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Comparative Study of Pollen Grains Morphology and Phytochemical Constituents of Some Saudi Arabian Date Palm (*Phoenix dactylifera* L.) Cultivars

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A B S T R A C T

Pollen morphological and phytochemical comparative study of nine Saudi Arabian date palm (*Phoenix dactylifera* L.) cultivars has been carried out. Pollen grains are generally small in size, monad, hetero-polar, asymmetric, elliptic in equatorial view, mono-sulcate. Exine sculpturing is micro-reticulate. Little differences have shown in the pollen dimensions, muri thickness and lumen size. The phytochemical screening of date palm pollen grains revealed the presence of high amounts of phenolics and flavonoids and displayed good antioxidant capacity. Additionally, High performance liquid chromatography (HPLC) analysis showed noticeable valuations in the distribution of different phenolic compounds among the nine pollen samples compared with 14 reference standards. Gallic acid, chlorogenic acid and caffeic acid were commonly detected in all methanolic pollen extracts. Moreover, 2, 5- dihydroxy benzoic acid and cinnamic acid are not detected in any of pollen samples. Pollen grains phytochemical screening beside their morphology may have a prospect value in the characterization of the studied Saudi Arabian male date palm cultivars. These results revealed that date palm pollen has antioxidant properties that could play a major role in human health and could provide dietary alternatives.

Introduction

Phoenix dactylifera L. is a monocotyledonous, dioecious, perennial tree belonging to Arecaceae -subfamily Coryphoideae- tribe Phoeniceae (Eoin, 2016). Nearly 3000 cultivars are distributed around the world (Zaid and De Wet, 1999). Cultivation of date palm trees has a very significant impact on the history of the ancient Middle East civilization. Date palm is an important fruit crop grown in the arid regions of Africa, Middle East and South Asia. Saudi Arabia is one of the leading date producing countries that cultivate about 400 cultivars (Bashah *et al.*, 1997). The entire tree of date palm is employed to provide food, shelter, fiber, furniture and many other products. Moreover, the date palm tree

successfully tolerates extremely adverse environmental conditions including drought, high temperature and salinity, which are the common criteria of desert lands. Date palm pollen grains (DPP) are a good economic and essential nutritional source becoming thus an excellent food supplements (Hassan, 2011 and Bishr and El-Desouky, 2012). DPP are also used as folk medicine. Regular consumption is beneficial in nephropathy, rheumatism, gastropathy and sexual debility (Elginidi *et al.,* 2015). Many other therapeutic properties for DPP extracts have been studied as an antioxidant and antimicrobial agents (Bassuny *et al.,* 2013 and Farouk *et al.,* 2015). More recently, Najla *et al.,* (2017), identified some bioactive compounds extracted from date palm seeds and pollen, and evaluated their antibacterial and antifungal properties. Accordingly, chemical components of DPP have gained a lot of attention in order to find out the main constituents responsible for such activities. Morphological characters of the female tree are usually taken into consideration for cultivar identification. However, the male trees identification is a difficult process because they are mostly identical to any female cultivar. However, farmers dealing with date palms can identify some male cultivars from their experience (Simozarg *et al.,* 2016).

Morphological characteristics of pollen grains can be useful characters in taxonomic studies because many pollen traits are influenced by the strong selective forces involved in various reproductive processes, including pollination, dispersal, and germination (Erdtman, 1952). In the meantime use of pollen morphology solely as a taxonomic character is challenging, and pollen characteristics must be considered in concert with other characteristics. Accordingly, the current study has been conducted to characterize the pollen morphology of nine cultivars of date palms grown in Saudi Arabia as well as detect and analyze their phytochemical constitution aiming to develop an additional pollen identification tools and to determine the antioxidant activity based on their total phenolics and flavonoids content, as well as on the content of individual phenolic compounds.

