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Research Article

Comparative Antioxidant Activities of Selected Apiaceous Plants Using EPR Technique

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ABSTRACT

Introduction: Electron Paramagnetic Resonance (EPR) spectroscopy is a unique technique able to identify and quantify free radicals in the complex biological matrices. In this study, free radical scavenging activity of aqueous and methanol extracts of fruits, shoots and roots of dill and parsley was examined using EPR technique, in addition to the determination of their contents of total polyphenols (TPC) and flavonoids (TFC). **Method**: The hydroxyl anion scavenging activity (HASA) of the tested extracts was determined using EPR spin trapping technique and hypoxanthine/xanthine oxidase system generating ($O^{\bullet}_{2}$). The TPC and TFC were determined using Folin-Ciocalteu and aluminum chloride colorimetric assays. **Results:** Both aqueous shoot of dill (ADSh) and methanol root of parsley (MPR) demonstrated the strongest inhibition of HASA. On the contrary, the aqueous extract of dill fruit at a concentration of 0.5 mg/ml was found to be pro-oxidant (49.43 %), but at 10 mg/ml demonstrated potent inhibition of HASA (98.12 %). TPC was found to be the highest in the methanol extract of parsley fruit (MPF, 88.62 ± 0.6 mg GAE/g) and the lowest in MPR (4.34 ± 0.050 mg GAE/g). On the other hand, TFC was the highest in MPF (584.29 ± 2.10 mg GAE/g) and the lowest in the methanol extract of dill fruit (MDF, 1.28 ± 0.02 mg rutin/g). **Conclusion**: In this study, EPR provided a direct insight that all tested apiaceous plants extracts showed effective HASA except two extracts that proved to be pro-oxidant at 0.5 mg/mL This study confirmed that there is no correlation between antioxidant potential and TPC and TFC.

Key Words: *Anethum graveolans*, Free radicals, *Petroselinum crispum*, Total phenolic content, Total flavonoid content.

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INTRODUCTION

Oxidative stress means imbalance shifted toward pro-oxidant leading to several diseases that target different pivotal body organs.[1] Natural antioxidants are constituents that can delay or prevent the lipid peroxidation or effectively interrupt the oxidizing chain reactions,[2] thus contributing to prevention and reduction of risk factors for several diseases such as cancer, diabetes, liver and cardiovascular disorders, aging processes and inflammation. Edible fruits and vegetables are rich sources of several antioxidant constituents such as polyphenols, flavonoids; and vitamins.[3] Recently, scientists are interested in investigating edible plants that preserve substantial antioxidant capacity.

Plants of family Apiaceae are commonly used for food, flavoring and medical purposes, and used as a household remedies since antiquity.[4] Parsley *(P. crispum*) is a culinary herb used in Egyptian cuisines and widely distributed throughout the world, and proved to have antioxidant activity.[5] Kolarovic *et al.*[6] previously examined the leaf and root juices of celery and parsley for their antioxidant potential. Also, aqueous extract of dill leaves (at 1 mg/mL) demonstrated potent scavenging activity of the DPPH• radical.[7]

Electron Paramagnetic Resonance (EPR) spectroscopy is a unique and precise technique able to detect, identify, and quantify free radicals in the complex biological matrices.[8] It is a technique for evaluating any molecule bearing lone pair of electrons. The basic significance of EPR are analogous to those of nuclear magnetic resonance (NMR).[9] Spin-trapping techniques (by EPR) can directly monitor either free radical short or long lived markers with unpaired electron and their scavenging by antioxidants.[10]

Our research aimed to study the hydroxyl radical scavenging potential of aqueous and methanol extracts of fruits, shoots and roots of two apiaceous plants using EPR. Besides, total phenolic and flavonoid contents in the extracts were determined.

MATERIALS AND METHODS

Chemicals and reagents

Folin-Ciocalteu reagent, gallic acid, and rutin standards were obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Aluminum chloride hexahydrate, methanol, and sodium carbonate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified using a Milli-Q system (Millipore).

