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Article

Screening of Polyphenols in Tobacco (*Nicotiana tabacum*) and Determination of Their Antioxidant Activity in Different Tobacco Varieties

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ABSTRACT: Tobacco (*Nicotiana tabacum*) is an herbaceous plant originating from South America and processed into cigarettes for consumption. Polyphenols are considered vital components of tobacco in view of their contribution to antioxidant properties. This study aimed to determine the phenolic compounds in different tobacco varieties by applying cold extraction with methanol and distilled water. The extracts were screened for phenolic compound diversity and distribution as well as their antioxidant potential in different tobacco varieties. The results showed that the methanolic extract of tobacco SP-28 exhibited the highest value in the total phenolic content (24.82 ± 0.07 mg GAE/$g_{d.w.}$) and total flavonoid content (4.42 ± 0.01 mg QE/$g_{d.w.}$), while the water extract of tobacco SN-2 exhibited the highest value in the total condensed tannin (1.12 ± 0.03 mg CE/$g_{d.w.}$). The radical scavenging capacities of tobacco SP-28 were relatively high in DPPH (18.20 ± 0.01 mg AAE/$g_{d.w.}$) and FRAP (3.02 ± 0.10 mg AAE/$g_{d.w.}$), whereas the ABTS value was the highest in tobacco SN-2 (37.25 ± 0.03 mg AAE/$g_{d.w.}$), and the total antioxidant capacity was the highest in tobacco SN-1 (7.43 ± 0.18 mg AAE/$g_{d.w.}$). LC-ESI-QTOF-MS/MS identified a total of 49 phenolic compounds, including phenolic acids (14), flavonoids (30), and other polyphenols (5) in four different tobacco varieties. Tobacco SP-28 showed the highest number of phenolic compounds, especially enriched in flavones. Our study highlights the antioxidant potential of tobacco extracts and reveals the phenolic distribution among different tobacco varieties that could support tobacco utilization in different pharmaceutical industries.

1. INTRODUCTION

Tobacco (*Nicotiana tabacum*) is one of the annual or limited perennial herbaceous plants in the *Solanaceae* family, which originated from South America.[1] There are more than 60 known tobacco plants in the genus of *Nicotiana*, but only two of them, *Nicotiana rustica* and *N. tabacum*, are known to be made into cigarettes.[2] Currently, tobacco becomes a very popular commercial plant because it is able to grow on relatively infertile land and is extremely profitable.[3] China has been the largest tobacco grower in the world, which achieved above 1 million hectares of cultivated area in 2017.[4] In recent years, the extraction of bioactive compounds from different plant and marine sources has become a popular trend.[5−8] Tobacco (*N. tabacum*) has been proven to contain a large number of biologically active ingredients, such as alkaloids and polyphenols,[9] which contain anti-oxidation, anti-inflammatory, and anti-fungal functions. The bioactive components in tobacco (*N. tabacum*) are mainly phenolics, flavonoids, terpenoids, alkaloids, and polysaccharides, which contribute to the functions of tobacco extracts.[10] Meanwhile, it also contains a lot of aromatic compounds such as limonene, indole, pyridine, and phytosterols.[9] The chemical composition of tobacco leaves is influenced by factors such as ripening, drying, fermentation, treatment processing, and storage. Polyphenols are important flavoring substances in tobacco, accounting for approximately 7% of dry weight, and their concentrations are determined by maturity, variety,[11] and the temperature of the air-curing process.[12]

Polyphenols are the most common antioxidants in the daily diet,[13] which can scavenge free radicals produced during cellular respiration and normal metabolism.[14] There are three main mechanisms of antioxidant action: regulation of the activities of antioxidant enzymes to reduce the production of oxygen radicals,[15] combination with free radicals to form

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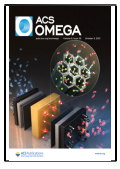

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phenolic oxygen radicals to stop the chain reaction,[16] and reduction of the Fenton reaction by chelating with metal ions.[17] The chlorogenic acid of tannin, scopolamine, hyoscyamine of coumarin, and rutin, flavone, and rhamnose of flavonoids are the main polyphenols in tobacco, among which chlorogenic acid, rutin, and scopolamine account for over 80% of the total content of polyphenols and are the most abundant polyphenols in tobacco leaves.[18] Polyphenols are not only influencing the growth of tobacco, but the phenolic compounds and their metabolites are also aroma substances of cigarettes.[12] Therefore, the content of polyphenols in tobacco determines the quality and flavor of cigarette products. The accumulation of scopolamine in tobacco plants may be a reaction of tobacco plants to adverse factors such as bacteria, mold, and chemical and mechanical damage.[18] The known pathways for the synthesis of polyphenols in tobacco can be generally divided into three: the shikimic acid pathway, the acetic acid−malonic acid pathway, and the acetic acid−mevalonate pathway.[19] A previous study shows that the content of polyphenols in different parts of tobacco also varies,[12] and the main trend is the concentrate of middle leaf > lower leaf > upper leaf.

In this study, the phenolic compounds were estimated by the total phenolic content (TPC) assay, total flavonoid content (TFC) assay, and total condensed tannin (TCT) assay. Also, different antioxidant methods were applied to determine the antioxidant of these tobacco sample powders such as total antioxidant capacity (TAC) assay, ferric reducing antioxidant power (FRAP) determination, and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis-3- ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging evaluation. Further, phenolic compounds were characterized by the LC-ESI-QTOF-MS/MS analysis and reported in several groups and subgroups. The Venn diagrams were sketched to illustrate the distribution of phenolic compounds in different tobacco varieties and Pearson's correlation coefficient and the principal component analysis (PCA) were applied to explain the differences between antioxidant assays and phenolic composition. This work reveals the differences of phenolics among four different tobacco varieties and provides comprehensive information about their phenolic composition and distribution to support the utilization of tobaccos in different industries.

2. RESULTS AND DISCUSSION

2.1. Polyphenol Estimation (TPC, TFC, and TCT). The phenolic compounds were extracted using cold extraction of methanol (MK-399, MSN-1, MSN-2, and MP-28) and distilled water (WK-399, WSN-1, WSN-2, and WSP-28). The phenolic estimation was carried out by TPC, TFC, and TCT assays. Tobacco has been shown to contain a variety of phenolic compounds.[20] These plant secondary metabolites are synthesized mainly through the shikimic acid and malonic acid pathways[21] and provide protection against abiotic stress and pathogen infection.[22] The main polyphenols in tobacco are chlorogenic acid and rutin (quercetin-3-rhamnosyl glucoside), which could be extracted from tobacco and tobacco waste.[23] The data in Table 1 showed that the TPC values of SP-28 were higher than the other four varieties. These results also tally with the fact that SP-28 tobacco is more resistant to stress and has higher bacterial tolerance. A higher concentration of total phenols (15.80 mg GAE/g) from the Oriental tobacco sample has been reported in a previous study,[24] which further proved the great potential of tobacco as a raw material of polyphenol products.

