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Phytochemical Investigation and *in vitro* Antioxidant Activity of Different Leaf Extracts of *Salix mucronata* Thunb.

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ABSTRACT

The present study was aimed to determine the phytochemical constituents of *Salix mucronata* Thunb. leaf extracts and their antioxidant activities. Dried leaf powder was extracted with MeOH, MeOH (85%), MeOH(70%) and distilled water. The different extracts were monitored for phytochemical screening. Total phenolic and flavonoid contents were measured by Folin-Ciocalteu and aluminum chloride assays. The antioxidant potential of tested extracts was evaluated using DPPH, ABTS and total antioxidant capacity (TAC) assays. The results showed that, MeOH (85%) extract exhibited high total phenolic and flavonoid contents (TPC=131.39±2.49 mgGAE /g ext. and TFC= 67.69±1.47 mg RE /g ext.). Also, MeOH (85%) extract showed high antioxidant activities (DPPH SC_{50}= 98.76±0.46 (μg/ml), ABTS= 45.83±0.32 mm Trolox® eq. /100 gm extract and TAC= 199.18±2.19mg equivalent of ascorbic acid /g ext.). On other hand, EtOAc fraction derived from MeOH (85%) extract exhibited the highest antioxidant activity; DPPH SC_{50}= 50.19±0.24 (μg/ml), ABTS= 76.22±1.61(mm Trolox® eq. /100 gm ext.) and TAC= 249.86±3.74 (mg equivalent of ascorbic acid /g ext.). This study demonstrated that, *S. mucronata* leaf is a good source of natural antioxidants. Also, there is a high correlation between the total phenolic content and the antioxidant activity.

INTRODUCTION

Reactive oxygen and nitrogen species (RON_S) such as hydroxyl radical ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), nitric oxide ($NO^{\bullet}$) and hypochlorous acid (HOCl) are highly reactive oxidants produced naturally in the human body through normal metabolic pathways or due to the exposure to external stimuli such as ionizing radiations, pollution, stress or even poor diet (Apak *et al.*, 2013). These RONs if not neutralized, they tend to attack the biomolecules like proteins, lipids, DNA and carbohydrates causing their damage and form harmful byproducts such as lipid peroxides in addition to causing the loss of enzyme activity, mutagenesis and carcinogenesis (Dupont and Hueckstead, 1992; Weidinger and Kozlov, 2015). Antioxidants are compounds that can prevent or minimize the oxidation of oxidizable products by scavenging the free radicals and reducing oxidative stress. The human body has many endogenous enzymatic antioxidant defenses such as catalase, superoxide dismutase, and glutathione peroxidase. These endogenous enzymatic antioxidant defenses protect the cells against the oxidative damage (Wannes *et al.*, 2010).In diseases such as cellular aging, carcinogenesis, coronary heart disease, diabetes and neurodegenerative infections. Therefore the external antioxidants especially from plant sources are very important to decrease the risk of these free radicals (Sulaiman *et al.*, 2013).So the increasing of dietary antioxidant intakes may help to support the human health (Martin-Puzon and Rivera, 2015).

Plants are rich sources of bioactive secondary metabolites, such as flavonoids, glycosides, saponins, terpenes, sterols, tannins, alkaloids and other metabolites. It has been reported that the most of these groups have antioxidant activity. *Salix* genus (Family Salicaceae) is known by its medicinal value and comprises 400 species worldwide. *Salix mucronata* Thunb. (Syn. *Salix safsaf* or *Salix subserrata*) is widely distributed along Nile Riverin Egypt (Al Sherif *et al.*, 2009).Many studies revealed that, *Salix* species contain many phytochemical constituents such as salicin (natural aspirin), flavonoids, terpenoids, lignans and phenolic acids. Most of these compounds showed biological and pharmacological effects. The isolated compounds from *Salix* genus such as salicin and salicylic acid are used for the treatment of fever, pain, and inflammation (Kim *et al.*, 2015).

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The present study was carried out to determine the phytochemical constituents and evaluate the antioxidant activity of different leaf extracts of *S. mucronata.*

MATERIALS AND METHODS

Plant materials

Fresh leaves of *S. mucronata* were collected from Sharkia governorate, Egypt. A voucher specimen of the whole plant was identified by Mrs. Rehab Mohamed Eid a botanist at Orman Garden Herbarium, Giza, Egypt. The leaves of the plant were dried in shade, grinded with electric mill to fine powder and kept in dry conditions for the extraction process.