Hypoxanthine (HX) reagent was prepared by dissolving 13.6 mg of HX (Sigma H-9377) in 9.7 ml of distilled water and 0.3 ml of 1.0 M NaOH to obtain a concentration of 10mM to be stored at -20°C. Xanthine Oxidase (XO) reagent was prepared by mixing 490 µl of XO with 10 µl PBS

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(phosphate buffer saline pH 7.2) freshly prepared. DMPO reagent (5,5-Dimethyl-1-pyrroline-N-oxide, DMPO) (Sigma MKBM3455V) was prepared by dissolving 28.5 mg of DMPO in 251.8 µl ethanol to give final concentration 1 mM and stored at -20°C. All chemicals used for determination of total phenols and total flavonoids were purchased from Sigma-Aldrich Co (St. Louis, Mo, USA). All reagents and solvents used were analytical grade.

Plant materials

Samples of dill *(Anethum graveolans)* and parsley (*Petroselinum crispum*) were collected in Spring from the Experimental Station of Medicinal and Aromatic Plants, Faculty of Pharmacy, Cairo University, Giza, Egypt. Plants were authenticated by Dr. A. Abdel El Mogali, A Professor in flora & phytotaxonomy researches, Agricultural research centre. The voucher specimens No. Ap 504 and Pet 514, respectively, were deposited at the herbarium of Pharmacognosy department, Faculty of Pharmacy, Cairo University.

Preparation of plant extract stock solutions

Fruits, shoots and roots of each plant were, separately pulverized using a cutter mill. Part of the powder of each plant part (100 g) was, separately extracted with milli Q water till exhaustion (500 ml x 3) with the aid of sonication bath, filtered through Whatman No.1 filter paper and evaporated at 40°C till dryness under reduced pressure (SENCO Technology Co., Shanghai, China). Part of the extract (100 mg) was dissolved in 10 ml distilled water with the aid of sonication and Tween 80 (2-3 drops) were added to obtain clear aqueous extract stock solution. Similarly, the above mentioned procedure was followed to prepare clear stock solutions of the methanol extracts of similar parts of each plant (100 g) using methanol (500 ml x 3). The extract stock solutions were transferred to amber colored bottles and stored at -4^{O}C until use.

Total polyphenol content (TPC)

TPC of the prepared extracts was determined using Folin-Ciocalteu colorimetric method following the method described by.[11]

Briefly, one mL of each extract stock solution or standard solution was mixed with 0.4 mL of Folin-Ciocalteu reagent. After 5 minutes, 4 mL of 7% sodium carbonate solution was added and the volume was made up to 10 ml with water and mixed well. After incubation in the dark for 90 min at room temperature, the absorbance against the prepared blank reagent (1mL water as a compensating liquid) was determined at 750 nm using an UV-VIS spectrophotometer (Varian Cary, California, USA). The phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per gram dry plant material (mg GAE/g dry plant material) on the basis of a standard curve of gallic acid (5-500 mg/L, y = 0.134x+0.0325, R^2=0.9986). All determinations were carried out in triplicate.

Total flavonoid content (TFC)

The aluminum chloride colorimetric method was used for the determination of TFC of the sample.[12] For TFC determination, rutin was used to make the standard calibration curve. Stock rutin solution was prepared by dissolving 1 mg rutin in 1.0 mL methanol, then the standard solutions of rutin were prepared by serial dilutions using methanol (50–200 μg/mL, y = 0.0034x+0.0842, R^2=0.9947).

TFC was determined by mixing 1mL of the extract solution (1 mg/mL) with 0.3 ml 5% $NaNO_2$, shaking, incubating for 5 min and adding 0.3 ml 10% $AlCl_3$. After 6 min 2 ml 1M NaOH was added and the absorbance was recorded at 430 nm. The concentration of flavonoids is expressed as mg equivalent rutin per gram dry plant material were deduced from a standard curve using rutin as the standard. All the determinations were carried out in triplicate and results were expressed as mean ± SD values.