Table 1. Polyphenol Content Estimation of Four Tobacco Varieties[a]

sample name	TPC (mg GAE/g)	TFC (mg QE/g)	TCT (mg CE/g)
WK-399	5.87 ± 0.05^{d}	0.25 ± 0.00^{d}	1.05 ± 0.02^{c}
WSN-1	6.91 ± 0.15^{d}	0.05 ± 0.00^{d}	1.19 ± 0.04^{b}
WSN-2	1.78 ± 0.23^{e}	0.12 ± 0.00^{d}	1.12 ± 0.03^{b}
WSP-28	12.93 ± 0.29^{b}	1.22 ± 0.00^{c}	2.98 ± 0.04^{a}
MK-399	11.15 ± 0.19^{b}	3.01 ± 0.01^{b}	1.14 ± 0.02^{b}
MSN-1	4.85 ± 0.08^{d}	1.13 ± 0.00^{c}	0.73 ± 0.00^{d}
MSN-2	8.53 ± 0.24^{c}	4.04 ± 0.01^{a}	0.80 ± 0.01^{c}
MP-28	24.82 ± 0.07^{a}	4.42 ± 0.01^{a}	0.95 ± 0.01^{b,c}

[a]GAE, gallic acid equivalents; QE, quercetin equivalents; CE, catechin equivalents; WK-399, WSN-1, WSN-2, and WSP-28 are distilled water extractions, while MK-399, MSN-1, MSN-2, and MSP-2 are methanol extractions. Superscripts a, b, and c reveal a significant difference between different samples in a column, which was analyzed by one-way ANOVA Tukey's HSD test ($P < 0.05$).

Flavonoids are formed from phenylalanine, tyrosine, and malonic acid and are commonly found in plants as glycosylated derivatives, such as rutin, which is abundant in tobacco.[25] The main structures of flavonoids consisted of a $C_6-C_3-C_6$ carbon skeleton.[26] It was found that the flavonoids were extracted significantly higher in methanol than water as an extraction solvent. Generally, chalcone synthase is the key to the synthesis of flavonoids and is regulated by five genes.[27] The expression intensity of these genes determines the difference in the concentration of flavonoids in different tobacco varieties.[20] Meanwhile, the tobacco flavonoid content is also affected by disease, temperature, light intensity, and other factors.[25] These reasons could illustrate why the value of the SN-1 tobacco total flavonoid content was merely 0.05 mg QE/g in water extraction and 1.13 mg QE/g in methanolic extraction. For total condensed tannins, the values of water extracts were significantly higher than methanolic extracts, which were 1.05 ± 0.02 mg CE/g in WK-399, 1.19 ± 0.04 mg CE/g in WSN-1, 1.12 ± 0.03 mg CE/g in WSN-2, and 2.98 ± 0.04 mg CE/g in WSP-28. A previous study has shown that this ingredient has inhibitory activity on the tobacco Mosaic virus.[28] Therefore, the higher tannins can be extracted from the highly antiviral tobacco varieties. This result is consistent with the characteristics of SP-28 tobacco.

2.2. Antioxidant Activities (TAC, DPPH, FRAP, and ABTS). Four different methods (TAC, DPPH, FRAP, and ABTS) were applied to identify the antioxidant activity of tobacco water and methanolic extract. DPPH and ABTS are stable free radicals that could determine the free radical scavenging capacity of antioxidants.[29] The DPPH radical scavenging result of this study is from 8.89 ± 0.08 mg AAE/g (MSN-1) to 18.20 ± 0.01 mg AAE/g (WSP-28) (Table 2). Other research also has determined that the tobacco extract is an excellent antioxidant that contains a strong ability to scavenge DPPH free radicals.[30] The scavenging effect is positively correlated with the concentration of the extract and is significantly superior to the vitamin C antioxidants commonly used in the food industry.[31] Moreover, the highest value exists in tobacco SP-28 again, which means that the samples with the highest total phenol content have the strongest free radical scavenging ability. A similar trend appeared in the ABTS test, which has shown that antioxidant

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Table 2. Determination of Antioxidant Activities by the Free Radical Capture Capacity[a]

sample	DPPH (mg AAE/g)	FRAP (mg AAE/g)	ABTS (mg AAE/g)	TAC (mg AAE/g)
WK-399	10.24 ± 0.05^{c}	2.39 ± 0.10^{b}	33.71 ± 0.26^{b}	5.89 ± 0.31^{a,b}
WSN-1	14.62 ± 0.06^{b}	2.25 ± 0.06^{b}	33.30 ± 0.03^{b}	7.43 ± 0.18^{a}
WSN-2	11.25 ± 0.04^{c}	1.45 ± 0.07^{c}	37.25 ± 0.03^{a}	6.12 ± 0.24^{a}
WSP-28	18.20 ± 0.01^{a}	3.02 ± 0.10^{a}	36.94 ± 0.14^{a}	3.29 ± 0.27^{c}
MK-399	10.39 ± 0.06^{c}	1.67 ± 0.05^{b,c}	25.00 ± 0.30^{c}	5.95 ± 0.19^{a,b}
MSN-1	8.89 ± 0.08^{d}	1.31 ± 0.07^{c}	26.25 ± 0.39^{c}	4.32 ± 0.11^{c}
MSN-2	12.17 ± 0.23^{b,c}	2.87 ± 0.17^{a}	26.79 ± 0.14^{c}	3.79 ± 0.23^{c}
MP-28	14.28 ± 0.09^{b}	1.75 ± 0.01^{b,c}	37.01 ± 0.13^{a}	6.58 ± 0.14^{a}

[a]AAE, ascorbic acid equivalents; WK-399, WSN-1, WSN-2, and WSP-28 are distilled water extractions, while MK-399, MSN-1, MSN-2, and MSP-2 are methanol extractions. Superscripts a, b, and c reveal a significant difference between different samples in a column, which was analyzed by one-way ANOVA Tukey's HSD test ($P < 0.05$).

activities of SP-28 extraction were 37.01 ± 0.13 mg AAE/g. Therefore, tobacco SP-28 extraction has greater potential as an antioxidant in the food industry.

