAS PADJADJARAN FAKULTAS KEDOKTERAN
KOMISI ETIK PENELITIAN KESEHATAN
HEALTH RESEARCH ETHICS COMMITTEE
Jl. Prof. Eyckman No. 38 Bandung 40161
Telp. & Fax. 022-2038697 email: kepk.fk.unpad@gmail.com

No.Reg.: 0616030303

PERSETUJUAN ETIK
ETHICAL APPROVAL

No: 701 /UN6.C1.3.2/KEPK/PN/2016

Komisi Etik Penelitian Kesehatan Fakultas Kedokteran Universitas Padjadjaran Bandung, dalam upaya melindungi hak asasi dan kesejahteraan subjek penelitian kesehatan dan menjamin bahwa penelitian yang menggunakan formulir survei/registrasi/surveilens/Epidemiologi/Humaniora/Sosial Budaya/Bahan Biologi Tersimpan/Sel Punca dan non klinis lainnya berjalan dengan memperhatikan implikasi etik, hukum, sosial dan non klinis lainnya yang berlaku, telah mengkaji dengan teliti proposal penelitian berjudul:

The Health Research Ethics Committee Faculty of Medicine Universitas Padjadjaran Bandung, in order to protect the rights and welfare of the health research subject, and to guaranty that the research using survey questionnaire/registry/surveillance/epidemiology/humaniora/social-cultural/archived biological materials/stem cell/other non clinical materials, will carried out according to ethical, legal, social implications and other applicable regulations, has been throughly reviewed the proposal entitled:

"*ORAL PRE-MALIGNANT AND MALIGNANT LESIONS DETECTION AMONG INDONESIANS*"

Nama Peneliti Utama *Principal Researcher*	:	Elizabeth Fitriana Sari
Pembimbing/Peneliti Lain *Supervisor/Other Researcher*	:	Assoc. Prof. Nicola Cirillo Prof. Michael McCullough
Nama Institusi *Institution*	:	Program Pascasarjana Program Studi Doktor University of Melbourne

proposal tersebut dapat disetujui pelaksanaannya.
hereby declare that the proposal is approved.

Ditetapkan di : Bandung
Issued in
Tanggal : 29 - 07 - 2016
Date

K e t u a,
Chairman,

Prof. Dr. Firman F. Wirakusumah, dr.,SpOG-K
NIP. 19480115 197302 1 001

Keterangan/notes:
Persetujuan etik ini berlaku selama satu tahun sejak tanggal ditetapkan.
This ethical clearance is effective for one year from the due date.
Pada akhir penelitian, laporan pelaksanaan penelitian harus diserahkan ke Komisi Etik Penelitian Kesehatan.
In the end of the research, progress and final summary report should be submitted to the Health Research Ethics Committee.
Jika ada perubahan atau penyimpangan protokol dan/atau perpanjangan penelitian, harus mengajukan kembali permohonan kajian etik penelitian.
If there be any protocol modification or deviation and/or extension of the study, the Principal Investigator is required to resubmit the protocol for approval.
Jika ada kejadian serius yang tidak diinginkan (KTD) harus segera dilaporkan ke Komisi Etik Penelitian Kesehatan.
If there are Serious Adverse Events (SAE) should be immediately reported to the Health Research Ethics Committee

Appendix 10. OC training for GDP and OPMD/OC screening program

Figure 10. 1 OC and OPMD screening in Bandung, West Java

Figure 10. 2 OC and OPMD screening in Jakarta, Special District of Jakarta

Figure 10. 3 OC and OPMD screening in Sorong, West Papua

Figure 10. 4 OC and OPMD screening in Pontianak, West Kalimantan

Figure 10. 5 OC and OPMD Screening in Aceh, Banda Aceh

Figure 10. 6 One-day standardise OC training for GDP in Bandung, West Java

Figure 10. 7 One-day standardise OC training for GDP in Jakarta, Special District of Jakarta

Figure 10. 8 One-day standardise OC training for GDP in Sorong, West Papua

Figure 10. 9 One-day standardise OC training for GDP in Pontianak, West Kalimantan