Materials and Methods

Pollen grains of nine date palm cultivars were obtained from Faculty of Food Science and Agriculture, King Saud University, Riyadh, Saudi Arabia. Pollen grains were examined using both light microscope (LM) and scanning electron microscope (SEM). Samples for LM examination were mounted on slides using glycerol jelly and observed under a Zeiss microscope. For SEM analysis, dried pollen grains were placed on copper stubs using double sided adhesive tape and coated with gold in a polaron JEC-1100E coating unit, then scanned and photographed with JEOL JSM-5300 SEM at Electron Microscope Unit – Faculty of Science – Alexandria University - Egypt. All measurements were based on 20 readings from each cultivar by using Image Tools software with high accuracy and the following parameters were recorded: pollen length, pollen width, colpus length, muri thickness and lumen area were measured. The terminology followed here is that of Erdtman (1952) and Punt *et al.,* (2007).

Phytochemistry of pollen grains

Preparation of extracts

The pollen samples (each 3 g) were individually extracted with 100 mL of methanol kept on a rotary shaker for 24 h. Thereafter, it was filtered and centrifuged at 5000 g for 15 min. The supernatant was collected and the solvent was evaporated to make the final volume one-fifth of the original volume (Tura and Robards, 2002). It was

stored at 4 °C in air tight bottles for further studies.

Determination of total phenolic content

The total phenolic content was determined with the Folin-Ciocalteu method (Wolfe *et al.,* 2003). The reaction mixture contained 200 µL of pollen methanolic extract, 800 µL of freshly prepared diluted Folin- Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The final mixture was diluted to 7 mL with deionized water. Mixtures were kept in dark at ambient conditions for 2 h to complete the reaction. Absorbance of samples and standard were measured at 765 nm using a T80 UV-Vis spectrophotometer - double beam. Total phenolic content was expressed as gallic acid (mg g^{-1} DW).

Determination of total flavonoid content

Total flavonoid content in the pollen extracts was estimated by the aluminum chloride, colorimetric assay method reported by Potter *et al.,* (2010). A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 mL of sample methanolic extract. After one hour at room temperature, the absorbance was measured at 420 nm using a T80 UV-Vis spectrophotometer-double beam. Total flavonoid content was calculated as rutin (mg g^{-1} DW).

DPPH free radical scavenging activity

Radical scavenging activity of pollen extracts against stable 2, 2diphenyl 2 picrylhydrazyl hydrate (DPPH) was determined by the method of Singh *et al.,* (2002). The change in colour was measured at 517 nm on a T80 UV-Vis spectrometer, double beam. One mL of aliquots of the extract (20-100 µg mL^{-1}) was added to methanol solution of DPPH (5 mL, 0.1 mM) and vortexed. The samples were kept in the dark for 20 minutes at room temperature and the decrease in absorbance was measured at 517 nm against a blank. The results were expressed as EC_{50}, which means the minimum concentration required for the antioxidant to reduce the initial concentration of the DPPH radicals by 50%. Radical scavenging activity was calculated by the following formula: % Inhibition = $[(A_B - A_A)/A_B] \times 100$ Where A_B = absorption of blank sample, A_A = absorption of test extract solution.

HPLC analysis

Isolation and quantification of individual phenolic compounds were obtained using a reversed-phase HPLC method described by Padda and Picha (2007). Twenty micro litter sample extract analyzed with a Exclipse XDB C_{18} (5 µm, 4.6 X 150 mm) column using a mobile phase consisting of 1 % (v/v) formic acid in aqueous solution: acetonitrile: 2-propanol (70:22:8), pH 2.5; flow rate: 0.75 mL/ min, temperature: 30 °C, UV detection at 320 nm: Agilent technologies 1200 series. Identification and peak assignment of the compound was based on comparison of its retention time with corresponding standard and by spiking of sample with the standard. Quantification of the compound was done using total peak area and each peak with external standard.

Statistical analysis

Statistical analysis was performed using Stat View 5.0 software. The phytochemical results were reported as the average of three repetitions ±SE (standard error).

Results and Discussion

General pollen grains characteristics (Table 1 and Figures 1 &2)

Identifying a particular cultivar is difficult because there are not reliable biological and

biochemical markers as well as the environmental factors that may affect the phenotype (Martínez-Gómez *et al.,* 2005). For the identification of cultivars, macro-morphological characters such as leaf, flower, and fruit parameters are utilized. Complement to these characters, the micromorphological characteristics of the pollen grains are one of the most important and valuable parameters for solving controversial taxonomical problems and distinguishing species and cultivars of fruit trees (Nikolić and Milatović, 2016).