The FRAP method can measure the antioxidant and reduction abilities of plant samples according to their ability to reduce Fe^{3+} to Fe^{2+}.[32] In FRAP, all values were significantly lower than the other assays, which showed that the highest value was merely 3.02 ± 0.10 mg AAE/g. This indicates that the tobacco extract has only a moderate reduction ability to trivalent iron. A study of tobacco and tobacco waste extract confirmed this view and showed that the tobacco extract was less effective at reducing Fe^{3+} ions than ascorbic acid.[30] This cements the point that the antioxidant activity of tobacco polyphenols is not completely realized by electron transfer. Some researchers reported that the total phenol content in tobacco leaves has a strong positive correlation with FRAP results.[33] In spite of this, such a kind of correlation was not found in this study; there was no consistency between the differences in iron reduction capacity and total phenol content among different tobacco varieties. In addition, tobacco SN-1 (6.12 ± 0.24 mg AAE/g) revealed a stronger antioxidant capacity than SP-28 in aqueous extraction. Thus, the antioxidant capacity is hard to predict from a result of one essay to another. According to a series of research results, different antioxidant activity determination methods give similar results in the detection of spices and seaweed extracts,[34] but there are great differences in the detection of fruit and vegetable samples.[35]

Although the total phenolic content of the methanol extract was much higher than that of water extracts, it did not show the same trend in the antioxidant activity test. This might be due to the different phenolic components and concentrations in different tobacco varieties. Thus, LC-ESI-QTOF-MS/MS was applied to characterize untargeted phenolic compounds present in these different tobacco varieties.

2.3. LC-ESI-QTOF-MS/MS Characterization of Tobacco Extraction. The extractions of four tobacco samples were analyzed by LC-ESI-QTOF-MS/MS and several chemicals were identified. In this study, negative ($[M - H]^-$) and positive ($[M + H]^+$) ionization modes are processed (see the Supporting Information). The polyphenols were tentatively characterized in Agilent LC/MS mass hunter qualitative software, which was based on the differences in m/z ratio in the MS spectra, and Personal Compounds Database and Library (PCDL) was also applied. Further analysis sorted out 49 compounds, which had over an 80 PCDL library score and the mass error < 5 ppm.

Table 3 shows that 49 polyphenols are present in solvent mixtures of aqueous and methanolic extracts in a ratio of 1:1 (v/v) of four different tobacco species (K-399, SN-1, SN-2, and SP-28). Phenolic compounds were classified into phenolic acids (14), flavonoids (30), and other polyphenols (5). Most of the phenolic compounds were included in flavonoids and phenolic acids and the flavonoids were more abundant. The variation in polyphenol compounds in the four tobacco species led to different free radical scavenging abilities between four kinds of tobacco varieties.

2.3.1. Phenolic Acids. Generally, most of the phenolic acid compound ionization was presented in negative mode; this was due to the fact that ESI^- mode was more sensitive to the characterization of phenolic acids.[36] The hydrogen atom donation ability provides phenolic acids radical scavenging activity, which makes these compounds be able to act as natural antioxidants.[37] Among, 14 kinds of phenolic acids were identified in the water and methanol extractions of four tobacco species. They were further classified as hydroxybenzoic (4), hydroxycinnamic (8), and hydroxyphenylpropanoic acids (2).

2.3.1.1. Hydroxybenzoic Acids and Hydroxyphenylpropanoic Acids. Gallic acid, 2,3-dihydroxybenzoic acid, and 2-hydroxybenzoic acid were detected in tobacco SN-2 at m/z 169.0135, m/z 153.0191, and m/z 137.0243. Gallic acid was also reported in mango by-products[38] and ginger.[37] Compound 2, which was extracted from tobacco SN-1, was tentatively identified as protocatechuic acid 4-*O*-glucoside, which generated a $[M - H]^-$ ion at m/z 315.0709. Protocatechuic acids were abundant in fruits of fishtail palm and jelly palm.[39] A previous study has shown that gallic acid and 2,3-dihydroxybenzoic acid exist in strawberry hops and juniper berries.[40] The product ions of 2,3-dihydroxybenzoic acid in MS/MS analysis indicated the loss of CO_2 (44 Da) from precursor ions.[41] Hydroxyphenylpropanoic acid components were only found in tobacco SP-28, which were dihydrocaffeic acid 3-*O*-glucuronide (RT = 19.003 min with m/z 357.0817) and dihydroferulic acid 4-*O*-glucuronide (RT = 23.973 min with m/z 371.0977). These ingredients are naturally versatile antioxidants with a wide range of potential medical and industrial applications.[42]

2.3.1.2. Hydroxycinnamic Acids. Ferulic acid was commonly found in foods including rice, oats, pineapple, coffee, and peanuts,[43] but it was rarely found in tobacco. In this study, two ferulic acid derivatives were detected in tobacco SN-1 and SP-28, which were 3-feruloylquinic acid (RT = 11.461 min with m/z 367.1032) and ferulic acid 4-*O*-glucuronide (RT = 20.530 min with m/z 20.530). Caffeic acid and its derivatives,

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Table 3. Polyphenols in Different Tobacco Samples Using LC-ESI-QTOF-MS/MS[a]