Figure 10. 10 One-day standardise OC training for GDP in Aceh, Banda Aceh

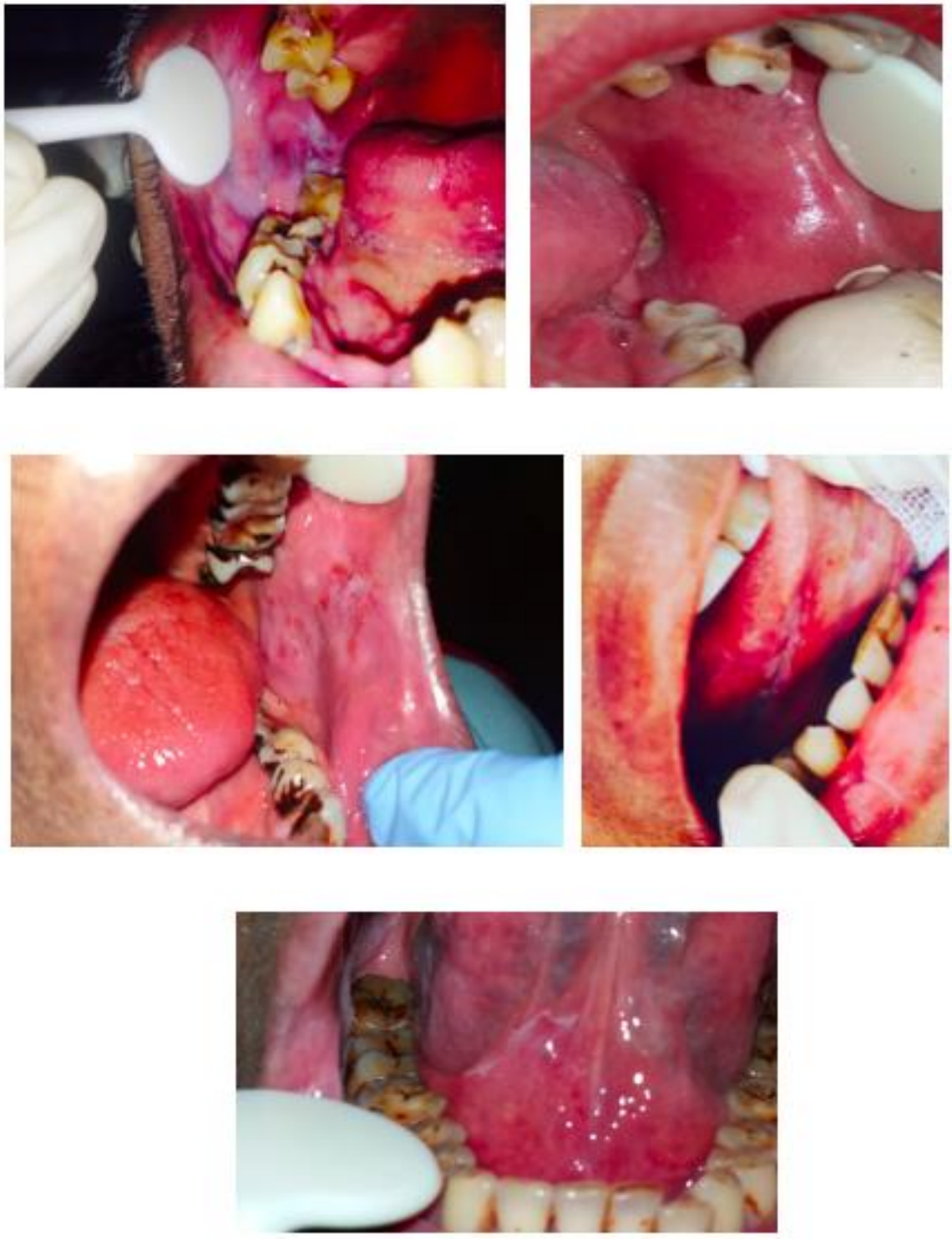


Figure 10. 11 OPMD cases found in the screening (Part-1)