The present study revealed that pollen morphology of date palm cultivars appeared to be relatively uniform. Regarding outline pollen grain elliptic in equatorial view, mean lengths of pollen grain ranged from 16.3 - 18.17µm whereas pollen width was 11.17-12.37µm. Based on the pollen length, the smallest mean pollen size recorded in cultivar Dikhiny (16.3 µm) while the largest size of pollen (18.17 µm) was recorded in cultivar Safawy. Pollen grains are mono sulcate, aperture extending almost the full length of the grain axis.

Aperture membrane is smooth, thin and narrow with inconspicuous margin. The present finding agreed with results of Soliman and Al-Obeed (2013) as well as Mohamed *et al.,* (2016). They observed almost the same pollen morphological characters within Saudi Arabian date palm cultivars. The exine sculpturing of all investigated cultivars is micro-reticulate, with simpli to duplibaculated muri. The mean muri thickness ranged from 0.08 µm to 0.16 µm. The most thickened muri (0.16 µm) was recorded in cultivar Sallag, whereas the thinnest one in cultivar Safry. Moreover, lumen sizes and shapes revealed differences among the studied cultivars. Cultivar Safry, showed the widest lumen (0.53 µm) while the narrowest (0.02 µm) is recorded in cultivar Khalas. Generally, the pollen features of the studied cultivars agreed with the previous observations of several workers (e.g. Erdtman, 1969; Harley, 1990; Rashid *et al.,* 2017). They mentioned that the monosulcate pollen type with reticulate to micro reticulate tectum occurred throughout the entire family Palmae.

Phytochemical analysis (Table 2 and Figures 3&4)

Date pollen cultivars, are recognized as an important source of compounds with pharmacological properties (Morais *et al.,* 2011). Chemical analysis of pollen grains has revealed the presence of a wide range of biochemically and nutritionally important substances (Almeida- Muradian *et al.,* 2005).

Phenolic compounds are plant secondary metabolites and a class of antioxidant agents that include flavonoids, stilbenes, coumarins, and phenolic acids (Tulipani *et al.,* 2008). In the present study, the phytochemical screening of the methanolic extracts of nine palm pollen grains cultivars were performed. Pollen extracts showed high amounts of phenolics and flavonoids content. The maximum values of phenolics were recorded in cultivars Safawy and Safry while Khadary and Safawy showed maximum flavonoids (Figure 3A).

The DPPH radical scavenging assay is used for the screening and evaluation of the antioxidants activity. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Zou and Wei, 2004 and Moreria *et al.,* 2008). The EC_{50} values of scavenging activities on DPPH radical in pollen extracts were illustrated in Figure 3B. This activity might be related to the nature of phenolic and flavonoids, thus, contributing to their electron transfer/hydrogen donating ability. Similar results were reported by Kumazawa *et al.,* (2004) and Czapecka *et al.,* (2005).

Table.1 General pollen features of the examined *Phoenix dactylifera* cultivars

No.	Characters / Cultivars	Mean ((µm)					Tectum
		Pollen length	Pollen width	Colpus length	Muri thickness	Lumina size	
1	Mabroom	17.12	11.55	16.8	0.14	0.08	Micro reticulate
2	Sallag	17.28	11.88	17.0	0.16	0.09	Micro reticulate
3	Khadary	17.20	11.92	16.5	0.15	0.20	Micro reticulate
4	Dikhiny	16.3	11.17	16.0	0.11	0.25	Micro reticulate
5	Maktumi	16.83	12.37	15.9	0.09	0.21	Micro reticulate
6	Succary	18.0	12.25	17.5	0.11	0.30	Micro reticulate
7	Safawy	18.17	12.27	18.0	0.09	0.11	Micro reticulate
8	Khalas	17.82	12.29	17.1	0.10	0.02	Micro reticulate
9	Safry	17.58	12.07	16.98	0.08	0.53	Micro reticulate

Table.2 HPLC analyses of phenolics constituents of the methanolic extracts of the nine pollen cultivars 1-9, names of cultivars are in Table 1