proposed compound	molecular formula	RT (min)	ionization (ESI^+/ESI^-)	molecular weight	theoretical (m/z)	observed (m/z)	error (ppm)	MS/MS product ion	sample
phenolic acids									
hydroxybenzoic acids									
1. gallic acid	$C_7H_6O_5$	11.133	$[M-H]^-$	170.0215	169.0142	169.0135	−4.1	125	SN-2
2. protocatechuic acid 4-*O*-glucoside	$C_{13}H_{16}O_9$	12.325	$[M-H]^-$	316.0794	315.0721	315.0709	−3.8	153	SN-1
3. 2,3-dihydroxybenzoic acid	$C_7H_6O_4$	15.181	$[M-H]^-$	154.0266	153.0193	153.0191	−1.3	109	*K-399, SN-2
4. 2-hydroxybenzoic acid	$C_7H_6O_3$	20.014	**$[M-H]^-$	138.0317	137.0244	137.0243	−0.7	93	*SP-28, K-399, SN-2
hydroxycinnamic acids									
5. verbascoside	$C_{29}H_{36}O_{15}$	4.228	$[M-H]^-$	624.2054	623.1981	623.1989	1.3	477, 461, 315, 135	SN-1
6. 3-feruloylquinic acid	$C_{17}H_{20}O_9$	11.461	$[M-H]^-$	368.1107	367.1034	367.1032	−0.5	298, 288, 192, 191	SN-1
7. caffeoyl glucose	$C_{15}H_{18}O_9$	12.666	$[M-H]^-$	342.0951	341.0878	341.0891	3.8	179, 161	SN-1
8. caffeic acid	$C_9H_8O_4$	15.871	**$[M-H]^-$	180.0423	179.0350	179.0341	−5.0	143, 133	*SP-28, SN-2
9. caffeic acid 3-*O*-glucuronide	$C_{15}H_{16}O_{10}$	19.297	$[M-H]^-$	356.0743	355.0670	355.0653	−4.8	179	*SP-28, SN-2
10. ferulic acid 4-*O*-glucuronide	$C_{16}H_{18}O_{10}$	20.530	$[M-H]^-$	370.0900	369.0827	369.0826	−0.3	193	SP-28
11. sinapic acid	$C_{11}H_{12}O_5$	22.639	**$[M-H]^-$	224.0685	223.0612	223.0605	−3.1	205, 163	*SP-28, K-399
12. *m*-coumaric acid	$C_9H_8O_3$	28.689	**$[M-H]^-$	164.0473	163.0400	163.0397	−1.8	119	*SP-28, K-399
hydroxyphenylpropanoic acids									
13. dihydrocaffeic acid 3-*O*-glucuronide	$C_{15}H_{18}O_{10}$	19.003	$[M-H]^-$	358.0900	357.0827	357.0817	−2.8	181	SP-28
14. dihydroferulic acid 4-*O*-glucuronide	$C_{16}H_{20}O_{10}$	23.973	$[M-H]^-$	372.1056	371.0983	371.0977	−1.6	195	SP-28
flavonoids									
dihydrochalcones									
15. 3-hydroxyphloretin 2′-*O*-glucoside	$C_{21}H_{24}O_{11}$	17.087	**$[M-H]^-$	452.1319	451.1246	451.1226	−4.4	289, 273	*SP-28, K-399, SN-2
16. phloridzin	$C_{21}H_{24}O_{10}$	31.058	$[M-H]^-$	436.1369	435.1296	435.1278	−4.1	273	SN-1
dihydroflavonols									
17. dihydromyricetin 3-*O*-rhamnoside	$C_{21}H_{22}O_{12}$	19.604	**$[M-H]^-$	466.1111	465.1038	465.1021	−3.7	301	*SP-28, K-399
18. dihydroquercetin 3-*O*-rhamnoside	$C_{21}H_{22}O_{11}$	31.493	$[M-H]^-$	450.1162	449.1089	449.1075	−3.1	303	*SN-1, SP-28
flavanols									
19. 4′-*O*-methylepigallocatechin	$C_{16}H_{16}O_7$	10.052	$[M+H]^+$	320.0896	321.0969	321.0963	−1.9	302	SP-28
20. (+)-catechin	$C_{15}H_{14}O_6$	13.897	**$[M-H]^-$	290.0790	289.0717	289.0707	−3.5	245, 205, 179	*SN-2, SP-28, K-399
21. procyanidin dimer B1	$C_{30}H_{26}O_{12}$	16.775	**$[M-H]^-$	578.1424	577.1351	577.1359	1.4	451	*K-399, SP-28
22. procyanidin trimer C1	$C_{45}H_{38}O_{18}$	19.173	**$[M-H]^-$	866.2058	865.1985	865.1990	0.6	739, 713, 695	*K-399, SP-28
23. (+)-gallocatechin	$C_{15}H_{14}O_7$	20.183	**$[M-H]^-$	306.0740	305.0667	305.0666	−0.3	261, 219	*SN-2, K-399
flavanones									
24. neoeriocitrin	$C_{27}H_{32}O_{15}$	22.819	$[M-H]^-$	596.1741	595.1668	595.1669	0.2	431, 287	SP-28
25. narirutin	$C_{27}H_{32}O_{14}$	30.646	$[M-H]^-$	580.1792	579.1719	579.1721	0.3	271	SN-1
flavones									
26. 6-hydroxyluteolin 7-*O*-rhamnoside	$C_{21}H_{20}O_{11}$	25.270	$[M-H]^-$	448.1006	447.0933	447.0935	0.4	301	*SP-28, SN-1
27. apigenin 6,8-di-*C*-glucoside	$C_{27}H_{30}O_{15}$	26.952	$[M-H]^-$	594.1585	593.1512	593.1524	2.0	503, 473	SP-28
28. rhoifolin	$C_{27}H_{30}O_{14}$	27.010	**$[M-H]^-$	578.1636	577.1563	577.1573	1.7	413, 269	*SP-28, SN-1
29. apigenin 6-*C*-glucoside	$C_{21}H_{20}O_{10}$	27.933	**$[M-H]^-$	432.1056	431.0983	431.0968	−3.5	413, 341, 311	*SN-1, SN-2, SP-28
30. diosmin	$C_{28}H_{32}O_{15}$	29.100	**$[M+H]^+$	608.1741	609.1814	609.1787	−4.4	301, 286	*SP-28, SN-1
31. chrysoeriol 7-*O*-glucoside	$C_{22}H_{22}O_{11}$	30.050	**$[M+H]^+$	462.1162	463.1235	463.1217	−3.9	445, 427, 409, 381	SN-1, *SP-28
flavonols									
32. myricetin 3-*O*-galactoside	$C_{21}H_{20}O_{13}$	16.784	$[M-H]^-$	480.0904	479.0831	479.0818	−2.7	317	SP-28
33. kaempferol 3-*O*-glucosyl-rhamnosyl-galactoside	$C_{33}H_{40}O_{20}$	24.110	$[M-H]^-$	756.2113	755.2040	755.2040	0.0	285	SP-28

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Table 3. continued

proposed compound	molecular formula	RT (min)	ionization (ESI^+/ESI^-)	molecular weight	theoretical (m/z)	observed (m/z)	error (ppm)	MS/MS product ion	sample
34. kaempferol 3-*O*-(2″-rhamnosyl-galactoside) 7-*O*-rhamnoside	$C_{33}H_{40}O_{19}$	26.474	$[M - H]^-$	740.2164	739.2091	739.2124	4.5	593, 447, 285	SP-28
35. kaempferol 3,7-*O*-diglucoside	$C_{27}H_{30}O_{16}$	28.515	**$[M-H]^-$	610.1534	609.1461	609.1451	−1.6	447, 285	*SP-28, SN-1, SN-2
36. myricetin 3-*O*-rhamnoside	$C_{21}H_{20}O_{12}$	29.834	**$[M-H]^-$	464.0955	463.0882	463.0863	−4.1	317	*SP-28, SN-1, SN-2
37. quercetin 3-*O*-(6″-malonyl-glucoside)	$C_{24}H_{22}O_{15}$	31.680	$[M + H]^+$	550.0959	551.1032	551.1008	−4.4	303	SN-2
isoflavonoids									
38. 6″-*O*-acetylglycitin	$C_{24}H_{24}O_{11}$	9.159	**$[M + H]^+$	488.1319	489.1392	489.1391	−0.2	285,270	SP-28
39. 3′-hydroxygenistein	$C_{15}H_{10}O_6$	17.278	$[M + H]^+$	286.0477	287.0550	287.0539	−3.8	269,259	*K-399, SP-28
40. 3′-hydroxydaidzein	$C_{15}H_{10}O_5$	22.627	$[M + H]^+$	270.0528	271.0601	271.0588	−4.8	253, 241, 225	K-399
41. 5,6,7,3′,4′-pentahydroxyisoflavone	$C_{15}H_{10}O_7$	29.837	**$[M + H]^+$	302.0427	303.0500	303.0490	−3.3	285, 257	K-399, *SP-28, SN-2
42. violanone	$C_{17}H_{16}O_6$	31.058	$[M - H]^-$	316.0947	315.0874	315.0862	−3.8	300, 285, 135	SN-1
43. 6″-*O*-malonylgenistin	$C_{24}H_{22}O_{13}$	31.447	**$[M + H]^+$	518.1060	519.1133	519.1114	−3.7	271	SN-1, *SP-28
44. glycitin	$C_{22}H_{22}O_{10}$	35.318	$[M + H]^+$	446.1213	447.1286	447.1274	−2.7	285	SP-28
other polyphenols									
hydroxybenzaldehydes									
45. 4-hydroxybenzaldehyde	$C_7H_6O_2$	31.363	**$[M - H]^-$	122.0368	121.0295	121.0292	−2.5	77	*SN-1, SP-28, K-399, SN-2
hydroxycoumarins									
46. coumarin	$C_9H_6O_2$	9.554	$[M + H]^+$	146.0368	147.0441	147.0445	2.7	103, 91	*SP-28, SN-2
tyrosols									
47. hydroxytyrosol 4-*O*-glucoside	$C_{14}H_{20}O_8$	24.382	$[M - H]^-$	316.1158	315.1085	315.1078	−2.2	153, 123	SP-28
lignans									
48. schisandrol B	$C_{23}H_{28}O_7$	5.936	$[M + H]^+$	416.1835	417.1908	417.1926	4.3	224, 193, 165	*SN-2, SP-28
stilbenes									
49. 3′-hydroxy-3,4,5,4′-tetramethoxystilbene	$C_{17}H_{18}O_5$	30.612	$[M + H]^+$	302.1154	303.1227	303.1217	−3.3	229, 201, 187, 175	SP-28

