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Assessing the Differences in Phenolics, Antioxidant and Anti-Tyrosinase Activities of Spent Coffee Ground Fractions

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ABSTRACT

Spent coffee ground (SCG) is among the high-volume by-products generated from coffee processing. The present study aimed to determine phenolic acids, antioxidant activity and tyrosinase inhibitory effect of fractions obtained from crude extracts of SCG. The fractionation was performed using different polarity-based solvents, resulting in n-hexane, ethyl acetate and aqueous extracts. Total phenolic content was evaluated by Folin – Ciocalteu method while individual phenolics were quantified using HPLC-DAD. Antioxidant activity was assessed by DPPH and ABTS assays. Inhibition of tyrosinase was determined using an in vitro bioassay. The ethyl acetate fraction was shown to have a higher amount of total phenolic content (227.17 mg GAE/g) compared to the other fractions and the methanol crude extract. It also exhibited higher antioxidant activity than the other fractions and was identified as the most effective tyrosinase inhibitor (87.59 ± 8.62 mg KAE/g). Gallic acid, chlorogenic acid, ferulic and salicylic acid had positive correlations with antioxidant activity, with Pearson's correlation coefficients ranging between 0.808 and 0.990. Through the correlation analysis, most of the monitored phenolic acids were shown to contribute to the inhibition of tyrosinase. The study has provided a better understanding of phenolic-rich SCG with the hope that it can further the use of this valuable by-product in food and cosmeceutical industries.

Keywords: Spent coffee, Phenolics, Tyrosinase, Antioxidant, Fractionation.

Introduction

Coffee is among the favorite beverages in the world, valued for its aroma and caffeine content. Global coffee production and consumption have generally been increasing over the past decade. In the year 2020/21, coffee consumption is estimated at 167 million 60-kg bags and is expected to rise by about 2% annually over the next years.[1] Spent coffee ground (SCG) is known as one of the high-volume by-products released from coffee processing and approximately six million tonnes of SCG are generated per year (680 kg/1000 kg of coffee beans).[2] Much effort has been taken to transform this waste into useful products, such as biofuels and fertilizers, and therefore to reduce its environmental impacts.[3] Sorbent materials derived from SCG have attracted great attention due to their potential applications to removal of heavy metals.[4-6] Some works have reported that SCG has a potential to be used as a nutrient-rich ingredient in bakery products, providing multiple health benefits. For example, SCG incorporated into biscuits contains sufficient amounts of dietary fiber and phenolics with abilities to exert antioxidant and antidiabetic potentials.[7] Research has also suggested applications of SCG in the production of alcoholic beverages.[8] It is because SCG inherits a pleasant aroma of coffee that can be very attractive for production of distilled spirits. Recently, several studies have focused on potential uses of SCG and its bioactive constituents in skin whitening products.[9] This is related to inhibition of tyrosinase which catalyzes the oxidation of L-DOPA (L-3,4-dihydroxyphenylalanine), playing an important role in the synthesis of melanin, also known as melanogenesis.

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Anti-tyrosinase potential and total phenolic content (TPC) of plant extracts were shown to be correlated.[10] Furthermore, evidence has indicated TPC of SCG extracts could have relationships with anti-tyrosinase activity, albeit not strong.[11] Beyond this, no studies on contribution of individual phenolics in SCG to the inhibition of tyrosinase have been conducted. Additionally, limited information about phenolic composition and bioactivities of SCG fractions prepared from crude extracts is available in the literature. In the present study, phenolics of SCG fractions obtained from methanolic extract and their correlation with tyrosinase inhibition activity were investigated. The study was to gain an insight into SCG bioactive constituents of SCG by determining phenolic profile of its fractions.

Materials and Methods

Chemicals

The standards with purity above 95%, including caffeic, chlorogenic, ferulic, cinnamic, p-coumaric, gallic, 2,4-dihydroxybenzoic, salicylic acids, mushroom tyrosinase (EC 1.14.18.1), L-DOPA and kojic acid were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Folin – Ciocalteu reagent was purchased from Merck KGaA (Darmstadt, Germany). Methanol, hexane, ethyl acetate (99.5%, ACS grade), ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2-diphenyl-1-picrylhydrazyl) were obtained from Fisher Scientific (Pittsburg, Pennsylvania, USA).

