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Antioxidant Activity, Lipoxygenase Inhibitory Effect and Polyphenolic Compounds from *Calotropis procera* (Ait.) R. Br

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ABSTRACT

The purpose for this study was to evaluate the biological, antioxidant, anti-inflammatory and anti-cancer properties of *Calotropis procera* alcoholic extract and its various solvent subfractions. The total antioxidant property was estimated by the Ferric Reducing Antioxidant Power (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH·), 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS·+) and β-carotene bleaching tests. Among the ethanol extract and three fractions, the ethyl acetate fraction showed the highest phenolic content (108.26 mg gallic acid g^{-1}) and the best antioxidant activity. The ascorbic acid equivalent antioxidant capacities of the ethyl acetate fraction were 0.26, 0.53, 0.17 mmol g^{-1} in FRAP, ABTS and DPPH assays, respectively. This study verified that the ethanol extract and ethyl acetate fraction from *Calotropis procera* have strong antioxidant activity that were correlated their high level of phenolic content. Furthermore, the same extract showed appreciable anti-inflammatory via lipoxygenase inhibitory activity (LOX), IC_{50} values ranged from 7.12-52.68 μg mL^{-1}. Moreover, ethyl acetate fraction showed the strongest cytotoxic effect (IC_{50} = 10.6 μg mL^{-1}) against MCF-7 breast cancer cell line.

Key words: Medicinal plants, *Calotropis procera*, antioxidant activity, anticancer activity, MTT assay, lipoxygenase inhibitory effects, polyphenolic compounds

INTRODUCTION

Phytochemicals have been of great interest as a source of natural antioxidants used for health promotion, food preservation, food flavoring and cosmetics since they are safer to consumption and more environmentally friendly than their synthetic counterparts (Shrikumar and Ravi, 2007). Plants are known for their health protective effect mostly attributed to their polyphenolic components, mainly flavonoids and phenolic acids, which possess antioxidant activity against the Reactive Oxygen Species (ROS) (Williams *et al*., 2004; Soobrattee *et al*., 2005). ROS induce oxidative damage to biomolecules like lipids, nucleic acids, proteins and carbohydrates. This damage causes the onset of many diseases such as rheumatoid, cirrhosis, arteriosclerosis, diabetes and cancer (Ebadi, 2006). Olalye and Rocha (2007) studied the effect of various plant extracts on the formation of lipid peroxidation end products, thiobarbituric acid reactive substances induced by

different pro-oxidants in rat liver homogenate. These authors suggested that the use of plant extracts in the treatment of various diseases was probably due to their components' ability to act as antioxidants.

Calotropis procera, a plant belonging to the Asclepiadaceae family, is widely distributed in Africa, Asia and the Middle East. This plant stands out now in ethnopharmacology as a source of interesting compounds that can be used in the future as new drugs (Silva *et al*., 2010). In Indian traditional medicine, different parts of the plant have been used in the treatment of various diseases including ulcer, tumors and hepatitis (Rana *et al*., 2004; Mathur *et al*., 2009). In the folk medicine of Algeria, *Calotropis procera* is used to treat respiratory diseases, rheumatism and asthma (Qureshi *et al*., 2001; Hammiche and Maiza, 2006). Recently, the white latex of this plant has been shown to have anticancer, anti-ulcer, anti-inflammatory, anti-hyperglycemic and hepatoprotective effects (Roy *et al*., 2005; Padhy *et al*., 2007; Chavda *et al*., 2010). *Calotropis procera* has been a source of novel broad components such as flavonoid quercetin, flavonoid glycosides, anthocyanins, cardioactive glycosides and triterpenoids (Hassan *et al*., 2006; Okiei *et al*., 2009).