In vitro hydroxyl radical (•OH) scavenging ability using EPR

Hydroxyl anion scavenging ability of the tested extracts was determined using EPR spin trapping technique and hypoxanthine/xanthine oxidase system generating ($O•^-_2$). As a specific spin trapping agent for $O•^-_2$ was used DMPO.[13] Briefly, the test samples were prepared to contain 5 µl HX, 10 µl EDTA, 30 µl extract solution, 5 µl XO and 10 µl DMPO, while control solution contained 30 µl PBS phosphate buffer saline (pH 7.2) instead of extract solution. The test solution was transferred to a capillary tube, which was then sealed with silicone sealant and introduced into the EPR cavity of a MiniScope MS400 Benchtop spectrometer (Magnettech, Berlin, Germany). The observed DMPO-OH• signals arising from DMPO-OOH• spin adduct were monitored over 15 min. Operating parameters for the EPR spectrometer were as follows: microwave power 5 mW, centre field 336 mT, sweep width 12 mT, modulation frequency 9.50 GHz, modulation amplitude usually 0.2 mT and a temperature of 22 °C. All scans were recorded using the same instrument settings and sample position and were carried out in diffuse room light. The area under curve for each kinetic measurement was then calculated over the 15 minutes period using OriginPro 9.0 software and hence taken to represent ROS yields due to oxidative stress. The free radical scavenging activity was expressed as the percentage inhibition of the hydroxyl radical EPR signal (due to DMPO-•OH adducts formation) in the above assay mixture and calculated according to the following formula:

100 - [(Sample amplitude/ Control amplitude) *100].

RESULTS

Determination of TPC and TFC content

The TPC of extracts prepared from the selected plants was determined using Folin-Ciocalteu colorimetric method. The results of TPC were reported as mg GAE/g dry weight plant. From the result depicted in Table 1 the highest TPC was for the methanol extract of parsley fruit (88.62 ± 0.60 mgGAE/g) followed by the aqueous dill fruit (20.30 ± 0.40 mgGAE/g), while the lowest TPC was for aqueous extract of parsley fruit (3.13 ± 0.09 mgGAE/g).

TFC of extracts prepared from the selected plants was determined as mg rutin equivalent/g dry weight plant. Table 1 showed that the highest content of TF was found in the methanol extract of parsley fruit (584.29 ± 2.10 mg rutin eq/g) followed by aqueous extract of parsley root (125.95 ± 0.80 mg rutin eq/g). Almost similar amount of TF were found in the methanol extract of dill root (125.39 ± 0.90 mg rutin eq/g), while the methanol extract of parsley root and of the aqueous dill fruit was found to contain the least amount of TF (1.72 ± 0.02 and 1.72 ± 0.05 mg rutin eq/g, respectively).

All determinations were carried out in triplicates, and results were expressed as mean ± SD values, eq/g: equivalent/gram.

In vitro hydroxyl radical (•OH) scavenging ability using EPR

EPR spin trapping is an established technique capable of scavenging, detection and distinguishing of short lived radicals (e.g. $O•^-_2$ and •OH) at room temperature.[14] Precision and accuracy of measurements made EPR technique one of the most strict research methods for evaluation of antioxidant activity of an extract differentiating between the different biological reactive radicals.[15] For production of hydroxyl radical ($OH^{\cdot}$) and evaluation of antioxidant efficacy of apiaceous extracts, the following reaction was implicated:

$$HX \xrightarrow{XO} X + OH^{\cdot}$$

The produced HO• radical reacts rapidly with either the added extract or the nitrogenic spin trap, DMPO to yield DMPO-OH• radical adduct which is a stable free radical species that is detectable by EPR spectroscopy.[16] The spin adduct formed is paramagnetic and shows an EPR spectrum with a hyperfine splitting parameter and g-value characteristic of the type of reactive free radical trapped. DMPO produces a spin adduct with an EPR signal that is a 1:2:2:1 quartet. Figure 1 illustrates the spectrum of the hydroxyl radical adduct with DMPO, showing this quartet signal.[10] The reaction were monitored and recorded for 15 minutes. Control HX/XO reaction (Figure 1) showed maximum EPR signal amplitude at 8 minutes (representing the peak of the reaction) which provides a practical time window to follow competition with extracts. As a result, concentration-dependent elimination of hydroxyl radical by aqueous and methanol extracts of fruits, shoots and roots of dill (Figure 2) and parsley (Figure 3) were analyzed by monitoring EPR signals intensity of the DMPO-HO• adduct. In most cases, a different radical species that is attributable to carbon centered radical adduct with DMPO appears upon the inclusion of all extracts (6 equal-intensity peaks). Table 2 shows a concentration-dependent relative to % hydroxyl radical (HO•) scavenging potency of tested extracts. Iswantini et al. inferred that extract proved to be a potential scavenger for free OH• radical when it has the percent inhibition value greater than 50%.[17]

At 10 mg/ml, all the tested extracts were quenched EPR signals from DMPO-HO• adduct (more than 85%), but at range 0.5-2 mg/ml signals scavenged to varying degrees. Methanol parsley roots extract (MPR) was perplexing in prohibiting formation of the DMPO-HO• adduct by 98.90 % at 10 mg/ml concentration. Such perception launched a further evaluation of the same extract but at low concentration. At 0.5 mg/ml, MPR extract could incessantly scavenge hydroxyl radical by at 70.20% as shown in Figure 2 and Table 2.

The aqueous dill shoot extract (ADSh) showed to be the 2nd highest percentage of quenching ·OH even at low concentration (98.60% at 10 mg/mL and 71.60 % at 0.50 mg/mL). Aqueous dill root extract (ADR), methanol parsley shoot extract (MPSh) and methanol dill shoot extract (MDSh) were implicated in scavenging hydroxyl radical by 95.40, 92.90 and 87.30 %, respectively at 2 mg/mL as shown in table 2 and figures 2 &3. Aqueous dill fruit extract (ADF) prone to be pro-oxidant extract

Table 1: Total phenol (TPC) and flavonoid content (TFC) in aqueous and methanol extracts of dill and parsley.

Plant	Part	Total phenolic content (mg gallic acid eq /g dry weight)		Total flavonoid content (mg rutin eq/g dry weight)	
		Aqueous	Methanol	Aqueous	Methanol
Dill	Fruit	20.30 ± 0.4	6.8 ± 0.10	1.72 ± 0.05	1.28 ± 0.02
	Shoot	14.20 ± 0.30	18.5 ± 0.50	39.90 ± 3.90	89.79 ± 0.80
	Root	8.727 ± 0.50	6.306 ± 0.2	107.442 ± 1.10	125.39 ± 0.90
Parsley	Fruit	3.13 ± 0.09	88.62 ± 0.60	111.58 ± 0.70	584.29 ± 2.10
	Shoot	6.96 ± 0.10	14.03 ± 0.20	14.59 ± 0.25	32.00 ± 0.40
	Root	7.01 ± 0.15	4.34 ± 0.05	125.95 ± 0.80	1.72 ± 0.02

Table 2: Effect of dill and parsley extracts on the EPR signal intensity of the DMPO-OH· spin adduct.