Peak Name	Amount (µg mL^{-1})								
	1	2	3	4	5	6	7	8	9
Gallic acid	8.21	2.60	1.80	2.93	4.08	2.36	1.85	1.48	1.46
Chlorogenic acid	60.17	41.32	33.28	63.01	25.18	1.55	130.05	42.89	128.90
Caffeic acid	68.83	72.20	29.43	72.69	26.13	34.38	127.74	52.72	187.25
3, 4- Dicaffeoyl quinic acid	-	-	16.72	-	6.16	27.06	25.17	-	-
2, 5- Dihydroxy benzoic acid	-	-	-	-	-	-	-	-	-
3, 5- Dicaffeoyl quinic acid	-	-	-	-	-	9.30	-	-	-
4, 5-Dicaffeoyl quinic acid	7.15	10.21	3.83	5.32	3.16	8.19	19.01	-	12.15
Catechin	-	-	-	-	22.27	-	-	50.84	-
Rutin	-	-	2.29	2.72	1.69	5.52	2.50	4.40	5.52
Phloridzin	-	1.51	2.43	1.80	-	2.07	3.57	1.02	5.39
Tannic acid	-	-	-	-	5.50	-	-	7.48	-
Geraniol	2.37	-	2.74	-	-	-	4.24	-	6.85
Quercetin	6.25	6.20	-	6.22	6.21	6.48	6.49	6.23	-
Cinnamic acid	-	-	-	-	-	-	-	-	-

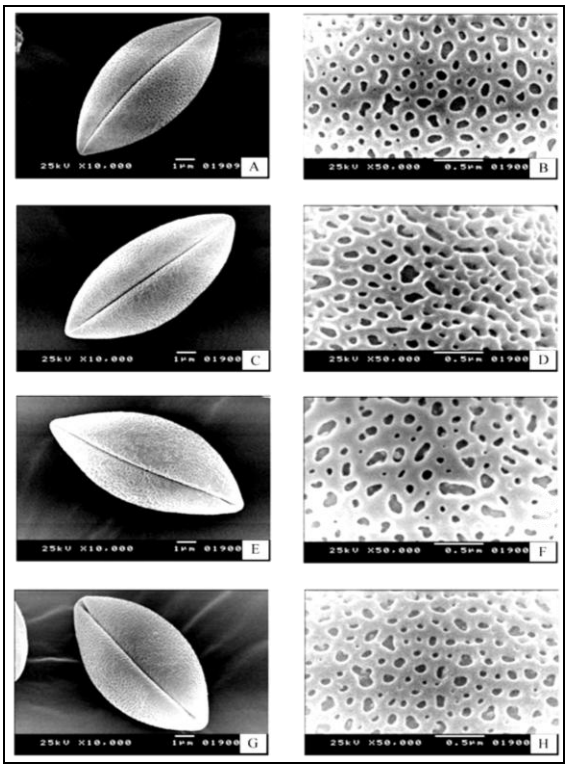

Fig. 1 Pollen shape and exine pattern of date palm cultivars: A, B, cultivar "1"; C, D, cultivar "2"; E, F, cultivar "3"; G, H, cultivar "4"

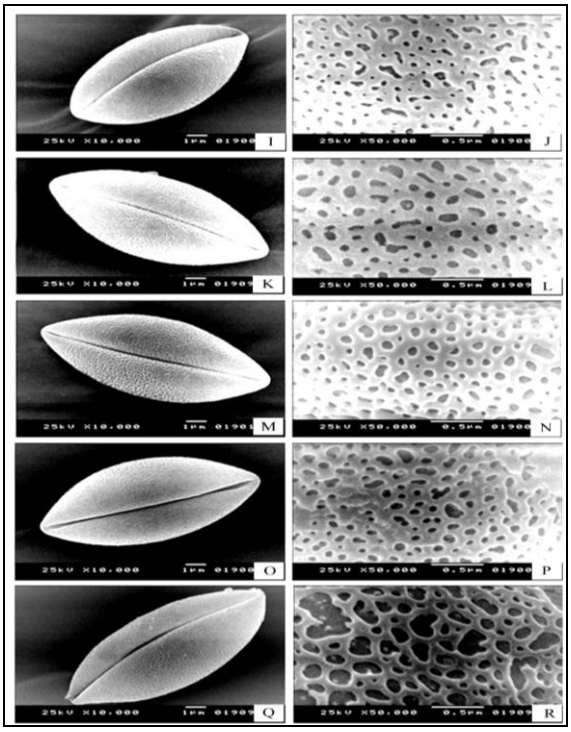

Fig. 2 Pollen shape and exine pattern of date palm cultivars: I, J, cultivar "5"; K, L, cultivar "6"; M, N, cultivar "7"; O, P, cultivar "8"; Q, R, cultivar