[a]Single asterisk (*): compounds are characterized in more than one sample, but data presented in the table belong to the asterisk sample. Double asterisk (**): compounds were detected in both positive ionization mode $[M + H]^+$ and negative ionization mode $[M - H]^-$, whereas the data were presented in single mode.

which normally are glycosides formed primarily with other sugars, are common in tobacco and its smoke.[44] In our study, the precursor ions found at m/z 341.0891 (compound 7), m/z 179.0341 (compound 8), and m/z 355.0653 (compound 9) represented the existence of caffeoyl glucose, caffeic acid, and caffeic acid 3-*O*-glucuronide. The MS/MS product ions at m/z 143 and m/z 133 were formed by caffeic acid losing $2H_2O$ and HCOOH.[40] The derivatives of caffeic, sinapic, and ferulic acids were also detected in edible parts of palm fruits,[39] black spices,[45] garlic, and cherry.[46]

2.3.2. Flavonoids. Flavonoids are the largest phenolic compound group in this study; 30 identified flavonoids were divided into flavanols (5), flavanones (2), flavones (6), flavonols (6), isoflavonoids (7), dihydrochalcones (2), and dihydroflavonols (2). It is worth noting that tobacco SP-28 contains almost all kinds of flavonoids that have been isolated. There is evidence that dietary intake of isoflavones and flavones is inversely associated with cancer risk.[47] Therefore, tobacco SP-28 extraction has the potential as a functional food additive.

2.3.2.1. Dihydrochalcones. In the present work, only two dihydrochalcones were present in tobacco samples. Compound 15 with precursor ions found at m/z 451.1226 in both positive and negative modes was identified as 3-hydroxyphloretin 2′-*O*-glucoside. This substance was also reported in black spices,[45] fruit peels,[48] and juniper berries.[49] The other dihydrochalcone compound was phloridzin (RT = 31.058 min with m/z 435.1278), which contained peak fragmentation at m/z 273 caused by the consecutive loss of glucoside.[40]

2.3.2.2. Flavanols and Flavanones. A total of five flavanols and two flavanones were divided from tobacco extractions. The procyanidin dimer B1 (RT = 16.775 min with m/z 577.1359) and procyanidin trimer C1 (RT = 19.173 min with m/z 865.1990) were only detected in tobacco K-399 and SP-28. More cyanidins were reported in previous studies, such as cyanidin 3-*O*-rutinoside, acylated cyanidin 3-*O*-(coumaroyl) rutinoside,[50] and cyanidin 3-*O*-rutinoside chloride.[25] The catechin and their derivatives were the most abundant components in group flavanols, among which 4′-*O*-methyl-epigallocatechin was identified with the precursor ion $[M + H]^+$ at m/z 321.0963. This compound has also been reported in *Elaeodendron transvaalense*, a kind of medicine plant located in southern African countries.[51]

2.3.2.3. Flavones, Flavonols, and Dihydroflavonols. There were six flavanols and six flavanones detected in tobacco samples except K-399. Several flavone and flavonol compo-

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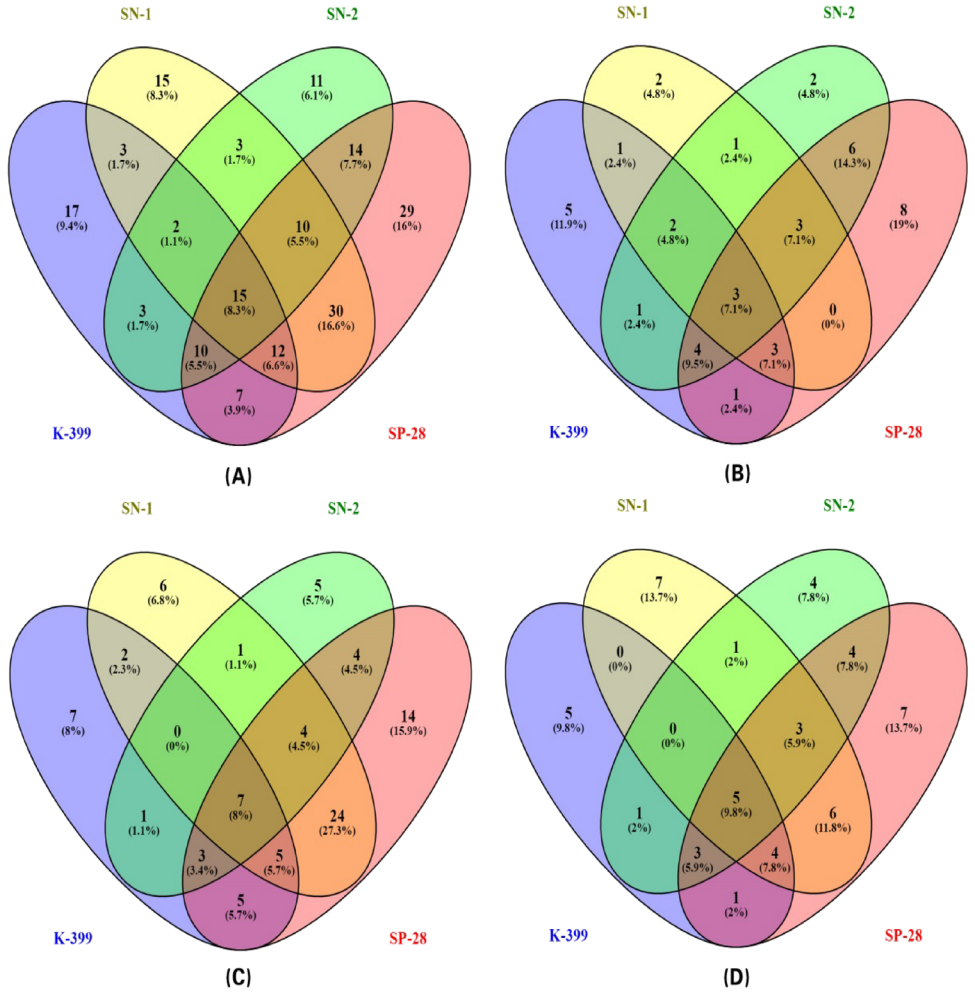