Sample collection

Spent coffee ground samples were obtained from well-known cafes during the period from October 2020 to December 2020 in Ho Chi Minh city, Vietnam. These samples were pooled and underwent a low-temperature drying process until moisture ≤ 5%.

Fractionation

About 10 g of the dried SCG sample were mixed with 100 mL of methanol in a flask. The mixture was shaken continuously for 24 hours at ambient temperature and filtered using a Whatman filter

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paper (GE Healthcare, Illinois, USA). The filtrate obtained was evaporated under reduced pressure. Approximately 1 g of the residue (MeOH) obtained was reconstituted in 90 mL of methanol/water (1:9, v/v). The solution was successively partitioned with hexane (40 mL × 5) and ethyl acetate (40 mL × 5), resulting in n-hexane (HEX), ethyl acetate (EA) and aqueous (AQ) fractions as shown in Figure 1.

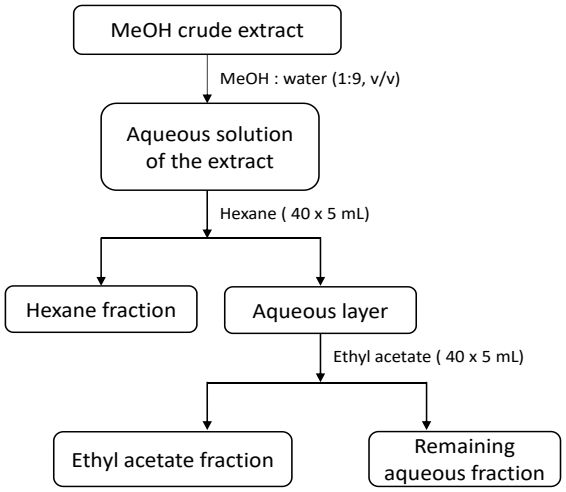

Figure 1: A scheme for fractionation of a methanolic SCG crude extract

Total phenolic content

Total phenolic content (TPC) of the crude extract and fractions obtained during the fractionation was evaluated by the Folin – Ciocalteu solution.[12] Gallic acid was employed as a reference standard. A mixture of a diluted extract (0.5 mg/mL) or standard, 2% sodium carbonate and 50% Folin – Ciocalteu solutions (1:1:1, v/v) was placed in a screw-capped tube and was incubated for 5 min at ambient temperature. The absorbance was measured at 760 nm with MeOH being used as a blank sample. The TPC values were estimated using the calibration curve (Absorbance = 0.0072 × Concentration + 0.1541; R^2 = 0.99) constructed for gallic acid (20 – 200 µg/mL) and the following equation: TPC = Concentration/0.5.

The results were shown as mg of gallic acid equivalent mg GAE/g of extract.

Analysis of phenolics

Each of the diluted extracts (0.5 – 1 mg/mL) placed in a vial was used for analysis of phenolics. Phenolics in the extracts were analyzed using a Shimadzu LC-2030 high-performance liquid chromatograph (HPLC)connected to a diode array detector. The separation was performed on a C18 HPLC chromatography column (250 × 4.6 mm, 5.0 µm particle size). A combination of methanol (solvent A) and 1% formic acid (solvent B) was employed as mobile phase. The flow rate was 0.8 mL/min with a step gradient as follows: 0 – 3 min (25% A); 3 – 5 min (25 – 40% A); 5 – 16 min (40 – 60% A); 16 – 21 min (60% A); 21 – 24 min (60 – 80% A), 24 – 27 min (80% A), 27 – 35 min (80 – 25% A). The column temperature was set at 40°C. Detection of phenolics was set at a wavelength of 275 nm.

Antioxidant activity

DPPH assay was performed using the method described by Xiao *et al*.[13] A DPPH solution (40 µg/mL) was prepared in 80% methanol. The reaction mixture consisting of test sample (0.5 mL) and the DPPH solution (0.75 mL) was shaken for half an hour at ambient temperature in a dark room and the absorbance at 517 nm was measured. ABTS assay was carried out using the method proposed by Leung *et al*.[14] Briefly, to release ABTS radical cation ($ABTS^{\bullet+}$), $K_2S_2O_8$ (2.45 mM) was mixed with ABTS (7 mM) at a ratio of 1:1 (v/v). After 12 – 16 hour incubation at an ambient temperature in the dark, the $ABTS^{\bullet+}$ solution was diluted with phosphate-buffered saline (pH = 7.4) to prepare a solution with absorbance of 0.75 ± 0.02 at 734 nm. A volume (100 µL) of the test sample was mixed with the $ABTS^{\bullet+}$ solution (3 mL), and the absorbance was measured.