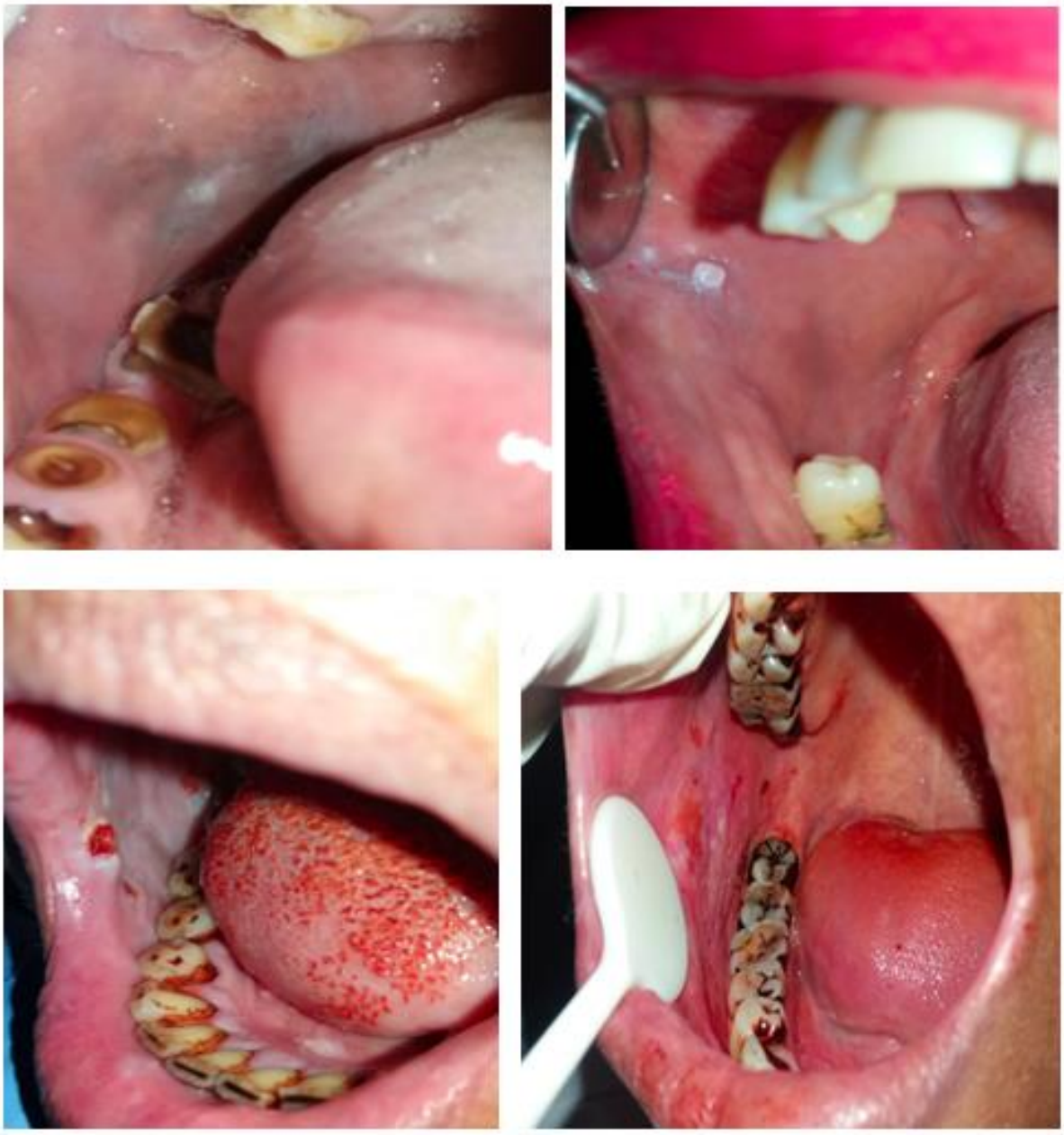


Figure 10. 12 OPMD cases found in the screening (Part-2)