Chemicals

DPPH (1, 1diphenyl-2-picryl hydrazyl radical),ABTS (2-2`azinobis (3-ethylbenzthiazoline-6-sulphonic acid) and Folin-Ciocalteu reagent were purchased from Sigma –Aldrich (Steinheim, Germany). Potassium persulphate and disodium hydrogen phosphate were purchased from Bio Basic Inc. (Canada). Ammoniummolybdate, sodium carbonate, sodium nitrite, sodium hydroxide and aluminum chloride were purchased from Merck (Darmstadt, Germany).Rutin, gallic acid, BHT (butylated hydroxy toluene) and ascorbic acid were purchased from Sigma –Aldrich (St. Louis, USA). α-tocopherol (Vitamin E) was purchased from Sigma –Aldrich (Gillingham, England).

Extraction process

Eight hundred grams of dried powder of*S. Mucronata* leaves were divided into four parts. Each part (200 g) was separately extracted three times with pure methanol, MeOH (85%), MeOH (70%) and distilled water respectively. Each extract was evaporated under vacuum till dryness using rotatory evaporator (BUCHI, Germany). The dried extracts were kept in dry vials for estimation of their chemical constituents as well as total phenolic and flavonoid contents. Also the antioxidant activity of these extracts was determined.

Fractionation process

The methanolic extract (85%) was defatted with petroleum ether. The defatted methanolic extract was successively fractionated with organic solvents such as chloroform ($CHCl_3$), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH) then these fractions were evaporated under reduced pressure till dryness.

Phytochemical screening

The Phytochemical screening of *S. mucronata* different extracts was carried out to detect the bioactive secondary metabolites in these extracts such as flavonoids (Shinoda test), alkaloids (Wagner's and Dragendorff's tests),sterols (Salkowski test), tannins (10% Lead acetate test), triterpenoids (Libermann-Burchard test), Saponins (Frothing test), cardiac glycosides (NaOH and Molisch tests) and phenols ($FeCl_3$ test) according to the reported methods described by Ayoola *et al.,* (2008); Boxi *et al.,* (2010); Bhatt and Dhyani (2012).

Total phenolic content

The total phenolic content was estimated using Folin-Ciocalteu method by measuring the intensity of the produced blue color (Singh *et al.,* 2012). Briefly, 0.5ml plant extract dissolved in methanol (200μg/ml) was added to 2.5ml of 10 fold diluted Folin-Ciocalteu reagent and 2ml sodium carbonate (7.5%). After 30 min incubation in dark with permanent shaking. The absorbance was measured at 760 nm against a standard solution of gallic acid. The total phenolic content (TPC) of the different plant extracts was measured as the mean of triplicate analyses and expressed as mg of gallic acid equivalent/g dry weight extract (mg GAE /g extract).

Total flavonoid content

The total flavonoid content was determined by using aluminum chloride colorimetric assay according to the method described by Barku *et al.,* (2013). The hydroxyl groups of flavonoids form a complex with aluminum chloride ($AlCl_3$).A pink color upon the reaction with sodium nitrite was appeared. 250μl of plant extract in methanol (500μg/ml) was mixed with 75 μl $NaNO_2$(5%) and1.3 ml distilled water. After 5min, 150 μl of $AlCl_3$(10%) was added. After 6 min, 0.5ml of 1M NaOH was finally added and the reaction mixture was diluted by 275 μl distilled H_2O. The absorbance was measured at 510nm after 15 min against a standard solution of rutin. The total flavonoid content (TFC) was expressed as mg rutin equivalent per gram extract (mg RE /g extract) and all experiments were carried out in triplicate.