The calculation of the DPPH or ABTS scavenging activities was performed as follows:

$$\% \text{ scavenging activity} = (A_b - A_s)/A_b \times 100\%$$

where, A_b and A_s are the absorbance of blank and samples measured at 734 nm (ABTS) or 517 nm (DPPH). Ascorbic acid was employed as a standard. IC_{50} values representing the sample concentration value required to trap 50% of DPPH radical were calculated.

Inhibition of tyrosinase

The SCG extracts were tested for inhibition of tyrosinase using the method of Cheraif *et al*.[15] A volume of tyrosinase solution (40 µL, 0.5 mg/mL) was mixed with 100 µL of each extract (1 mg/mL) or a blank and 100 µL of PBS (pH 7.4), left for incubation for 15 min at ambient temperature. L-DOPA (40 µL, 0.5 mM) as substrate was added in the mixture and the change in absorbance at a wavelength of 475 nm was monitored. The tyrosinase inhibition activity was presented as kojic acid equivalents (mg KAE/g extract).

Statistical analysis

All the experiments were conducted in triplicate and data were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) test and a significance level at $p < 0.05$was carried out to assess statistically significant differences in means of TPC, phenolic concentrations and antioxidant activity among the extracts. Correlations between antioxidant and phenolic concentrations were assessed to better understand how individual phenolics contribute to antioxidant activity of the samples. Principal component analysis was used as a mathematical tool to differentiate the extracts based on their phenolic contents. Statistical analyses were carried out using XLSTAT version 2016 (Addinsoft, Paris, France).

Results and Discussion

Total phenolic content

In the study, the total amount of phenolics in the crude extract and fractions was estimated. As seen in Figure 2, significant differences in TPC were noted among the SCG extract and fractions. The EA fraction was found to have the highest phenolic content (227.17 mg GAE/g) followed by the AQ extract, whereas the HEX extract showed the lowest phenolic content (60.22 mg GAE/g). It has been demonstrated that ethyl acetate has a much greater extraction capacity for phenolics in plants compared to hexane.[16] In respect of SCG, TPC of ethyl acetate extracts was previously reported to be twice as much as that of hexane extracts.[17] In the present study, the results also indicated the EA fraction was richer in phenolics compared to the MeOH extract. Prior research into coffee silver skin showed similar results in which ethyl acetate fractions had 35% higher TPC than aqueous-ethanolic extracts.[18] Beyond these, limited information about differences in phenolic contents between SCG extracts and their fractions is available while data about effects of different solvents on extraction of SCG phenolics abound in the literature. From this point, the findings in the present study would be useful for further assessment of bioactive constituents and health-promoting aspects of SCG.

Phenolics

In addition to TPC, phenolic profile of the fractions was determined to gain a better understanding of this class of compounds in SCG. Eight phenolics, including gallic, chlorogenic, caffeic, p-coumaric, ferulic, 2,4-dihydroxybenzoic (DHBA), salicylic and cinnamic acids, were screened for the samples. The results obtained were processed using chemometric tools which help intuitively visualize similarities and dissimilarities among the phenolic profiles. As described in Table 1, gallic acid was exclusively detected in the EA fraction at an average concentration of 56.76 µg/g of extract. This implies that the compound

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may have dissolved completely into ethyl acetate. Unlike gallic acid, the other phenolics were all found to be present in all the samples examined. Chlorogenic acid was detected in the fractions at the levels following the order: EA > AQ > HEX. The results also indicated that the EA fraction had about two times higher content of chlorogenic acid compared to the HEX fraction. Similarly, caffeic acid, ferulic acid and salicylic acid were shown to distribute mostly in the EA fraction. Particularly, the level of salicylic acid in the EA fraction (7865.01 µg/g) was approximately 95 times as high as those in the other fractions. The findings of the study showed that the EA fraction was the richest in phenolic acids. In general, significant differences in concentrations of the phenolic acids detected were noted for the three examined fractions. Principal component analysis (PCA) was employed to underscore variation in phenolic composition among the fractions. The first principal component (F1) was strongly associated with gallic acid, ferulic acid, salicylic acid, explaining up to 62.13% of the total variability. The second principal component (F2) could be defined by caffeic acid, p-coumaric acid and cinnamic acid, accounting for 37.16% of the total variability. As displayed in Figure 3, the samples of AQ and HEX fractions are situated on the right half of the plot (quadrant II and quadrant III, respectively) while those of EA fraction are located on the left haft. The biplot graphically shows the distribution of phenolic acids among the fractions which were described above.