Figure 1. Venn diagrams of polyphenol components determined in different tobacco samples. (A) Total phenolic compound distribution in different tobacco species and (B) relations of phenolic acids present in different tobacco samples. (C) Flavonoid distribution in tobacco samples and (D) other phenolic distribution situation in all four different varieties of tobaccos.

nents identified were in the form of glycosides, most of which were combined with rhamnoside and glucoside. Compounds 33, 34, and 35, which had the precursor ion $[M - H]^-$ at m/z 755.2040, 739.2124, and 609.1451, were tentatively characterized as kaempferol 3-*O*-glucosyl-rhamnosyl-galactoside, kaempferol 3-*O*-(2″-rhamnosyl-galactoside) 7-*O*-rhamnoside, and kaempferol 3,7-*O*-diglucoside. In addition, many other kaempferol derivatives have been reported, such as astragalin and nicotiflorin.[25] The precursor ion $[M - H]^-$ at m/z 285 was generated by losing a neutral hexose $[M\text{-}C_5H_{10}O_5]^-$ of the ion $[M - H]^-$ at m/z 285. The other groups of flavonols detected in this study were myricetin 3-*O*-galactoside (RT = 16.784 min with m/z 479.0818) and myricetin 3-*O*-rhamnoside (RT = 29.834 min with m/z 463.0863) in negative mode. Apigenin derivatives have also been found in air-cured tobacco in a previous study.[52] In tobacco SN-1, SN-2, and SP-28, compound 29 was identified as apigenin 6-*C*-glucoside, which had the precursor ion $[M - H]^-$ at m/z 431.0968.

2.3.2.4. Isoflavonoids. Compounds 38, 40, and 44 with precursor ions $[M + H]^+$ at m/z 489.1391, m/z 271.0588, and m/z 447.1274 were tentatively characterized as 6″-*O*-acetylglycitin, 3′-hydroxydaidzein, and glycitin. These three isoflavonoids were rarely found in tobacco samples but frequently observed in legume plants, such as soybean[53] and black bean.[54] Moreover, compound 42 was identified as violanone, which detected precursor ions at m/z 315.0862 in negative ionization mode. This substance was found in tobacco samples for the first time.

2.3.3. Other Polyphenols. Five other polyphenol compounds were detected in these four kinds of tobaccos, which were divided into hydroxybenzaldehydes, hydroxycoumarins, tyrosols, lignans, and stilbenes. The product ions of coumarin (RT = 9.554 min with m/z 147.0445), which were located at m/z 103 and m/z 91, were generated by losing CO_2 and two CO.[55] 4-Hydroxyfenzaldehyde was the only detected hydroxybenzaldehyde (RT = 31.363 min with m/z 121.0292), which exists in all four tobacco varieties. This compound was found in both negative and positive ionization modes, which contained peak fragmentation at m/z 77. In this group, tobacco SP-28 contained all species of polyphenols including hydroxytyrosol 4-*O*-glucoside, schisandrol B, and 3′-hydroxy-3,4,5,4′-tetramethoxystilbene.

2.4. Phenolic Content Distribution in Tobacco. Various polyphenols exist in tobacco samples that have conjugated structures in forms, and there are differences in their distribution in different tobaccos. Therefore, analyzing the variability of these polyphenols species in different tobacco samples at the same time would be a complex task. The Venn diagrams (Figure 1) were sketched in this study to offer a synopsis of different phenolic compound distributions, which

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Table 4. Correlation between the Phenolic Content (TPC, TFC, and TCT) and Antioxidant Activities (DPPH, FRAP, and ABTS) Performed as Pearson's Correlation Coefficients (r)

variable	TPC	TFC	TCT	DPPH	FRAP	ABTS
TFC	0.724[a]					
TCT	0.165	−0.216				
DPPH	0.484	0.063	0.790[b]			
FRAP	0.113	0.082	0.563	0.629[a]		
ABTS	0.246	−0.302	0.458	0.601	0.153	
TAC	0.079	−0.188	−0.422	−0.141	−0.457	0.253

[a]Significant level, $P \leq 0.05$ of correlation. [b]Significant level, $P \leq 0.01$ of correlation.

were labeled with different colors in tobacco SN-1 (yellow), SN-2 (green), SP-28 (red), and K-399 (blue).

The Venn diagrams showed that SP-28 contained 29 unique compounds, which account for 16% of the total phenolic compound. Meanwhile, tobacco SN-1, SN-2, and K-399 contain 17 (9.4%), 15 (8.3%), and 11 (6.3%), respectively. The maximum value of overlapping total phenolic compounds that were distributed in tobacco SP-28 and SN-1 was 30 (16.6%), among which 24 (27.3%) of them belonged to flavonoids. The minimum value of overlapping total phenols present in tobacco SN-1, SN-2, and K-399 was 2 (1.1%). Moreover, there were 15 total phenolic compounds commonly existing in four different tobacco species. Previous researchers found that the composition of polyphenols could be applied as a parameter to characterize the class of tobaccos.[56] This indicates that tobacco SP-28 is the most abundant polyphenol tobacco species. It has been shown in the literature that phenols are responsible for tobacco resistance to pathogens and can influence aromatic properties.[24] As a result, tobacco with high stress resistance and a superior aroma would contain higher phenolics.

In other studies, flue-cured tobacco leaves contain more flavonoids than phenolic acids,[57] and this pattern is also reflected in the present study. The Venn diagram shows that all tobacco species presented more phenol species in most overlapping phenols and all unique phenols. The maximum unique phenolic acids and flavonoids were still located in tobacco SP-28, which were 8 (19%) and 14 (15.9%), respectively. One obvious difference is that there was no common overlapping phenolic acid among tobacco SP-28 and SN-1, while this area had the highest similarity of flavonoids, which was 24 (27.3%). The other trend was that seven phenols were commonly found in four tobaccos, which were distributed in one phenolic acid, five flavonoids, and other phenolics.

2.5. Correlation between the Phenolic Content and Antioxidant Activities. The correlation of the phenolic content (TPC, TFC, and TCT) and antioxidant activities (DPPH, FRAP, ABTS, and TAC) was evaluated by Pearson's correlation test, and the correlation coefficients are presented in Table 4. Additionally, the similarities and differences between methods applied to estimate antioxidant activity and measure the phenolic content were investigated by principal component analysis (PCA), and these are summarized in Figure 2.