Lipoxygenases are the key enzymes in the biosynthesis of leukotrienes that play an important role in several inflammatory diseases such as arthritis, asthma, cancer and allergic diseases (Rackova *et al*., 2007). According to the study, there is little information about the biological activity of ethanolic extract of *Calotropis procera* leaves and its different partition subfractions. The main aims of this study are to evaluate the antioxidant potential, lipoxygenase inhibitory activity and to screen phenolic content of the aqueous ethanolic extract of *Calotropis procera* leaves and its different partition subfractions. The antioxidant potential of extracts was evaluated in relation to the scavenging of two stable nitrogen–centered radicals, 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) and 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonate) (ABTS+). The reducing power of extracts was also evaluated by Ferric Reducing Antioxidant Power (FRAP) assay as well as anti-bleaching of β-carotene activity. Moreover, the anti-inflammatory of calotropis procera extracts via lipoxygenase inhibitory activity (LOX) and their cytotoxicity on MCF-7 breast cancer cell line were evaluated.

MATERIALS AND METHODS

Chemicals: Ascorbic acid, ferric chloride, Folin-Ciocalteu reagent, dibutyl hydroxytoluene (BHT), 2,4,6-tripyridyl triazine, gallic acid, sodium carbonate, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,4,6-tripyridyl triazine, 2, 2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) and β-carotene were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from common commercial suppliers.

Plant material: *Calotropis procera* was collected in June 2010, from Falaj Hazza area in the town of Al Ain in the southern part of the United Arab Emirates. The harvested specimens were properly identified and voucher specimens were deposited at the Herbarium of the Department of Biology, Faculty of Sciences, UAE University.

Preparation of plant extract: A sample of ground, air-dried aerial part of *Calotropis procera* (10 g) was macerated with 200 mL of 80% (v/v) aqueous ethanol for 72 h at 4°C. The resulting mixture was then filtered and divided into two parts. The first part was evaporated to dryness under reduced pressure in a rotary evaporator at 40°C to give ethanol crude extract. The other part was concentrated under reduced pressure and was suspended in water to give a volume of 50 mL. The resulting mixture was extracted with n-hexane (2×100 mL) to remove fatty materials. The

aqueous residual layer was extracted sequentially with ethyl acetate, n-butanol using the same protocol. The crude ethanol extract as well as the four fractions (n-hexane, ethyl acetate, n-butanol and aqueous residue) were dried, weighed, dissolved in DMSO and kept at -20°C for further studies.

Total phenolic content, antioxidant activity and lipoxygenase inhibition assays: Total phenolic content was determined by the method of Singleton *et al.* (1999) using the Folin-Ciocalteu reagent. FRAP assay was determined according to the method described by Nenadis *et al.* (2007). ABTS assay is based on the reduction of the blue-green 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonate) radical cation (ABTS·+) by antioxidants to its colorless ABTS form according to a method described by Erel (2004).

The ability of a compound to donate a hydrogen atom was assessed on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical according to a procedure based on Nenadis *et al.* (2007). The ability of plant extract to prevent bleaching of β-carotene was assessed according to a procedure based on Lim and Quah (2007). Lipoxygenase (EC 1.13.11.12 type 1-B) (LOX) was assayed according to the method described by Wu (1996).

Cytotoxicity: The cytotoxicity of *Calotropis procera* extracts was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan) assay on MCF-7 human breast cancer cell line model. Cells were grown in the DMEM medium (GIBCO-BRL) supplemented with 10% fetal calf serum (GIBCO-BRL, USA), 100 units mL^{-1} penicillin-streptomycin (GIBCO-BRL, USA) and non-essential amino acid (GIBCO-BRL, USA). The cells were maintained at 37°C in 5% CO_2 incubator. After reaching confluency, the cells were sub cultured into 96 wells culture plates, allowed to grow for 1-2 days to a density of 2×104 cells/well and treated with different concentrations of *Calotropis procera* extracts (final concentrations 10-500 μg mL^{-1} in 0.05% DMSO) for 24 h. MTT solution with DMSO served as a negative control and with SDS as a positive control. The MTT [3-(4, 5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide] assay was performed according to the manufacturer (Promega, USA) instructions to examine the cytotoxic effect of these compounds. Briefly, 20 μL of MTT reagent (Cell Titer 96-Aquous non-radioactive cell proliferation Assay, Promega, USA) was added to the each well and incubated the cells for one to two hours. This assay is a colorimetric assay for cell viability based on the cellular cleavage of the yellow tetrazolium salt, MTT, into the purple formazan crystals that is soluble in cell culture medium and is measured at 490 nm directly in 96-well assay plates. Absorbance is directly proportional to the number of the living cells in culture. Viability was calculated as a percentage of the control cells. All experiments were carried out in triplicates. The IC_{50} value was defined as the concentration of plant extract necessary to inhibit the growth to 50% of the control.