ASSAYSFOR ANTIOXIDANT

DPPH scavenging method

DPPH(1, 1diphenyl-2-picryl hydrazyl radical) is a stable violet colored radical which converts to yellow color on reduction. The decrease in the optical density was measured spectrophotometrically at 517nm according to the procedure described by Alam *et al.,* (2013).In this assay, 1.5ml of a serial concentrations of various plant extracts in methanol was added to 1.5ml of a freshly prepared DPPH solution (DPPH was dissolved in methanol and absorbance was adjusted to 0.1±0.05).The tubes were kept in dark for 30 min followed by measuring the absorbance against blank sample at 517 nm. Ascorbic acid, vitamin E and BHT were used as standards and all experiments were carried out in triplicate. The DPPH scavenging activity of the extracts was calculated and SC_{50} (Concentration of sample required to scavenge 50 % of DPPH radicals) value was determined from this equation:

DPPH scavenging activity (SA) %= $[(A_{control} - A_{sample})/A_{control}] \times 100$

Where A_{sample} is the absorbance of a sample solution, and$A_{control}$ is the absorbance of the control solution (containing all of the reagents except the test sample).

ABTS assay

The ability of various extracts to quench $ABTS^{\cdot+}$ cationic radical (2-2`azinobis (3-ethylbenzthiazoline-6-sulphonic acid) in reference to Trolox® (water soluble analogue of vitamin E) was detected as described by Kaur *et al.,* (2011). The $ABTS^{\cdot+}$ was firstly generated by overnight interaction between ABTS (7mM) and potassium persulphate (2.45 mM) then it was kept in dark at 5 °C in refrigerator. The intense colored ABTS stock solution was diluted by ethanol with ratio 1:70 and its absorbance was adjusted to 0.7±0.01 at 734nm. Finally 100μl (200μg/ml) of each plant extract was mixed with 1ml of ABTS solution in micro cuvette and the reduction in absorbance was measured exactly after 2.5 min against blank sample. Trolox® standard solution (final concentration 0-15μM) in methanol was prepared and assayed at the same conditions. The absorbances of the resulting oxidized solutions were compared with Trolox® standard calibration curve. Results were expressed in terms of mmol Trolox® equivalent per 100 g dry weight of plant extract.

Total antioxidant capacity (TAC) assay

The total antioxidant capacity was estimated by Phosphomolybdate assay. This method was based on the reduction of Mo (VI) to Mo (V) by extracts forming a green phosphate Mo (V) complex under acidic condition. The method was carried out according to Abdel-Gawad *et al.*,(2014). Briefly, 0.5ml of plant extract in MeOH (500μg/ml) was added to 5ml reagent (0.6M sulphuric acid, 28mM disodium hydrogen phosphate and 4mM ammonium molybdate).

The tubes were capped and incubated in a 95°C water bath for 90 min. After the incubation period, the tubes were cooled to reach room temperature and the absorbance was measured at 695nm against blank (5ml reagent in addition to 0.5ml methanol under the same conditions).The total antioxidant activity was expressed as mg equivalent of ascorbic acid/g plant extract. All experiments were carried out in triplicate.

STATISTICAL ANALYSIS

The statistical analyses were performed using SPSS (16) software and Microsoft Excel program version 2010. The results were given as means ± standard deviation (SD) and all experimental analyses were carried out in triplicate.

RESULTS AND DISCUSSION

Phytochemical screening

Plant cells produce two types of metabolites, primary metabolites (carbohydrates, lipids and proteins) and secondary metabolites (alkaloids, phenolics, essential oils, terpenes, sterols, flavonoids, tannins, etc.). Literature survey showed that the natural compounds have the major role in treatment of several diseases (Aggarwal and Shishodia 2006; Ndam *et al.,* 2014). Also, it has been reported that, *Salix* extracts, contains many phenolic and flavonoid compounds.

These natural groups are used to treat different diseases. Therefore, in the present study, preliminary phytochemical screening of different extracts of *S. mucronata* [MeOH, MeOH (85%), MeOH (70%) and water] was carried out to identify the major chemical constituents and the ability of these constituents to scavenge free radicals in tested extracts. The results in table1 showed that the different extracts have high amounts of flavonoids, phenols and moderate amounts of tannins, sterols, triterpenoids and cardiac glycosides. The results also exhibited that MeOH (85 %) extract have high phenolic and flavonoid contents, so this extract was defatted with petroleum ether and successively fractionated with different organic solvents $CHCl_3$, EtOAc and *n*-BuOH.