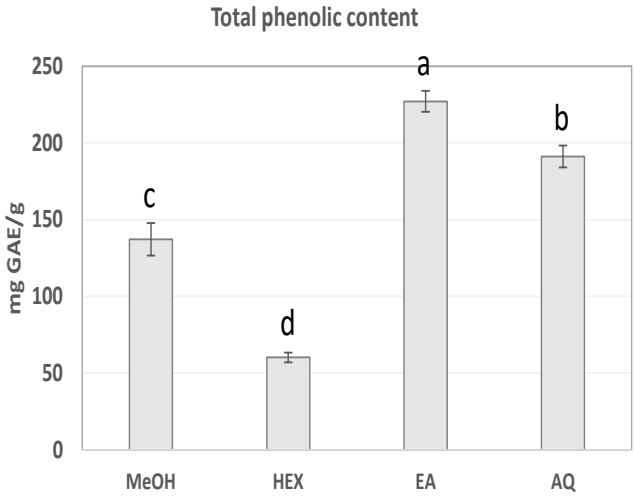

Figure 2: Total phenolic content of the SCG crude extract and fractions. Different letters indicate statistically significant differences among the samples ($p < 0.05$).

Table 1: Comparison of phenolic concentrations (µg/g of extract) among the SCG fractions

	SCG fractions		
Phenolic acid	**HEX**	**EA**	**AQ**
Gallic acid	n.d.	56.76 ± 5.16	n.d.
Chlorogenic acid	582.53 ± 24.18 c	1683.21 ± 97.99 a	1060.70 ± 64.72 b
Caffeic acid	110.48 ± 2.99 c	247.02 ± 6.58 a	215.39 ± 7.57 b
p-Coumaric acid	189.85 ± 7.37 c	226.79 ± 3.72 b	299.59 ± 17.39 a
Ferulic acid	61.54 ± 5.43 b	778.42 ± 36.94 a	67.17 ± 1.36 b
DHBA*	524.43 ± 31.09 b	387.22 ± 18.06 c	731.72 ± 17.34 a
Salicylic acid	83.78 ± 5.56 b	7865.01 ± 623.06 a	135.90 ± 7.23 b
Cinnamic acid	9.51 ± 0.45 c	19.31 ± 0.78 b	43.84 ± 2.04 a

*: 2,4-dihydroxybenzoic acid n.d.: not detected
Different letters for the same phenolics show statistically significant differences among the extracts ($p < 0.05$)

Antioxidant activity

Free radical scavenging activity of the SCG fractions were determined and compared with the crude extract and ascorbic acid was used as a positive control for the assays. The activity is estimated via IC_{50} values which correspond to the concentrations of SCG extracts able to trap 50% of free radicals in the reaction mixture. A lower IC_{50} value is indicative of a greater antioxidant activity. Figure 4 displays the EA fraction exerted the strongest antioxidant activity compared with the other fractions. The antioxidant activity of the EA fraction estimated via ABTS assay was even higher than that of ascorbic acid (76.16 ± 3.40 and 167.77 ± 13.68 µg/mL, respectively). As discussed above, the samples of this fraction were also richer in phenolic acids than those of the other fractions. For DPPH radical scavenging activity, the HEX fraction showed considerably lower IC_{50} values in comparison with the AQ fraction (13.06 ± 0.59 and 14.87 ± 0.78 µg/mL, respectively), signifying its stronger activity. However, no significant differences in ABTS activity were observed for these two fractions (174.11 ± 7.48 and 185.10 ± 13.72 µg/mL, respectively). Both assays revealed that the all the fractions exerted higher antioxidant activity compared to their crude extracts. EA fractions were previously reported to have stronger free radical scavenging activity (DPPH) than their crude extracts obtained from coffee silver skin.[18] Previously, coffee cherry extracts prepared from ethyl acetate reportedly presented higher DPPH activity than those from ethanol and hexane.[19]