Extract conc. (mg/ml)	% of OH· radical scavenging by the tested extracts											
Plant	Dill						Parsley					
Extract type	Aqueous			Methanol			Aqueous			Methanol		
Organ	Fruit	Root	Shoot	Fruit	Root	Shoot	Fruit	Root	Shoot	Fruit	Root	Shoot
0.50	49.43	n.d	71.60	59.78	54.40	n.d	67.95	n.d	63.50	58.18	70.20	n.d
1.00	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
2.00	n.d	95.40	n.d	n.d	n.d	87.30	n.d	58.30	n.d	n.d	n.d	92.90
10.00	98.12	97.10	98.60	95.39	95.40	89.90	96.19	88.20	97.70	98.48	98.90	81.30

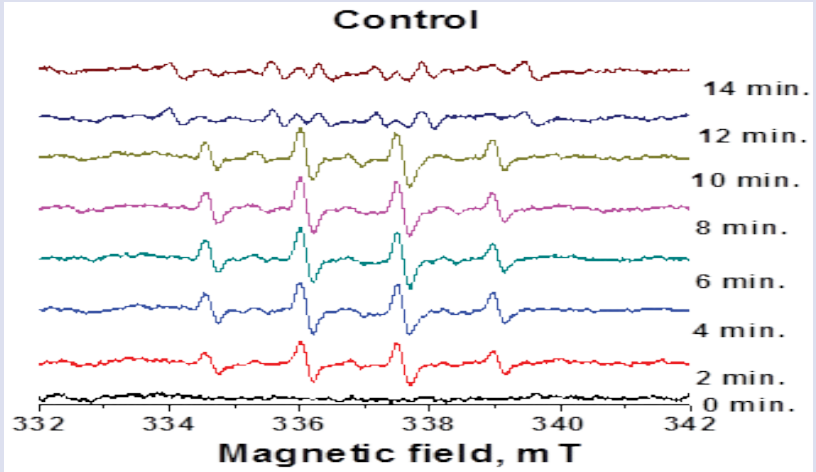

Figure 1: Time evolution of the X-band EPR spectra of control due to trapping of hydroxyl radical (HO) by DMPO (1 mM) within 15 minutes.