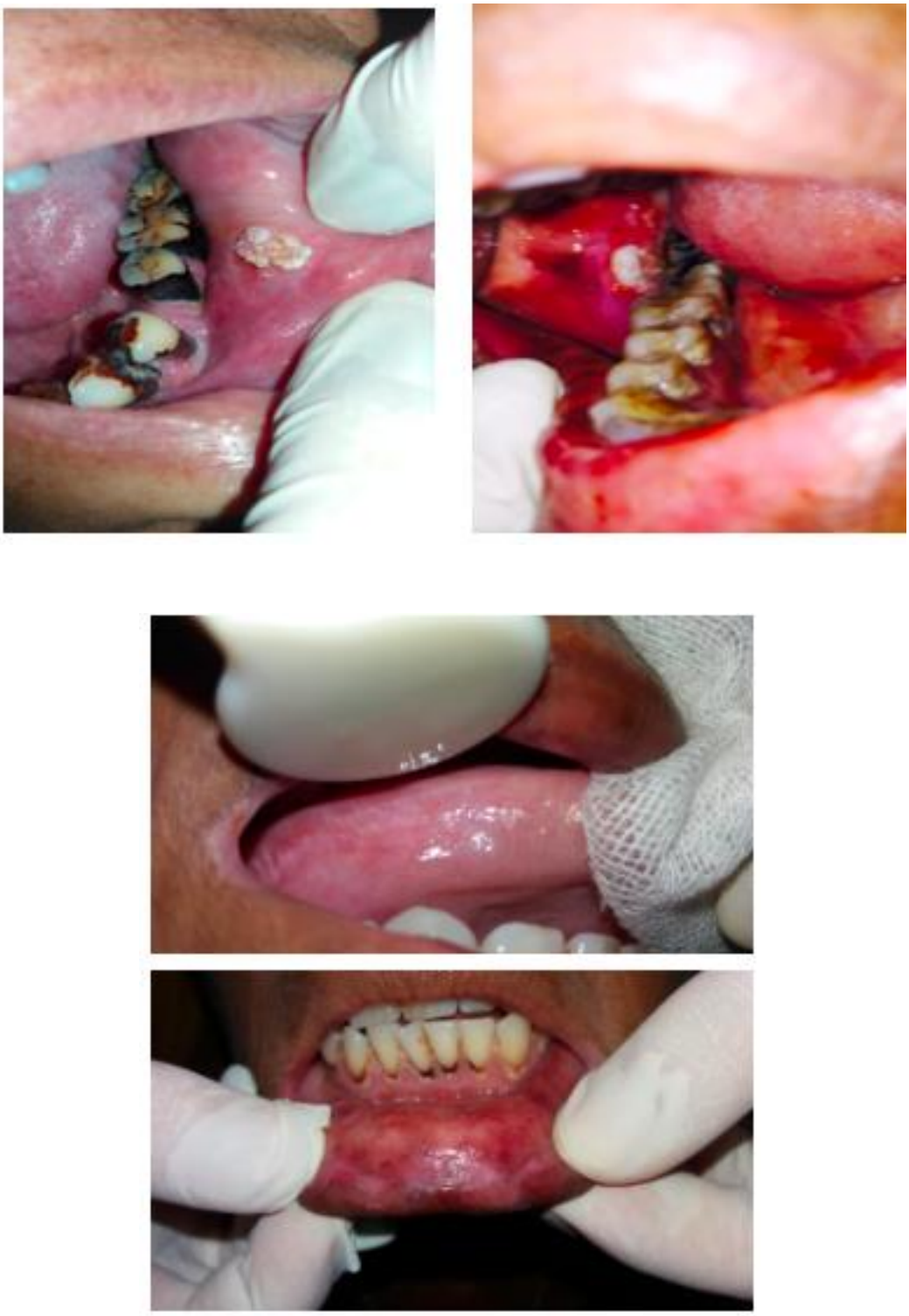


Figure 10. 13 OPMD cases found in the screening (Part-3)