Only two antioxidant assays showed a positive significant correlation, which were DPPH and FRAP with the Pearson's correlation coefficient $r = 0.629$ ($P < 0.05$). A previous study has proved a significant positive correlation existing between FRAP and other antioxidant assays.[58] Furthermore, a highly significant positive correlation has been found between TCT and DPPH with Pearson's correlation coefficient $r = 0.79$ ($P <$

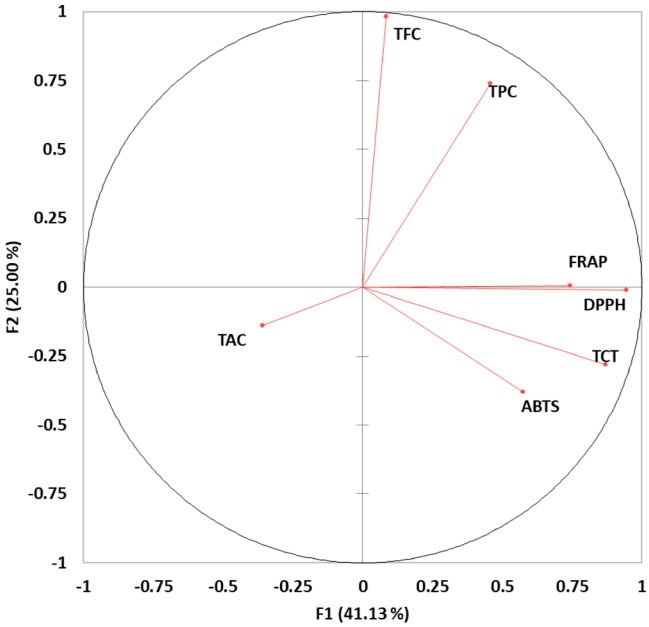

Figure 2. Principal component analysis (PCA) of the phenolic content (TPC, TFC, and TCT) and antioxidant determination assays (DPPH, ABTS, FRAP, and TAC) of four tobacco species.

0.01), which revealed the same trend in the studies of Li et al.[59] and Wang et al.[60] The reason is that the phenolic compounds in tobacco are able to offer H to DPPH free radicals to form DPPH-H.[59] The TPC assay was detected having a significant positive correlation with antioxidant activity including ABTS, DPPH, and FRAP.[61] However, these correlations did not reach a significant level in this study. Other antioxidant assays were also strongly correlated with each other in a previous report[62] but not presented in this study. This was due to the finding that phenols in extracts of tobacco samples have different scavenging abilities to DPPH, ABTS free radicals, and reducing Fe^{3+}-TPTZ. Apart from the above, the TPC has shown a significant positive correlation with the TFC; this supported the finding in phenolic content distribution in tobacco that flavonoids occupy a dominant position in tobacco polyphenols.

Figure 2 indicates that there was 66.14% of total variability present in the initial data, which was kept by the first two factors F1 and F2. The distance between two assays presents the proximity level of them; the closer the two vectors are, the more significant the correlation is. For instance, the distances of assays FRAP and DPPH and assays DPPH and TCT were very close, so it contained significant positive correlations, which are shown in Pearson's correlation coefficient table (Table 4). In short, this study highlights the antioxidant

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potential of tobacco extraction and reveals the polyphenol diversity among different tobacco species, which could support tobacco by-products utilized as additives in different industries for bioactive product development.

3. METHODOLOGY

3.1. Chemical and Reagents. Several chemicals of analytical grade that were used for extraction and characterization were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) including 2,2′-azino-bis (3 ethylbenzothiazoline-6-sulfonic acid) (ABTS), Folin and Ciocalteu's phenol reagent, 2,2′-diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, vanillin, hexahydrate aluminum chloride, ferric chloride, gallic acid, quercetin, and 2,4,6-tripyridyl-*s*-triazine (TPTZ). The supplier of hydrochloric acid, glacial acetic acid, hydrated sodium acetate, methanol, and anhydrous sodium acetate was Thermo Fisher Scientific Inc. (Waltham, MA, USA). Sodium carbonate (anhydrous) was supplied by Chem-Supply Pty Ltd. (Adelaide, SA, Australia), while sulfuric acid (98%) was purchased from RCI Labscan (Rongmuang, Thailand). Deionized water (resistivity, 18.2 MΩ/cm) was prepared by a Millipore Milli-Q Gradient Water Purification System (Darmstadt, Germany) and the filtration was processed by a 0.22 μm type Millipak Express 20 filter (Milli-Q, Darmstadt, Germany).

3.2. Sample Preparation and Extraction. Four kinds of tobacco samples (K-399, SN-1, SN-2, and SP-28) of *N. tabacum* were collected from various regions of Swabi, KPK, Pakistan. The plant specimen was identified by a botanist in the Department of Botany, University of Swabi KP, Pakistan. The drying procedure was processed under shade at room temperature, ground into uniform powder, and stored at −20 °C. Then, the powder samples were cold-extracted with organic solvents methanol (MK-399, MSN-1, MSN-2, and MSP-28) and distilled water (WK-399, WSN-1, WSN-2, and WSP-28). The methanolic extracts were concentrated by a rotary evaporator at low temperature (50−55 °C) and the water extracts were concentrated by a water bath. Finally, the aqueous and methanol extracts were filtrated by a syringe filter (0.45 μm, Thermo Fisher Scientific Inc., Waltham, MA, USA) and the supernatant was used for further analysis, conducted at the Department of Agriculture and Food Systems, The University of Melbourne in Australia.

3.3. Antioxidant Activity Determination. *3.3.1. Determination of the Total Phenolic Content (TPC).* The total phenolic content of tobacco extracts was determined by following the protocol of the Folin−Ciocalteu method[63] with some modifications. Twenty-five microliters of extract and 25 μL of Folin−Ciocalteu's reagent (1:3 diluted with water) were mixed in a 96-well plate (Corning Inc., Corning, NY, USA), followed by 5 min of incubation at 25 °C. Then, 200 μL of water and 25 μL of 10% (w/w) sodium carbonate were added to dilute and another 1 h of incubation was required. Finally, the absorbance at 725 nm was measured in a microplate reader, and gallic acid (0−200 μg/mL) in ethanolic solution was added for standard curve generation. The result was presented in mg gallic acid equivalents/$g_{d.w.}$.

3.3.2. Determination of the Total Flavonoid Content (TFC). The total flavonoid content was measured by the $AlCl_3$ colorimetry-based method.[38] The tobacco extract (80 μL) was mixed with 80 μL of 2% aluminum chloride and 120 μL of 50 g/L sodium acetate (water solution) in a 96-well plate, followed by 2.5 h of incubation at 25 °C. The absorbance at 440 nm was measured in a microplate reader, and quercetin methanolic solution (0−50 μg/mL) was added for standard curve generation. Each sample was processed in triplicate, and the result was presented in mg quercetin equivalents.