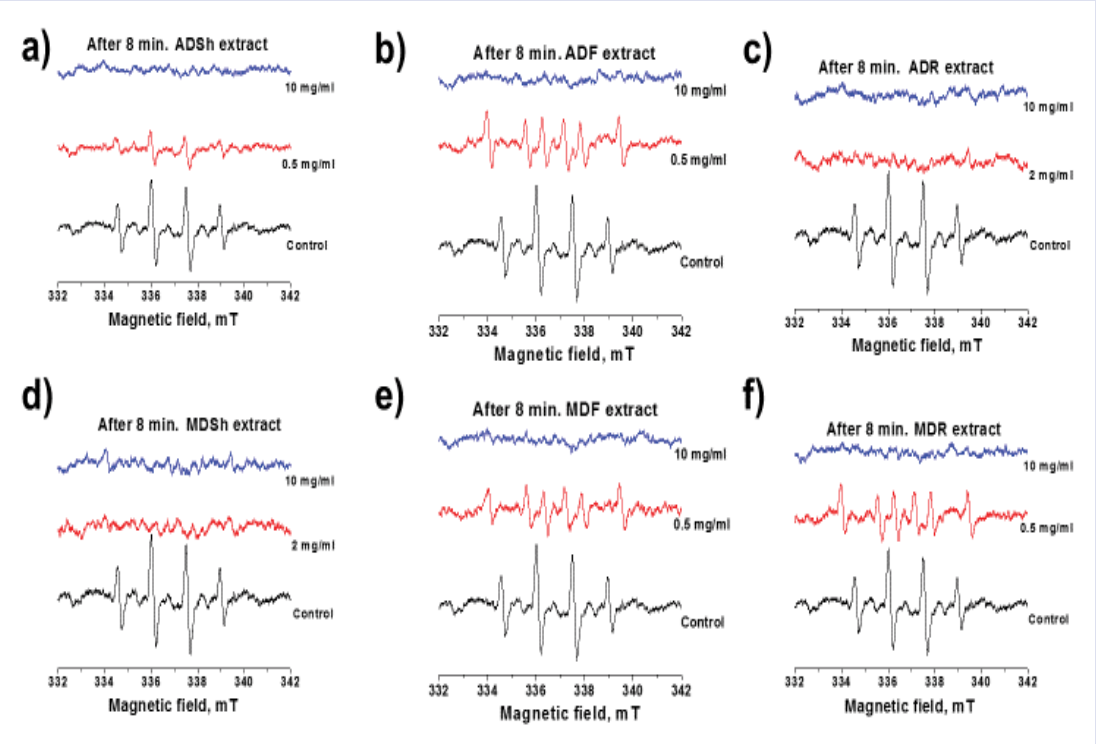

Figure 2: Time evolution of the X-band EPR spectra due to trapping of hydroxyl radical (HO˙) by DMPO (1 mM) of dill extracts at different concentrations: a) ADSh extract, b) ADF extract, c) ADR extract, d) MDSh extract, e) MDF extract, f) MDR extract after 8 minutes. MDR; methanol dill roots, ADSh; aqueous dill shoots and ADF; aqueous dill fruits, ADR; aqueous dill roots, MDF; methanol dill fruits, MDSh; methanol dill shoots.