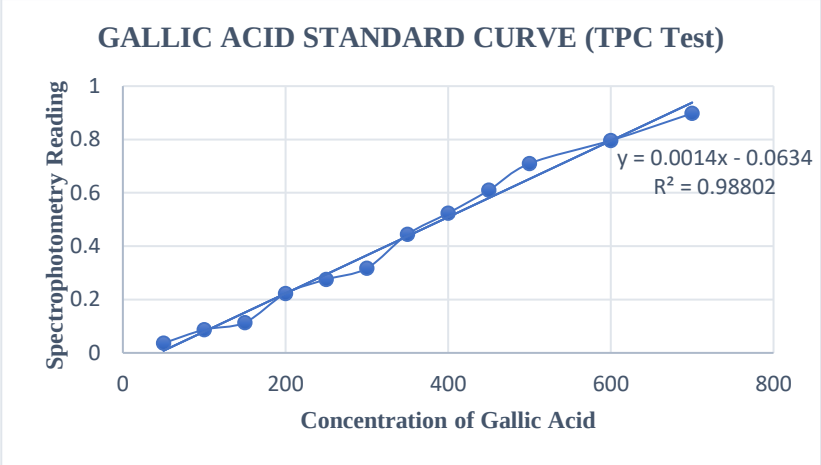
Appendix 11. Additional data

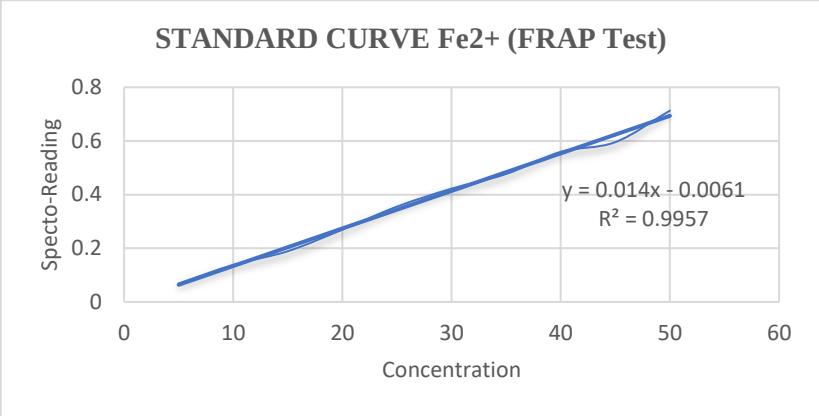
Table 11. 1 LC-MS data of phenolic compounds extracted from various Indonesian BQ

Sl No	Name of Compound	Retention Time	m/z
Phenolic Acid			
1	Gallic Acid	7.187	**169**, 125
2	Protocatechuic Acid	12.459	**153**, 109
3	p-hydroxybenzoic Acid	19.92	**137**, 93
4	Catechin	22.353	**289**, 245
5	Syringic Acid	26.881	**197**, 153/182
6	Epicatechin	28.206	**289**, 245
7	Trans - sinapic acid	38.006	**223**, 164
8	Ferulic Acid	37.005	**193**, 134
9	Resveratrol	52.888	**227**, 185
10	Quercitin	64.985	**301**, 151
Anthoxanthins & Stillbenes			
11	5-hydroxymethyl furfural	2.064	
12	Caftaric Acid	13	**311**, 179
13	Caffeic Acid	25.881	**179**, 135
14	p-coumaric Acid	33.518	**163**, 119
15	Epicatechin gallate	37.117	**441**, 289
16	Polydatin	38.101	**389**, 227
17	Quercitin 3-o galactoside	40.611	**463**, 301
18	Quercitin 3-o glucuronide	46.81	**477**, 301
19	Quercitin 3-o rhaminoside	47.311	**447**, 301
20	Kaempferol	66.586	**285**, 249
21	Kaempferol 3-o glucoside	46.86	**447**, 284/285
22	Quercitin 3-O Glucoside	41.633	**463.2**, 301

Table 11. 2 Samples' weight before and after lyophilised

Sample	Weight Before Lyophilized (gram)	Weight After Lyophilized (gram)	Contain water (%)
Areca Nut (BA)	20	18.3	8.3
Areca Nut (NS)	20	16.8	15.9
Areca Nut (WK)	20	17.1	14.5
Areca Nut (WP)	20	6.2	69.9
Betel Leaf (BA)	20	2.8	85.8
Betel Leaf (NS)	20	3.1	84.3
Betel Leaf (WK)	20	2.7	86.5
Betel Stem Inflorescence (WP)	20	5.9	70.7
Husk (BA)	20	5.3	73.7
Husk (NS)	20	5.6	72.0
Husk (WK)	20	7.6	62.2
Husk (WP)	20	5.1	74.0
Slaked lime – Paste form (BA)	0.8	0.4	47.8
Slaked lime – Paste form (NS)	0.8	0.4	56.7
Slaked lime – Paste form (WK)	0.8	0.4	47.8
Slaked lime – Powder form (WP)	0.8	0.8	0