Identification of main polyphenolic compounds: HPLC analyses were performed according to the method of Abad-Garcia *et al.* (2007) with an Agilent 1200 LC system consisting of degasser, quaternary pump (G1311A), auto sampler (G1329A), column heater (G1316A) and Diode Array Detector (DAD) (G1315C).

Statistical analysis: The data are expressed as Mean±SEM. Correlation analysis of antioxidants versus the total phenolic content was carried out using the regression analysis, with SPSS version 10 statistical programs (SPSS Inc., Chicago, IL, USA). When significant differences by ANOVA were detected, analysis of differences between the means of the extracts was performed by using Turkey's multiple comparison test. Significant difference was determined at $p<0.05$.

RESULTS AND DISCUSSION

Antioxidant and free radical scavenging activity: It is clear that due to the complex nature of the different phytochemical classes present in plants, the antioxidant capacities of plant extracts cannot be evaluated using a single method. In the present work, the FRAP, ABTS, DPPH· and β-carotene methods were used to assess the antioxidant activities of plant extracts.

FRAP assay: FRAP assay depends on the reduction of ferric ions to the ferrous ions by the antioxidant agents. Table 1 shows that all fractions of *Calotropis procera* extract exhibited some degree of electron donation capacity. A statistically significant difference ($p<0.05$) was found between extracts. The ethyl acetate fraction exhibited the highest antioxidant potency (0.26 mmol ascorbic acid equivalent g^{-1}) followed by crude ethanol extract (0.09 mmol ascorbic acid equivalent g^{-1} dry extract). The antioxidant potency of the extracts in FRAP assay was in the following order ethyl acetate fraction >crude ethanol extract butanol fraction>water fraction.

ABTS radical scavenging assay: ABTS assay expressed as ascorbic acid equivalent g^{-1} dry extract varied from 0.53 mmol g^{-1} for ethyl acetate fraction to 0.21 mmol g^{-1}extract for water fraction. A statistically significant difference ($p<0.05$) was found between extracts ethyl acetate fraction was better than the synthetic antioxidant BHT (0.42 mmol g^{-1}). The ethyl acetate fraction showed the strongest activity with IC_{50} = 61.36 μg mL^{-1} which is better than BHT antioxidant with IC_{50}= 77.85 μg mL^{-1}. The activity of the extracts were in the following order ethyl acetate fraction>BHT>crude ethanol extract>butanol fraction>water fraction (Table 1). The measured ABTS radical scavenging ability of the ethyl acetate fraction of *Calotropis procera* is higher than those reported for other plants such as peanuts (81.3 μmol g^{-1}), pistachios (75.9 μmol g^{-1}) and almonds (25.4 μmol g^{-1}) (Yang *et al*., 2009).

DPPH radical scavenging assay: The DPPH radical- scavenging assay is a widely used method for evaluating the ability of antioxidants (or plant extracts) to scavenge the stable free radical generated from DPPH which changes its color from purple into yellow upon accepting a hydrogen radical (H·) from the antioxidant species to form the stable DPPH-H molecule (Lim and Quah, 2007). The results of the DPPH assay expressed as ascorbic acid equivalent g^{-1} dry extract and as IC_{50} values (μg mL^{-1}) for the four fractions of *Calotropis procera* extract are shown in Table 1. A statistically significant difference ($p<0.05$) was found between extracts. The best free radical scavenging activity was exerted by ethyl acetate fraction (IC_{50}= 119.50 μg mL^{-1}) which contained the highest amount of total phenolics. The lowest radical scavenging activity was exhibited by the

Table 1: Total antioxidant activity of ethanol extract and soluble fractions from *Calotropis procera* expressed as ascorbic acid equivalents (mmol $g^{-1}$of dry extract). BHT was nsed as positive control