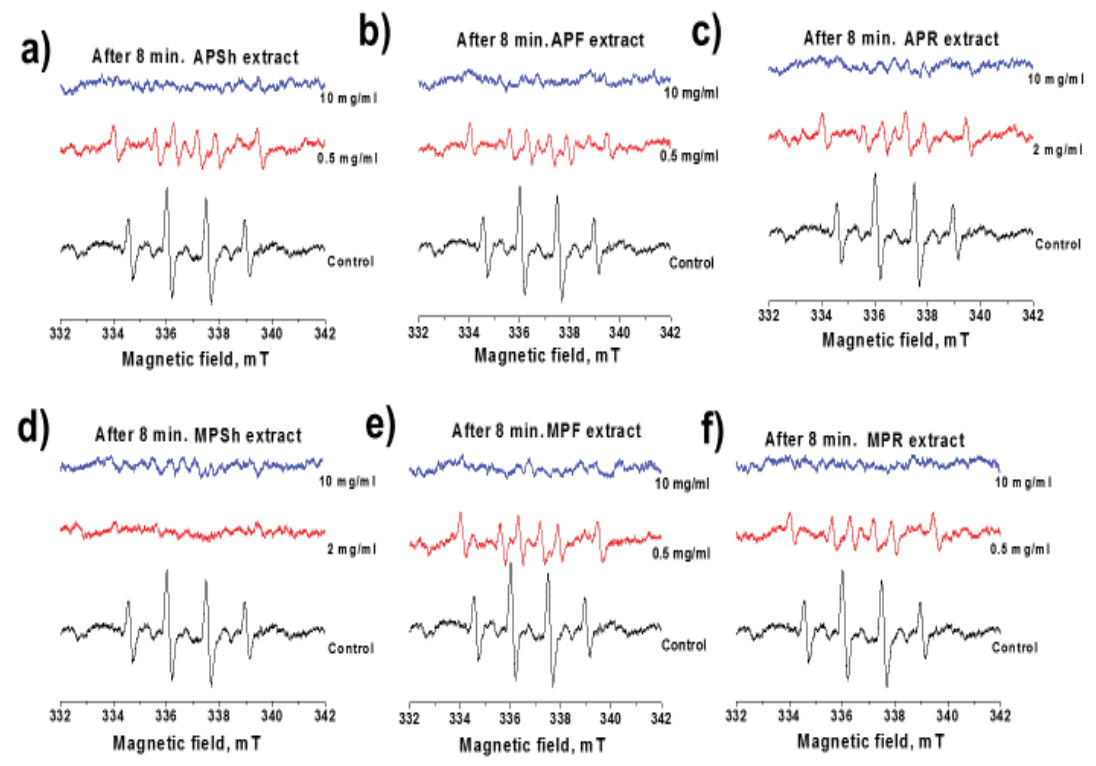

Figure 3: Time evolution of the X-band EPR spectra due to trapping of hydroxyl radical (HO˙) by DMPO (1 mM) of parsley extracts at different concentrations: a) APSh extract, b) APF extract, c) APR extract, d) MPSh extract, e) MPF extract, f) MPR extract after 8 minutes. MPR; methanol parsley roots, APSh; aqueous parsley shoots and APF; aqueous parsley fruits, APR; aqueous parsley roots, MPF; methanol parsley fruits, MPSh; methanol parsley shoots.

at 0.50 mg/mL concentration, notwithstanding with its inhibition percentage at 10 mL/mg (98.12 %) as shown in Figure 2.

DISCUSSION

Total phenolic and flavonoids contents are found to positively correlated with total antioxidant capacity of apiaceous plants.[17] However, in the current study we found by EPR spectroscopy that ADSh and MPR were to be a venerable hydroxyl radical scavenger even in low concentration, although it's TPC and TFC weren't the highest. Also, the low hydroxyl radical quenching capacity at low concentration attributed to ADF was expected as it possesses the low total phenol content but moderate flavonoid content was observed. On the contrary, the marked antioxidant potential of ADR (>97%) may be partially attributed to its flavonoid content. MPR and MPSh have varying antioxidant potential as it was a remarkable scavenger at 10mg/mL, while at 0.5 mg/ml provokes partial inhibition to hydroxyl radical, although both showed the lowest phenolic and flavonoid contents. APR, ADF, MDF and MPF were perplexing as it inclined to be a pro-oxidant extract at 0.5 mg/mL concentration although it was a potent free radical quencher at 10 mg/ml concentration although they have degree of variation in their phenolic and flavonoid contents. It was unbelievable that MPF, APR and MDR possess the highest flavonoid content and tends to be pro-oxidant in 0.5-2 mg/ml although it was a potent hydroxyl radical scavenger at 10 mg/ml concentration.

CONCLUSION

In this study, EPR provided a direct insight that all tested apiaceous plants extracts are effective scavengers of hydroxyl radicals at concentration range 0.5-10 mg/ml except ADF and MDR extracts that proved to be pro-oxidant at 0.5 mg/mL concentration range. Our results suggest therefore that both extracts may not provide safe natural antioxidant. Also, the relation between contents of phenolic, flavonoid and their efficacy in limiting DMPO-OH• adduct was also investigated. The knowledge gained from the results confirmed that there is no correlation between antioxidant potential and phenolic and flavonoid contents. Further phytochemical investigation is necessary to highlight the mechanism by which these extracts exhibit antioxidant versus pro-oxidant activities in relation to active constituents (or even phytochemical classes) of bioactive components implicated in that activity. The current results encouraged the authors to investigate the protective biological effect of the most active extract (ADSh) for pivotal organ in further future study. Family apiaceae fruits weren't plant part of choice to be used as antioxidant in low doses.

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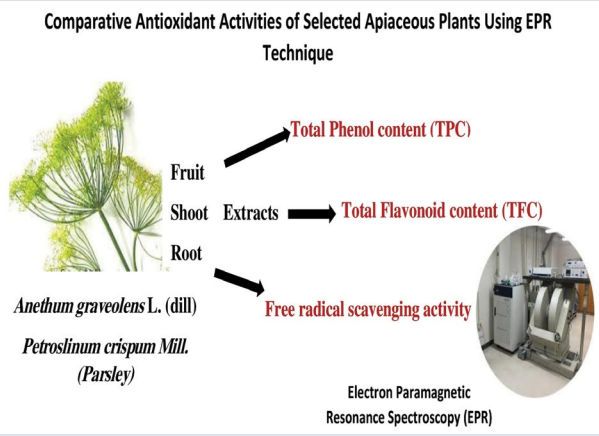
GRAPHICAL ABSTRACT



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