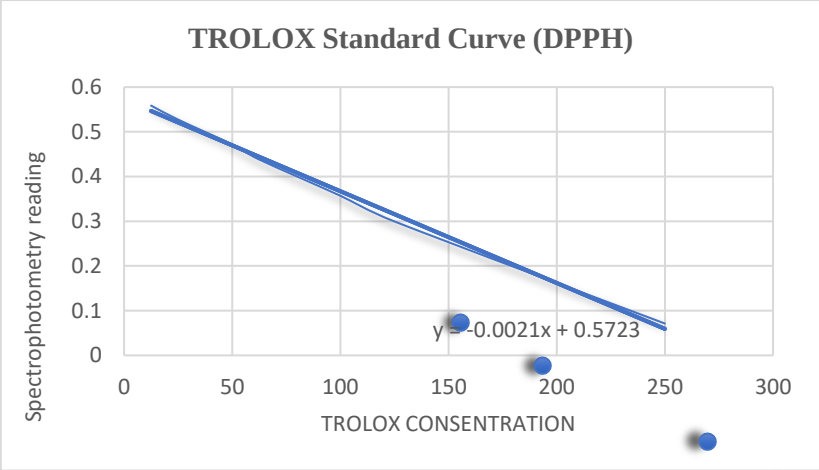
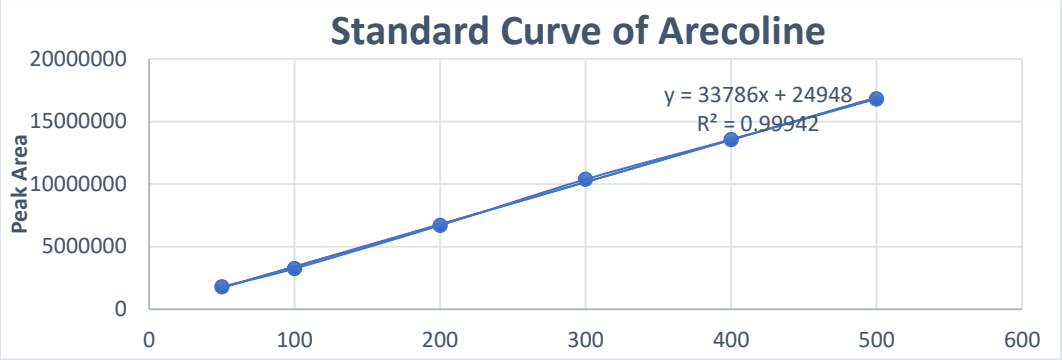
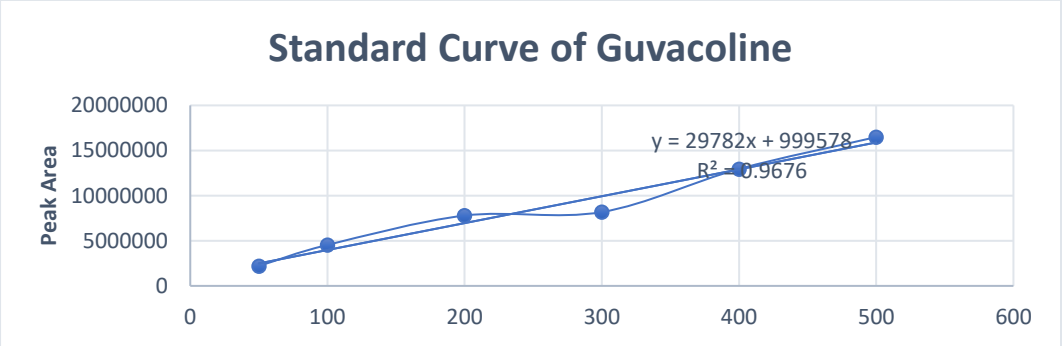


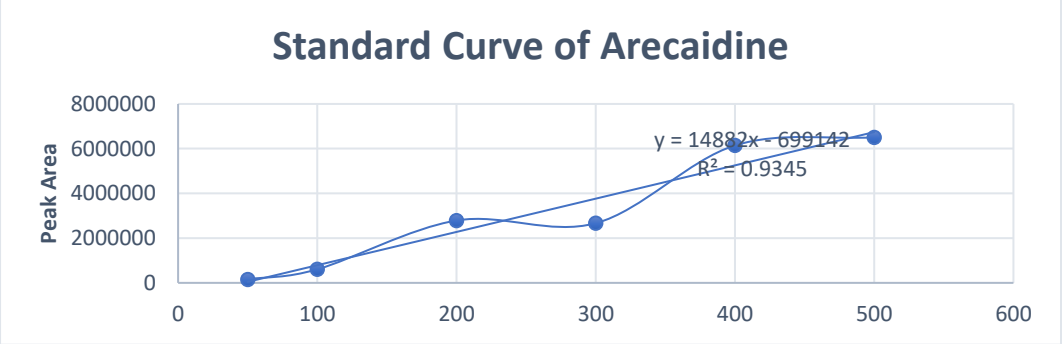
Figure 11. 1 Standard curve for TPC, FRAP, and TROLOX