3.3.3. Determination of the Total Condensed Tannin (TCT). The total tannin content measurement was based on a previously reported method.[60] Twenty-five microliters of tobacco extract was mixed with 150 μL of 4% vanillin solution and 25 μL of 32% sulfuric acid in a 96-well plate and incubated for 15 min at 25 °C. Finally, the absorbance at 500 nm was measured in a microplate reader, and catechin (0−1000 μg/mL) in methanolic solution was added for standard curve generation. The measurements were repeated three times, and the result was presented in mg catechin equivalents.

3.3.4. 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) Antioxidant Assays. The process was based on the published protocol of Zhu et al.[64] DPPH solution was diluted with analytical grade methanol to 0.1 M. Forty microliters of the extraction was added into 260 μL of DPPH radical methanol solution in a 96-well plate and incubated for 30 min at 25 °C. The standard curve was generated by ascorbic acid solution with 0−30 μg/mL. For accuracy, each sample was measured in triplicate, and results were expressed in mg ascorbic acid equivalents.

3.3.5. Ferric Reducing Antioxidant Power (FRAP) Assay. The antioxidant power was also determined by the ferric reducing capability assay, which was based on the reported method of Hong et al.[65] The FRAP reagent was composed of 300 mM acetate buffer, 10 mM TPTZ, and 20 mM ferric chloride in a volume ratio of 10:1:1. Twenty microliters of tobacco extract was mixed with 280 μL of FRAP reagent in a 96-well plate, followed by 10 min of incubation at 37 °C. Absorbances of samples were measured by a microplate reader at 593 nm, and ascorbic acid (0−50 μg/mL) solution was added as a reference for standard curve generation. Measurements done three times were expressed in mg AAE (ascorbic acid equivalents).

3.3.6. 2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic Acid (ABTS) Radical Scavenging Assay. The $ABTS^+$ radical cation decolorization assay was applied to measure the ABTS antioxidant activity of tobacco extracts.[66] The $ABTS^+$ stock solution was prepared by mixing 7 mM ABTS and 140 mM potassium persulfate solutions, followed by 16 h of incubation in the dark environment. Then, the $ABTS^+$ solution was diluted with ethanol until it achieved the absorbance of 0.70 ± 0.02 at 734 nm. Finally, the absorbance of samples, which included 10 μL of sample extract and 290 μL of prepared $ABTS^+$ solution, followed by another 6 min of incubation, was measured at 500 nm in a microplate reader. The ascorbic acid aqueous solution with the concentration of 0−200 μg/mL was applied for standard curve generation. The measurements were repeated three times, and the result was presented in mg AAE.

3.3.7. Total Antioxidant Capacity (TAC) Assay. The total antioxidant capacity measurement method was based on the published protocol of Prieto et al.[67] The tobacco extracts were pipetted (40 μL) and added into 260 μL of phosphomolybdate reagent, which was prepared by mixing 0.6 M sulfuric acid, 0.028 M sodium phosphate, and 0.004 M ammonium molybdate. The following procedure was incubation of 300 μL samples at 95 °C for 10 min and then cooling to room temperature. Finally, the absorbance of sample solution at 695 nm was measured and compared with the standard curve, which was structured by ascorbic acid with a predetermined gradient concentration.

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3.4. Polyphenol Identification by LC-ESI-QTOF-MS/MS Analysis. The identification of the polyphenol content was carried out according to the previously published method of Suleria et al.[48] A liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) was connected with a mass Q-TOF liquid chromatograph and continued to connect to a double mass spectrometer by an electrospray ionization (ESI) source. Separation was processed in a Synergi Hydro-RP 80 Å, LC Column 250 mm × 4.6 nm, 4 μm (Phenomenex, Torrance, CA, USA) with a stable column temperature at 25 °C and a sample temperature at 10 °C. Mobile phase A was 2% acetic acid in 98% water, and mobile phase B was a mixture of acetonitrile/water/acetic acid (100:99:1, v/v/v). The whole gradient program was set to 85 min in length, with a mobile phase flow of 0.8 mL/min, and the volume of sample injection was 5 μL. Mass spectrum parameters were executed as follows: nebulizer gas pressure, 45 psi; 250 °C sheath gas with a flow rate of 11 L/min; 300 °C N_2 with a flow rate of 5 L/min. The software of Agilent (Agilent Technologies, Santa Clara, CA, USA) was specialized for data acquisition and analysis. The peak identification was executed in both negative ($[M - H]^-$) and positive ($[M + H]^+$) ionization modes. The working voltages were 3.5 kV (capillary) and 500 V (nozzle), and the mass spectra ranged from 50 amu to 1300 amu. Agilent LC-ESI-QTOF-MS/MS Mass Hunter workstation software (Qualitative Analysis, version B.03.01, Agilent) was applied for data acquisition and analysis performance.

3.5. Statistical Analysis. The chemical composition of each sample will be represented as the mean ± standard deviation of the three independent repetitions. Data differences between four different tobacco species were analyzed by one-way ANOVA Tukey's HSD test, and the statistically significant level was set as $P < 0.05$. The group differences between the aqueous extract and the methanolic extract were also tested. The correlation between antioxidant assays and phenolic compound estimation methods was evaluated by Pearson's correlation coefficient at $P < 0.05$ and the principal component analysis (PCA) was executed by XLSTAT-2019.1.3 (Addinsoft Inc., New York, NY, USA).

4. CONCLUSIONS

In conclusion, all tobacco samples, SN-1, SP-28, K-399, and SN-2, contain a wide variety of polyphenols and are able to scavenge free radicals efficiently, showing strong antioxidant potential. According to the result of LC-ESI-QTOF-MS/MS, 49 phenolic compounds were characterized; some variations in polyphenols existed in different tobacco species. The polyphenols of tobacco SP-28 were the most abundant, which might be the reason that the ability to scavenge free radicals is better than the other tobacco samples. These identified polyphenols revealed the potential value of tobacco by-products. This project would promote tobacco by-product recycling and offer new raw materials for food industries and pharmaceuticals. Further studies about tobacco extraction toxicological, bioavailability, and animal studies are required for developing tobacco by-products as commercial ingredients.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c03275.

LC-ESI-QTOF-MS/MS basic peak chromatography for the characterization of phenolic compounds of tobaccos (PDF)

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X.Z. and H.A.R.S. conceptualized the study. X.Z., A.R., A.B., and M.S. provided the methodology. Y.S.A., F.A.A., and H.A.R.S. validated the data. O.B., X.Z., and A.R. conducted the formal analysis. M.H.M. and H.A.R.S. performed the investigation. X.Z and H.A.R.S. provided the resources, curated the data, and prepared the original draft of the manuscript. All authors read the paper and agreed to the process for publications.

Notes

The authors declare no competing financial interest.

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