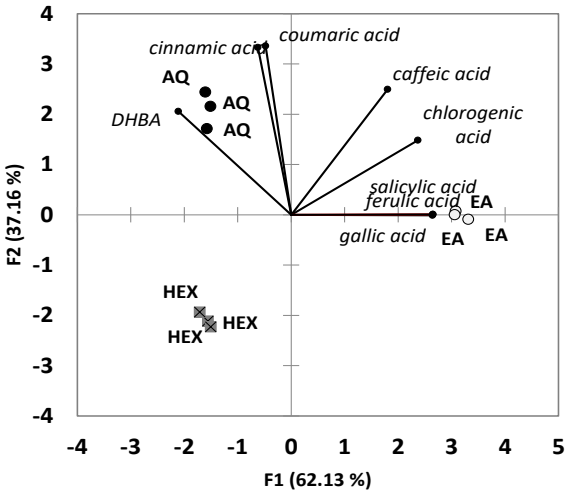

Figure 3: Principal component analysis of the phenolic data of the SCG fractions. The figure depicts a biplot obtained from the data matrix of phenolic acids and fractions.

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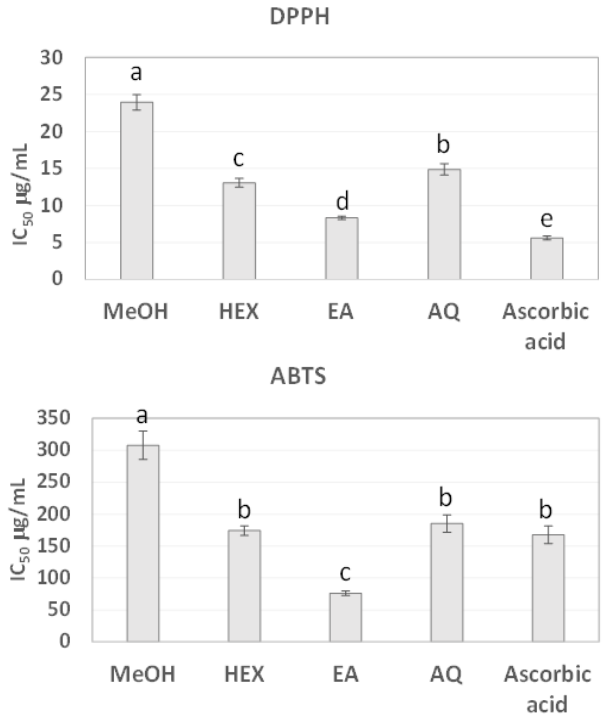

Figure 4: Free radical scavenging potentials (DPPH and ABTS) of the SCG fractions. Different letters indicate statistically significant differences among the samples ($p < 0.05$).

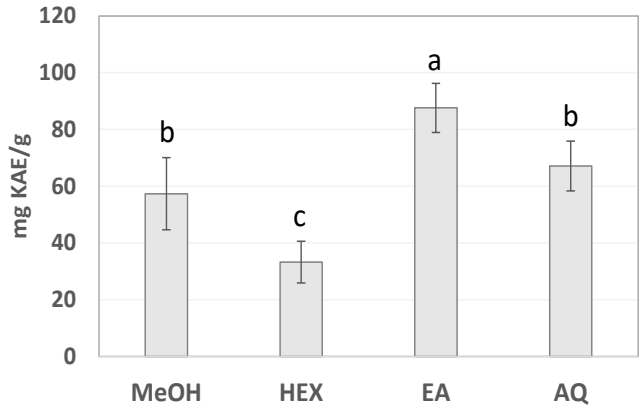

Figure 5: Tyrosinase inhibitory effect of the SCG fractions and extract. KAE denotes kojic acid equivalent. Different letters indicate statistically significant differences among the samples ($p < 0.05$).

To our knowledge, while data on SCG crude extracts abound in the literature, information about phenolics and antioxidant activity of SCG fractions is very limited. Our work is the first to report phenolic composition and free radical scavenging potentials of fractions obtained from SCG extracts.