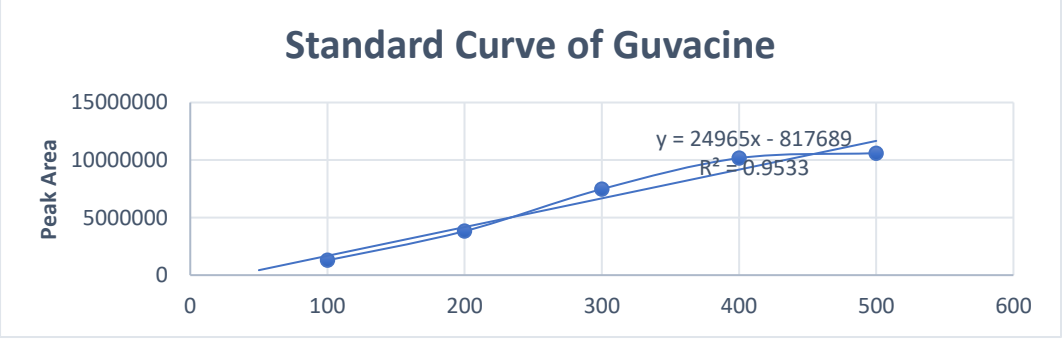
A



B



C



D

Figure 11. 2 Standard curve of arecoline (A), guvacoline (B), arecaidine (C), and guvacine (D) in HPLC platform

Table 11. 3 Raw data for preliminary study of cytotoxicity effect of BQ component on OKF-6

Sample	Concentration	Day 1		Day 2		Day 3	
		Mean	SD	Mean	SD	Mean	SD
WP HUSK	100	133.2	12.7	105.9	3.7	106.0	1.5
	250	111.7	6.2	100.4	4.4	113.5	4.8
	500	108.1	2.1	95.2	4.2	107.1	6.7
	1000	101.2	12.0	113.0	10.8	120.2	5.7
WP MIX	100	126.4	7.0	111.5	4.4	99.4	12.4
	250	115.3	12.5	100.0	4.8	101.9	5.7
	500	112.9	12.5	101.4	7.3	99.3	3.9
	1000	117.2	13.2	108.9	3.5	111.1	7.3
WP-AN	100	99.6	1.8	71.5	0.3	48.1	1.0
	250	93.0	5.2	57.7	2.8	30.2	2.7
	500	68.9	7.8	49.1	0.5	27.1	0.2
	1000	66.3	6.0	45.2	2.3	23.3	1.9
WP SI	250	44.8	4.5	50.8	1.3	26.3	1.3
	100	97.3	9.0	96.1	2.3	77.6	8.5
BA-AN	100	105.1	12.2	106.1	9.2	36.3	2.7
	250	103.1	18.6	84.4	10.5	24.3	4.5
	500	95.6	5.8	52.6	2.5	16.7	0.6
	1000	55.1	9.6	38.6	1.5	22.4	1.1
BA Mix	100	119.7	18.6	148.2	8.1	75.3	7.9
	250	127.4	8.1	142.1	3.1	88.4	7.0
	500	123.4	1.1	134.6	0.4	88.0	9.6
	1000	125.2	6.0	145.8	5.1	88.8	8.0
BA-Husk	100	110.4	3.6	144.4	8.9	87.1	6.8
	250	119.5	4.2	120.5	8.9	82.7	5.2
	500	121.1	11.0	123.8	1.2	81.0	12.7
	1000	125.6	12.8	114.9	7.6	85.4	8.4
BA-Leaf	100	133.1	11.9	126.7	17.4	95.0	12.6
	250	119.4	7.7	112.6	7.3	91.8	11.3

Table 11. 4 Number of cells counted by automated cell counter for validation

Sample	Concentration μg/ml	Total cell number	Living cell	CV%
Banda Aceh Areca Nut	250	70100	28741	41
	250	66766	32047	48
	250	71767	32295	45
	500	75067	25000	33.3
	500	76766	26667	34.7
	500	78400	20000	25.51
West Papua Areca Nut	100	91733	45100	49.2
	100	95100	35000	36.8
	100	115000	55350	48.13
	250	151666.7	26700	17.6
	250	178333.3	13333	7.5
	250	166667	15000	8.9
Control		68433	63400	92.65
		90336.7	80000	88.56
		70100	65100	92.87