"9"

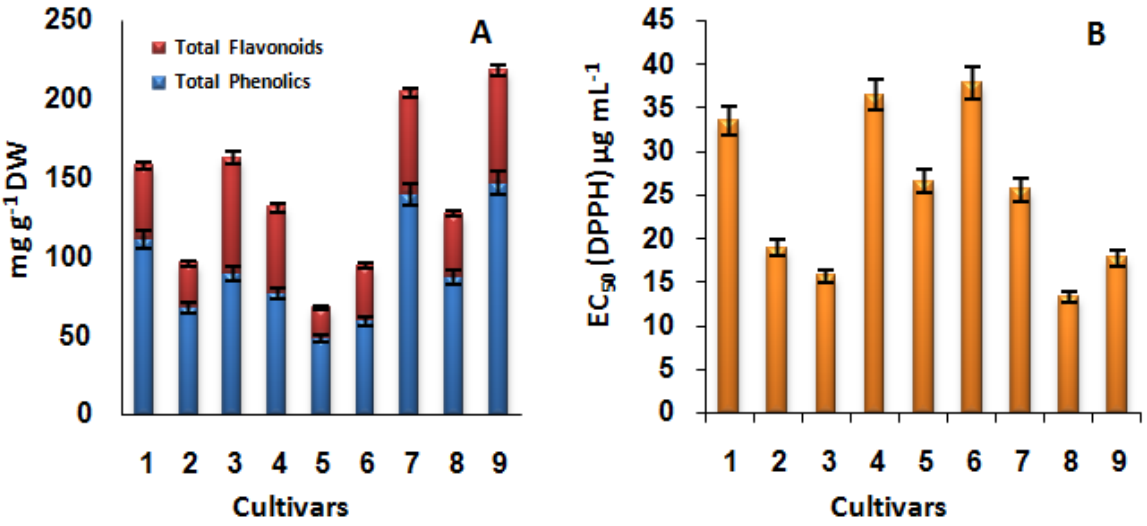

Fig. 3 Total phenolics and flavonoids content (A) and EC_{50} values of DPPH radical scavenging activity (B) of methanolic extracts of the nine pollen date palm cultivars, names of cultivars are in Table 1. Mean value± standard error (n =3)