	FRAP assay	ABTS assay		DPPH assay		β-Carotene assay
Extract	TAC (mmol g^{-1})	TAC (mmol g^{-1})	IC_{50} (μg mL^{-1})	TAC (mmol g^{-1})	IC_{50} (μg mL^{-1})	IC_{50} (μg mL^{-1})
Ethanol Extract	0.09±0.01	0.31±0.01	107.90±3.01	0.06±0.01	501.50±1.50	102.60±0.95
Ethyl acetate	0.26±0.01	0.53±0.01	61.36±0.43	0.17±0.02	119.50±0.05	72.00±2.00
n-Butanol	0.06±0.01	0.24±0.01	135.00±5.00	0.05±0.01	793.08±3.00	153.00±3.00
Water	0.05±0.01	0.21±0.01	173.56±5.40	0.04±0.02	933.00±17.0	191.50±1.50
BHT	-	0.42±0.01	77.85±0.85	0.43±0.01	87.98±7.60	36.85±1.15

Values are Mean±SE of three experiments

water fraction (IC_{50} = 933.0 μg mL^{-1}). The overall trend of the scavenging abilities of the extracts were in the following order BHT>ethyl acetate fraction>crude ethanol extract>butanol fraction>water fraction. The IC_{50} of ethyl acetate fraction of *Calotropis procera* was found to be more effective than aqueous-methanol extracts from basil (IC_{50} = 500 μg mL^{-1}), oregano (IC_{50} = 320 μg mL^{-1}) and rosemary (IC_{50} = 180 μg mL^{-1}) (Kosar *et al*., 2005).

β-Carotene bleaching test: β-carotene undergoes rapid bleaching in the absence of antioxidants. The presence of antioxidants hinders the extent of bleaching by neutralizing the linolic hydroperoxyl radical formed. A statistically significant difference ($p<0.05$) was found between extracts. The ethyl acetate fraction (IC_{50} = 72.0 μg mL^{-1}) and ethanol extract (IC_{50} = 102.6 μg mL^{-1}) showed the highest ability to prevent bleaching of β-carotene, followed by butanol fraction (IC_{50} = 153.0 μg mL^{-1}) and water fraction (IC_{50} = 191.5 μg mL^{-1}). The IC_{50} of ethyl acetate fraction of *Calotropis procera* was found to be more effective than aqueous-ethanolic extract from chicory leaves (IC_{50}>100 μg mL^{-1}) (Conforti *et al*., 2009). The inhibition of bleaching of β-carotene ability of ethyl acetate fraction and ethanol extract are much higher than those reported for some other plants such as mint (IC_{50}>100 μg mL^{-1}) and radish (IC_{50}>100 μg mL^{-1}) (Confortia *et al*., 2008).

Total phenolic content: The total phenolic content of the plant extracts was measured with the Folin-Ciocalteu reagent assay and the results are shown in Table 2. A statistically significant difference ($p<0.05$) was found between extracts. The values varied from 13.98 to 108.26 mg gallic acid g^{-1} of dry extract. Ethyl acetate fraction contained the highest amount of phenolics (108.26 mg gallic acid g^{-1}), followed by ethanol crude extract (25.30 mg gallic acid g^{-1}) and butanol fraction (15.97 mg gallic acid g^{-1}), whereas the lowest level was found in the water fraction (13.98 mg gallic acid g^{-1}). Present results of TP and DPPH scavenging activity of *Calotropis procera* were higher than those of Ramesh *et al*. (2009). The total phenolic content and DPPH antioxidant activity of methanol leaf extract were 3.6 mg gallic acid g^{-1} and IC_{50} = 208 μg mL^{-1}, respectively.