Inhibition of tyrosinase

The results indicated that the EA fraction was the most effective tyrosinase inhibitor, followed by the AQ fraction (Figure 5). As discussed earlier, the EA samples also contained highest levels of phenolics and exerted the strongest free radical scavenging capacities. The anti-tyrosinase activity of the EA (87.59 ± 8.62 mg KAE/g of extract) was 30% higher than that of the AQ fraction. There is no significant difference in the effects between the AQ and MeOH samples (67.09 ± 8.78 and 57.36 ± 12.71mg KAE/g of extract, respectively). Previously, methanolic extracts of SCG were found to inhibit tyrosinase and the effect was about 58.42 mg KAE/g of extract,[11] comparable to the present study. As regards aqueous extracts, the aforementioned research revealed this type of samples showed no tyrosinase inhibition. The HEX fraction, which was identified as having the lowest phenolic content as described earlier, presented the weakest inhibitory effect on tyrosinase (33.25 ± 7.36 mg KAE/g extract). A recent study conducted by Kiattisin *et al.* (2019) has indicated that coffee cherry extracts obtained from ethyl acetate are more effective on inhibiting tyrosinase compared to those from hexane.

Correlation analysis

In this study, the calculation of Pearson's correlation coefficients was performed to assess the contribution of phenolic acids to antioxidant and anti-tyrosinase potentials of SCG fractions (Table 2). The reciprocal of IC_{50} obtained from DPPH and ABTS assays was used for the calculation. The results demonstrated that gallic acid, chlorogenic acid, ferulic and salicylic acid present in the fractions were positively correlated with the antioxidant activity determined by DPPH and ABTS assays. Prior research reported association between the presence of gallic acid in SCG and increased degrees of antioxidant activity using DPPH and superoxide dismutase-like assays.[20] Despite often having positive correlation with antioxidant activity of plant extracts, TPC showed no correlation with DPPH or ABTS in the present study. This could be due to several factors, such as 1) TPC estimated following the Folin – Ciocalteu method is not representative of an absolute measurement of the phenolic amount in the samples, 2) phenolics belonging to different classes are likely to exhibit different antioxidant capacities attributed mainly to their chemical structure, 3)other phytochemicals, such as tocopherols and pigments, as well as their synergistic, antagonistic or additive effects in the extracts may affect the measured antioxidant activity.

With respect to anti-tyrosinase activity, the results revealed TPC was positively correlated with the inhibitory effect on tyrosinase ($r = 0.941$, $p < 0.01$). With exceptions of p-coumaric acid, DHBA and cinnamic acid, the studied phenolic acids were demonstrated to contribute to inhibition of the enzyme. It is suggested that inhibition of tyrosinase could partly be dependent on hydroxyl groups attached on phenolic molecules which have impacts on enzymatic activity.[21] This could explain why stronger inhibitory effect on tyrosinase was observed for the EA fractions as described in the previous section. The findings of the present study corroborate those reported on relationships between TPC and anti-tyrosinase potential of phenolic content in plant extracts.[10] Along with phenolic content, free radical scavenging potential estimated by ABTS assay was shown to be moderately associated with anti-tyrosinase activity in the present study ($r = 0.722$, $p < 0.05$).

Table 2: Pearson's correlation coefficients between total phenolic content, phenolic acids, antioxidant and anti-tyrosinase activities in the fractions of SCG

	DPPH	ABTS	Tyrosinase
Gallic acid	0.969*	0.983*	0.766**
Chlorogenic acid	0.808*	0.885*	0.918*
Caffeic acid	0.543	0.643	0.934*
p-Coumaric acid	-0.330	-0.204	0.434
Ferulic acid	0.975*	0.990*	0.763**
DHBA	-0.883	-0.808	-0.227
Salicylic acid	0.970*	0.985*	0.768**
Cinnamic acid	-0.394	-0.273	0.394
TPC	0.529	0.633	0.941*
DPPH	1	0.982	0.611
ABTS		1	0.722**

*,** Significant differences at $p < 0.01$ and $p < 0.05$, respectively

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Conclusion

This study is the first work to provide an insight into phenolic acids, antioxidant and tyrosinase inhibition activities of extracts obtained from spent coffee ground fractionation. The findings showed that spent coffee ground fractions were rich in bioactive phenolic acids and possessed strong antioxidant and anti-tyrosinase properties. The study has given a better understanding of a by-product released from coffee processing with the hope that it can further be utilized for development of valuable products in food and cosmeceutical industries. From this perspective, the study will not just open an avenue for the recycling of spent coffee ground but also help divert waste away from landfill.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

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