The results in table 1 exhibited that, EtOAc and *n*-BuOH fractions have high amounts of flavonoids, tannins, phenols, cardiac glycosides, moderate amounts of sterols and saponins as well as small amounts of alkaloids. The presence of these secondary metabolites in the tested plant indicates that *S. mucronata* may be potent antioxidant due to the high ability of phenolic compounds to scavenge the free radicals which are associated with many diseases (Ayoola *et al.,* 2008; Ram *et al.,* 2015; Florence *et al.,* 2015).

Table 1: Preliminary Phytochemical screening of *S. mucronata* leaf extracts and fractions derived from MeOH (85%) extract.

Phytochemical constituents	Tests	MeOH ext.	MeOH (85%) ext.	MeOH (70%) ext.	Water ext.	Fractions of MeOH (85%) ext.			
						$CHCl_3$ fraction	EtOAc fraction	*n*-BuOH fraction	Residue fraction
Flavonoids	Shinoda test	++	+++	++	+	+	+++	+++	+
Alkaloids	Wagner's test	+	+	--	--	--	+	+	--
	Dragendorff's test	+	+	--	--	--	+	+	--
Tannins	10% Pb acetate test	++	++	++	+	+	+++	+++	-
Sterols	Salkowski test	+	++	+	--	++	++	++	--
Triterpenoids	Libermann-Burchard test	+	++	+	--	+	++	++	--
Cardiac glycosides	NaOH test	++	++	+	+	+	++	+++	+
	Molisch test	++	++	++	++	+	++	+++	+
Phenols	$FeCl_3$ test	++	+++	++	+	++	+++	+++	+
Saponins	Frothing test	+	+	+	--	-	+	++	--

(+++): high amount, (++): moderate amount, (+): small amount, (-): Absent.

Total phenolic contents

The total phenolic content was determined using Folin-Ciocalteu assay; this spectrophotometric assay allows the estimation of all phenolics present in the plant extracts. The results in table 2showed that MeOH (85%) extract has the highest total phenolic content (131.39±2.49 mg GAE/g ext.), followed by MeOH (70%) extract (129.92±0.84 mg GAE/g ext.) whereas, the water extract had the lowest phenolic content (89.49±1.15 mg GAE/g ext.).

On the other hand, EtOAc and *n*-BuOH fractions derived from MeOH (85%) extract in table 3 exhibited the highest total phenolic contents (259.46±2.23 and162.99±3.91 mg GAE/g ext.) respectively.$CHCl_3$fraction had moderate content of phenolics (95.74±2.23 mg GAE/g ext.) whereas, the residue fraction showed the lowest phenolic content (66.34±0.64 mg GAE/g ext.).

It has been reported that the phenolic compounds isolated from medicinal plants are very reactive in neutralization of free radicals by donating odd electron or hydrogen atom due to the presence of phenolic hydroxyl groups (Song *et al.,* 2010; Casquete *et al.,* 2015).

Table 2: Yield, total phenolic and flavonoid contents of various leaf extracts of *S. mucronata.*

Extract	Yield %	Total phenols (mggallic acid equivalent (GAE) / g ext.)	Total flavonoids (mg rutin equivalent (RE) / g ext.)
MeOH ext.	20.03	128.08±0.85	46.51±0.8
MeOH(85%) ext.	18.75	131.39±2.49	67.69±1.47
MeOH (70%) ext.	16.4	129.92±0.84	62.65±0.78
Water ext.	11.1	89.49±1.15	28.72±0.74

The results were expressed as the mean ± standard deviation (SD) of three independent experiments.

Total flavonoid content

Flavonoids consist of a large group of polyphenolic compounds. They are highly active radical scavengers of most oxidizing molecules, including singlet oxygen, and various free radicals which implicated in several diseases (Saeed *et al.,* 2012). So, the presence of flavonoids in the plant extracts increases their ability to scavenge or deactivate free radicals (Kaur and Mondal, 2014).