Correlation between antioxidant activity and phenolic content: The correlation coefficient (R^2) between the antioxidant values and total phenolic content of ethanol extract of the plants and its various fractions was determined (Table 3). The significant linear correlation (p-value<0.01) was

Table 2: Total phenol content of ethanol extract and soluble fractions from *Calotropis procera* expressed as gallic acid equivalents (mg g^{-1} of dry extract)

Extract	Yield (%)	Total phenolic content (mg g^{-1})
Ethanol extract	8.0	25.30±0.07^{b,}
Ethyl acetate	1.6	108.26±8.26^{a,c,d}
n-Butanol	6.4	15.97±0.49^{b}
Water	6.0	13.98±0.79^{b}

Values are Mean±SE of three experiments. Values with on letter are significantly different ($p<0.05$)

Table 3: Linear correlations between the amount of total phenolic content and antioxidant activities of ethanol extract and soluble fractions from *Calotropis procera*

Assay	Correlation (R^2)	Significant
FRAP activity	0.99	p<0.001
ABTS·+ scavenging activity	0.98	p<0.001
DPPH· scavenging activity	0.95	p<0.001
β-carotene bleaching inhibition	0.79	p<0.05

confirmed between total phenolic content and related FRAP, DPPH and ABTS and β-carotene bleaching inhibition antioxidant activities of medicinal plant extracts. Therefore, the presence of phenolic compounds contributed significantly to the antioxidant activity of the tested extracts. This result is in agreement with the previous reports of Lim and Quah (2007) and Wojdylo *et al.* (2007).

LOX inhibition assay: LOX catalyzes deoxygenating of polyunsaturated fatty acids to yield cis, trans-conjugated diene hydroperoxides. All plant extracts exhibited strong LOX inhibitory activity with IC_{50} ranging from 2.75 to 23.65 μg mL^{-1} (Fig. 1). The ethyl acetate fraction (IC_{50} = 1.41 μg mL^{-1}) and ethanol extract (IC_{50} = 2.75 μg mL^{-1}) showed the highest ability to inhibit LOX activity, followed by butanol fraction (IC_{50} = 13.85 μg mL^{-1}) and water fraction (IC_{50} = 23.65 μg mL^{-1}). LOX inhibition of CP extracts was higher than that of other plants such as Oregon grape (761 mg mL^{-1}) (Rackova *et al.*, 2007). These results indicate that *Calotropis procera* has high anti-inflammatory effect which can be presumably related to polyphenolic content and antioxidant property of extract.

Cytotoxicity: The four *Calotropis procera* fractions were evaluated for cytotoxicity activities against MCF-7 breast cancer cell line. Cell growth was measured using the MTT reduction assay procedure. The inhibitory effect of cell growth was observed to be concentration dependent (Fig. 2). Ethyl acetate fraction showed strongest cytotoxic effect (IC_{50} = 10.6 μg mL^{-1}), followed by butanol fraction (IC_{50} = 15.95 μg mL^{-1}) and ethanol crude extract (IC_{50} = 55.20 μg mL^{-1}), whereas the lowest effect was found in water extract (IC_{50} = 483.65 μg mL^{-1}). This result is in agreement with the previous study that has demonstrated a cytotoxicity activity of root extracts from *Calotropis procera* against Hep2 cancer cells (Mathur *et al.*, 2009). These authors demonstrated that ethyl acetate extract of root was much more effective in inducing cell death compared to ethanol and hexane extracts. The water extract had no cytotoxic effects.

Identification of main polyphenolic compounds: HPLC analysis revealed the presence of various compounds in *Calotropis procera* extracts (Table 4). Quantitative and qualitative identification of the various plant extracts were performed against authentic standards of seven

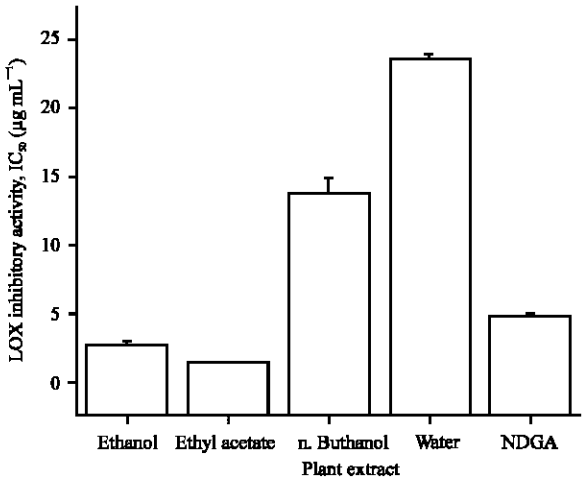

Fig. 1: LOX inhibitory activities of ethanol extract and soluble fractions from *Calotropis procera* expressed as IC_{50} (μg mL^{-1}). Values are Means±SE of three experiments