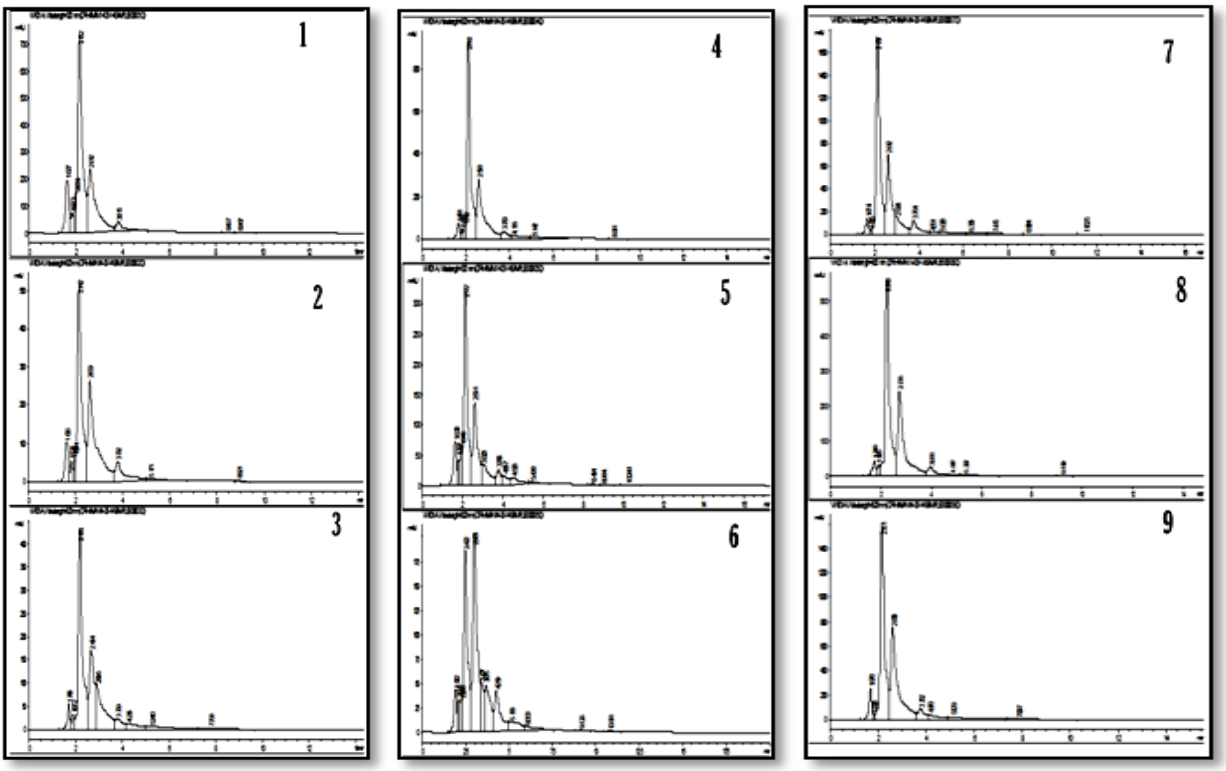

Fig. 4 HPLC chromatogram for methanolic extracts of the nine pollen cultivars, names of cultivars are in Table 1

Compared with 14 reference standard phenolic and flavonoid compounds, the HPLC- analysis results for the methanolic extract of the studied pollen samples showed a noticeable valuation in the distribution of different phenolic and flavonoid compounds (Table 2 and Figure 4). Gallic acid, chlorogenic acid and caffeic acid were commonly detected in all pollen extracts. While 3, 5-dicaffeoyl quinic acid was only detected in pollen cultivar Succary, while, 2,5-dihydroxy benzoic acid, cinnamic acid are not detected in any of pollen samples. Geraniol only detected in cultivars Mabroom, Khadary, Safawy and Safry. However, 4, 5-dicaffeoyl quinic acid was detected in all cultivars except Khalas. Rutin was commonly detected in all pollen samples except cultivar Mabroom. Serra Bonvehí *et al.,* (2001), stated that rutin could be an indicator of the quality of bee pollen, which might reflect long periods of storage or excessive heating during the drying process. Quercetin was detected in all pollen samples except cultivars Khadary and Safry. However, tannic acid was only detected in the pollen extract of cultivar Khalas. Catechin is only detected in cultivars Maktumi and Khalas.

Generally, the phytochemical analyzes of the studied pollen grains, showed considerable antioxidant activity and a great diversity of phenolics and flavonoids. The present result, agreed with Pietta (2000) as well as Freire *et al.,* (2012). They emphasized the significant role that the phenolics and flavonoids play in the antioxidant capacity of pollen. Moreover, the obtained data are consistent with the previous study of Abed El-Azim *et al.*, (2015), who reported that the pollen of the date palm have six compounds which were identified as caffeic acid, gallic acid, coumaric acid, chlorogenic acid, catchin and quercetin. Additionally, many types of flavonoids have been identified in date pollen palm. Abbas and Ateya (2011) isolated five flavonoids compounds (rutin, luteolin-7-O-b-D-glucoside, apigenin, isorhamnetin-3-O-glucoside and naringin for the first time from the pollen. Moreover, Daoud *et al.,* (2015) found that the pollen of Tunisian date palm cultivars contain high concentrations of flavonoids, and four types of flavonoids which include quercitin, rutin, catechin and epicatechin. Recently, AL-Samarrai *et al.*, (2017) found that Iraqi pollen date palm contain many types of flavonoids (lincoceric acid, chlorogenic acid, ferulic acid, naringin, apigenin-7-O-beta glycopyranoside, letulin and letulin-7-O-beta glycosides).

Pollen features joining with the phytochemical profile may provide a valuable additional tool of potential value in the characterization of the studied Saudi Arabian date palm cultivars. Moreover, the analysis of date palm pollen grains exhibited considerable phenolic and flavonoids content that displayed good antioxidant activities.

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