The results in table 2 showed that the total flavonoid contents of different leaves extracts of *S. mucronata* were arranged in the order, MeOH (85%) extract has the highest flavonoid content (67.69±1.47mg RE/g ext.) followed by MeOH (70%) extract (62.65±0.78 mg RE / g ext.), MeOH extract (46.51±0.8 mg RE/g extract). The water extract has the lowest content (28.72±0.74 mg RE/g ext.). On the other hand, the different fractions of MeOH (85%) extract (Table 3)has total flavonoid contents arranged in the following order; EtOAc fraction has the highest content (121.8±1.82 mg RE/g ext.) followed by *n*-BuOH fraction (58.65±2.21mg RE/g ext.) and $CHCl_3$ fraction (39.22±2.75 mg RE/g ext.). The residue fraction had the lowest total flavonoid content (20.52±1.37 mg RE/g ext.). Therefore, the ethyl acetate fraction possesses the highest total flavonoid content.

Table 3: Yield, total phenolic and flavonoid contents of different fractions derived from MeOH (85%) extract of *S. mucronata.*

Extract	Yield %	Total phenols (mggallic acid equivalent (GAE) / g ext.)	Total flavonoids (mgrutin equivalent (RE) / g ext.)
$CHCl_3$ fraction	3.46	95.74±2.23	39.22±2.75
EtOAc fraction	1.18	259.46±2.23	121.8±1.82
n-BuOH fraction	5.97	162.99±3.91	58.65±2.21
Residue fraction	5.64	66.34±0.64	20.52±1.37

The results were expressed as the mean ± standard deviation (SD) of three independent experiments.

ASSAYS FOR ANTIOXIDANT

DPPH scavenging method

DPPH (1, 1diphenyl-2-picryl hydrazyl radical) is a stable free radical having a maximum absorbance at 517 nm in methanol and its color changed from purple to yellow after accepting an electron or proton radical from antioxidant molecules (antioxidant extracts) to become a stable diamagnetic molecule(Singh *et al.,* 2016).The results in table 4 showed that MeOH (85%) extract is the most free radical scavenger extract(SC_{50}= 98.76±0.46 μg/ml) followed by MeOH (70%) extract (SC_{50}= 102.52±1.6 μg/ml). The water extract showed the lowest antioxidant activity (SC_{50}= 209.78±2.04 μg/ml). Also, the results in table 5exhibited that EtOAc and *n*-BuOH fractions derived from the methanol extract (85%) had the more potent antioxidant activity (SC_{50}= 50.19±0.24 and72.19±0.52 μg/ml) respectively. The residue fraction showed the lowest antioxidant activity (SC_{50}= 213.68±1.17μg/ml). From this study it was appeared that, EtOAc fraction is the most active fraction because it contains high amount of phenols. These results are in full agreement with the previous studies on other plants which mean that the plant phenolic compounds are very important due to their free radical scavenging ability (Enechi *et al.,* 2013; Bera *et al.,* 2015).

Table 4: DPPH scavenging activity, ABTS radical scavenging activity and total antioxidant capacity of various leaf extracts of *S. mucronata.*

Extract	DPPH scavenging activity SC_{50} (μg/ml)	ABTS radical scavenging activity (mm Trolox® eq. /100 gm ext.)	Total antioxidant capacity (mg equivalent of ascorbic acid / g ext.)
MeOH ext.	131.62±2.51	41.41±1.07	158.47±1.44
MeOH(85%) ext.	98.76±0.46	45.83±0.32	199.18±2.19
MeOH (70%) ext.	102.52±1.6	43.29±0.66	170.73±3.12
Water ext.	209.78±2.04	27.69±0.64	111.74±2.59
Ascorbic acid	13.58±0.34	--	--
Vitamin E	23.12±0.21	--	--
BHT	17.74±0.076	--	--

The results were expressed as the mean ± standard deviation (SD) of three independent experiments.

ABTS assay

ABTS (2-2`azinobis [3-ethylbenzthiazoline-6-sulphonic acid]) assay is a powerful assay used to determine the chain-breaking antioxidants in case of lipid peroxidation and antioxidant activity of hydrogen donating antioxidants. This assay involves the oxidation of ABTS to form an intensely-green colored nitrogen-centered $ABTS^{\bullet+}$. These free radical cations have maximum absorption at 734 nm and stable in a wide range of pH (Singh *et*

al., 2016; Zheng *et al.*, 2016).The results in table 4 revealed that MeOH (85%) extract of *S. mucronata* exhibited the highest antioxidant activity (45.83±0.32 mm Trolox®eq. / 100 gm ext.).Whereas, the water extract of the plant showed the lowest antioxidant activity (27.69±0.64 mm Trolox® eq. / 100 gm ext.).