possible polyphenolic compounds (gallic acid, vanillic acid, epicatechin, p-coumaric acid, ferulic acid, quercetin-3-β-D-glucoside and rutin). Ethanol crude extract was found to contain all of these compounds, with gallic acid, quercetin-3-β-D-glucoside, rutin and p-coumaric acid being the highest in concentration (1.15, 0.82, 0.47, 0.61 mg g^{-1} extract, respectively). On the other hand, the seven previously mentioned polyphenolic compounds were detected in the ethyl acetate fraction, with quercetin-3-β-D-glucoside, rutin and p-coumaric acid being the highest in concentration (2.06, 2.64 and 2.36 mg g^{-1} extract, respectively). The n-butanol fraction was found to contain gallic acid, rutin and epicatechin in lower concentrations (0.27, 0.86 and 0.50 mg g^{-1} extract, respectively) as the major components. Finally, only gallic acid was detected in the water fraction in min

Table 4: Contents of main polyphenolic compounds of ethanol extract and soluble fractions from *Calotropis procera*

Extract	Retention time (min)	Compound identified	Contents (mg g^{-1} extracts)
Ethanol extract	10.12	Gallic acid	1.15±0.030
	29.29	Vanillic acid	0.16±0.003
	39.07	(-)-Epicatechin	0.57±0.002
	49.29	p-coumaric acid	0.61±0.001
	92.02	Quercetin-3β-D-glucoside	0.82±0.002
	93.31	Rutin	0.47±0.003
Ethyl acetate	10.09	Gallic acid	0.53±0.001
	29.17	Vanillic acid	0.36±0.003
	39.11	(-)-Epicatechin	0.63±0.020
	49.15	p-Coumaric acid	2.36±0.008
	57.87	Ferulic acid	0.05±0.002
	92.49	Quercetin-3β-D-glucoside	2.06±0.001
	93.11	Rutin	2.60±0.009
n-Butanol	10.09	Gallic acid	0.27±0.005
	39.82	(-)-Epicatechin	0.50±0.006
	93.92	Rutin	0.86±0.002
Water	10.22	Gallic acid	0.09±0.005

Values are Mean±SE of three experiments

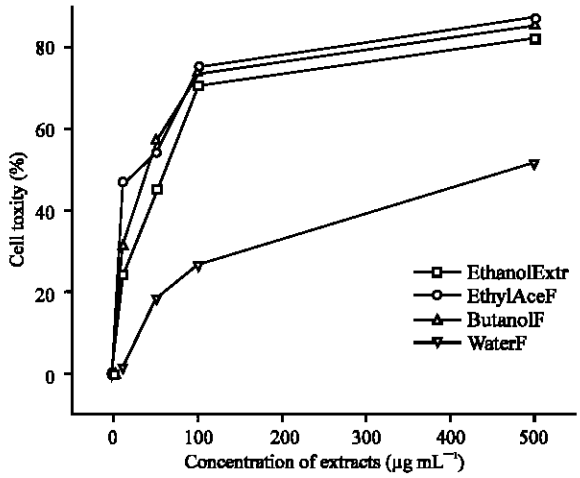

Fig. 2: Cytotoxic effect of ethanol extract and soluble fractions from *Calotropis procera* on MCF-7 breast cancer cells. Cell toxicity was measured by MTT assay. Data are means of percentage changes of control

concentrations (0.09 mg g^{-1}, respectively). From these results, it can be seen that the polyphenolic compounds were concentrated in the ethyl acetate fraction as compared with the n-butanol and water fractions. The results of the present study demonstrated for the first time the characterization of several phenolic compounds of *Calotropis procera*.

CONCLUSION

The investigation of *Calotropis procera* alcoholic extract partition fractions yielded promising antioxidant, anti-inflammatory and anti-cancer properties. Generally, ethyl acetate fraction showed the highest activity in all the studied assays. Based on the observed trends obtained in this research, bioactivities were presumably attributed to their polyphenolic compositions.

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