The methanol (70%) and pure methanol extracts exhibited a moderate activity (43.29±0.66 mm Trolox® eq. / 100 gm ext. and 41.41±1.07 mm Trolox® eq. / 100 gm ext.) respectively. Also, the different fractions derived from MeOH (85%) showed antioxidant activity as shown in table 5 and the activity in order, EtOAc fraction is the highest antioxidant activity (76.22±1.61 mm Trolox® eq. / 100 gm ext.) followed by*n*-BuOH and chloroform fractions (57.57±0.76 and 29.37±1.04 mm Trolox® eq./100 gm ext.) respectively. whereas the residue fraction showed the lowest antioxidant activity (21.02±0.67 mm Trolox® eq./100 gm ext.).

Total antioxidant capacity (TAC) assay

The phosphomolybdenum method was used to estimate the total antioxidant capacity of *S. mucronata* different extracts. Literature survey revealed that the natural antioxidants reduce Mo (IV) to Mo (V) generating the green phosphate/Mo (V) compounds.

These compounds have an absorption maxima at 695 nm (Nandhakumar andIndumathi2013; Zhao *et al.*, 2014).The results in the present study (Table 4) showed that, MeOH (85%) extract is the highest total antioxidant capacity (199.18 ± 2.19 mg equivalent of ascorbic acid/g ext.) followed by MeOH (70%) extract (170.73±3.12 mg equivalent of ascorbic acid / g ext.) and MeOH extract (158.47±1.44 mg equivalent of ascorbic acid / g ext.). The water extract exhibited the lowest total antioxidant capacity (111.74 ± 2.59 mg equivalent of ascorbic acid / g ext.).

On the other hand, the results in table 5revealedthat EtOAc fraction is the highest total antioxidant capacity (249.86 ± 3.74 mg equivalent of ascorbic acid / g ext.) due to its highest phenolic content. The residue fraction showed the lowest antioxidant capacity (106.14±1.9 mg equivalent of ascorbic acid / g ext.).

Table 5: DPPH scavenging activity, ABTS radical scavenging activity and total antioxidant capacity of different fractions derived from MeOH (85%) extract of *S. mucronata.*

Extract	DPPH scavenging activity SC_{50} (*µ*g/ml)	ABTS radical scavenging activity (mmTrolox® eq. / 100 gm ext.)	Total antioxidant capacity (mg equivalent of ascorbic acid / g ext.)
$CHCl_3$ fraction	182.5±1.98	29.37±1.04	119.22±20
EtOAc fraction	50.19±0.24	76.22±1.61	249.86±3.74
n-BuOH fraction	72.19±0.52	57.57±0.76	233.45±1.57
Residue fraction	213.68±1.17	21.02±0.67	106.14±1.9
Ascorbic acid	13.58±0.34	--	--
Vitamin E	23.12±0.21	--	--
BHT	17.74±0.076	--	--

The results were expressed as the mean ± standard deviation (SD) of three independent experiments.

RELATIONSHIP BETWEEN PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY

The relationship between the total phenolic content and the antioxidant activity of *S. mucronata* extracts and different fractions derived from the MeOH(85%) extract showed a positive correlation between their total phenolic contents and the antioxidant activities with relation coefficients (r^2)= 0.76, 0.81 and 0.96 for DPPH, TAC and ABTS respectively. Accordingly in this study, there is a linear and significant relationship between the antioxidant capacity and the total phenolic content. These results are in full agreement with previous several studies on other plant extracts (Kaur and Kapoor, 2002; El-Hashash *et al.*, 2010; Hyun *et al.*, 2013) and revealed that *S. mucronata* different extracts can serve as a good sources of natural antioxidants.

CONCLUSION

The present study demonstrated that MeOH (85%) extract of *S. mucronata* leaves has the highest total phenolic content and antioxidant activity. Also, EtOAc and *n*-BuOH fractions derived from MeOH (85%) extract have high total phenolic content and antioxidant capacity. There is a high positive correlation between the antioxidant and total phenolics. Owing to the high content of total phenolics and antioxidant capacity of EtOAc and *n*-BuOH fractions recommend for further isolation and identification of their chemical constituents using advanced chromatographic and spectroscopic tools.

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