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Comparative Study in Antioxidant Activity and Secondary Metabolites between *In Vitro* and *Ex Vitro* Cultures of *Physalis angulate* L.: An Edible and Medicinal Plant

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Abstract: This study was carried out in order to evaluate the free-radical scavenging activity and determine secondary metabolites in the samples of seedlings growing *in vitro*, as well as in the different parts (stems, leaves, fruits and seeds) of the natural plant *Physalis angulata* L. using GC/MS technique. Methanolic extract of *in vitro* seedlings had a significant free radicals scavenging activity through the method of DPPH free radical compared to fruits extract. The GC/MS analysis showed the presence of 25 compounds in seedlings, 27 in stems, 43 in leaves, 46 in fruits and 25 in seeds. Most of the compounds diagnosed in the seedlings sample were not found in natural plant samples. This indicates the importance of *in vitro* cultures in the production of new compounds that are medically and economically effective.

Keywords: *In vitro* cultures, *Physalis angulata* L., DPPH radical scavenging, Medicinal plants, Phytochemicals

Solanaceae is one of the important plant families including various species with different nutritional and medicinal benefits that distributed throughout temperate, tropical and subtropical regions around the world. *Physalis angulata* L. is one of these plants that belonging to this family from the America, and introduced to Africa, Asia, the Pacific and Europe (Nurtit Silva and Agra 2005, Raju et al 2007, Am and Nidavani 2014). *P. angulata* L. is an annual herbaceous plant that grows wild or planted which has an erect and branching stem up to a height of about 80 cm with green serrated leaves and bearings pale yellow flowers. The fruits are covered with a green-colored balloon calyx which turns pale when fruits ripen. Fruits have a cherry-shape and color ranging from yellow to bright orange (Mejia and Rengifo 2000). This plant is cultivated to take advantage of its nutritional value, as well as its medicinal importance in folk medicine to treat rheumatism, hepatitis, cancer, leukemia, prostate and bladder disorders, diabetes, asthma, malaria and other diseases, due to the containment of this plant on different active secondary metabolites, such as terpenes, alkaloids, phenols and essential oils, most of which act as antioxidants and anti-inflammatory and this is due to their effective role in preventing damage to living cells as a result of exposure to oxidative stress resulted from increased levels of free radicals (Kindscher et al 2012, Zubair et al 2014, Zhang and Tong 2016).

Since the pharmaceutical industry did not rely on natural plant resources as a source of the drug, due to a number of reasons, including limited availability of plants and productivity fluctuations due to different environmental conditions, as well as the destruction of the natural environment of plants and the difficulty of applying technology in agricultural operations (Eba 2005). Hence, and in order to make use of secondary metabolites, scientific research institutions and pharmaceutical laboratories in the world are seeking to use the *in vitro* cultures in manufacture and production of these compounds and convert them into therapeutic drugs than relying on plants directly to extract the various compounds, as well as that productivity is throughout the year and in controlled conditions without being affected by environmental conditions or the season of plant growth (Tiwari et al 2011, Ahmed et al 2013). The earlier studies indicated some plant tissues induced from different explants and continuously cultivated produced some of the medically important secondary metabolites, or by exposing the strains of cells and tissues to some biotic or abiotic elicitors to increase the production of the required compounds or to get new synthesized compounds that are important economically or medically (Shanmuga et al 2015, Hussain et al 2017, Lystvan et al 2018, Mastuti and Rosyidah 2019, Souza et al 2019). However, there have been few studies on the method of comparison between *in vitro* cultures and other samples of native plants tissues in antioxidant activity and their content of secondary metabolites which form a good relationship for the selection of cell lines with high productivity of secondary metabolites. Additionally, there are no studies on this plant in Iraq. Therefore, the present study aimed to investigate the antioxidant activity and secondary metabolites in the *in vitro* and *ex vitro* cultures of *P. angulata* L. plant.

MATERIAL AND METHODS

The experiments were carried out in the plant tissue laboratory- Al-Mussaib Technical College and laboratories of the Environment and Water Research Center, Ministry of Science and Technology.

Collection of plant material and in vitro cultures conditions: Seeds were collected from mature fruits of *P. angulata* L. plants growing in the vegetables field and were well washed with tap water to remove the viscous material from them and then were dried in the air and were used as explants. A group of seeds was planted in a field to obtain growing plants under natural environmental conditions. The other group was taken to the plant tissue culture laboratory where divided into two groups, one soaked in distilled water for 24 hours and the other group was left without soaking and before culturing all the seeds were sterilized with 96% ethanol for 90 seconds, then rinsed with sterile distilled water for 30 seconds. They were cultured in glass vessels containing of 25 ml of half- strength hormone-free basal MS medium (Murashige and Skoog 1962), which prepared by dissolving of 4.43g of powdered MS medium (manufactured by HI Media, India) in 2000 ml of distilled water and enriched with sucrose (5%, w/v) and agar (0.6%, w/v). The pH of the medium was adjusted to 5.7±0.1 with 0.1 N from NaOH or HCl and then autoclaved at 121°C and 1.04 kg cm^{-2} for 15 min. The cultures were incubated in the growth room at 26±3°C and 16h photoperiod. Twelve replicates for each treatment were taken with 10 seeds per replicate. The percentage of seed germination, fresh and dry weights of seedlings were calculated after four weeks of culture.

Estimation of antioxidant activity using DPPH free radical method: To estimate the antioxidant activity of *in vitro* germinating seedlings and fruits (fleshy tissue only), a required weights (300 mg of dried samples) were involved in hot extraction by maceration with ethanol (75%) in distilled water. The antioxidant activity of crude extracts was assessed according to the method of Olugbami et al (2015), with some modification by quantifying the scavenging ability to stable free radical 1,1- Diphenyl-2-picrylhydrazyl (DPPH). The assay was carried out on Shimadzu UV- Visible spectrophotometer at 518 nm using ascorbic acid as positive control. The DPPH radical has a deep violet color due to its unpaired electron and radical scavenging ability can be followed spectrophotometrically by absorbance reduced at 518 nm when the pale yellow non-radical form is produced. Based on this assay, equal volumes (0.5 ml) of DPPH (0.4µm, prepared by dissolving 0.01576 mg in 100 ml of absolute methanol) and 1.0 ml of each concentration (62.5, 125, 250, 500 and 1000 µg ml^{-1}) from crude extracts were mixed and allowed to stand for 30 min. Then, the absorbance was read at 518 nm and converted into percentage radical scavenging activity as follows:

Scavenging activity (%) = A_{518}Control – A_{518}Sample/ A_{518}Control ×100

Where A_{518} Control is the absorbance of DPPH radical +methanol; A_{518} Sample is the absorbance of DPPH radical with an extract or standard. Ascorbic acid was used as a standard reference. This experiment repeated triplicate.

Gas Chromatography-Mass Spectrometer (GC/MS) analysis conditions: The required samples were harvested from *in vitro* germinating seedlings, as well as samples of stems, leaves, fruits and seeds obtained from natural plants that growing in the field. The samples were dried in the air and then crushed into fine powder using mortar and pestle. About 200 mg of dried powder were taken from each sample and mixed with 2 ml of chloroform. The mixture was then sonicated (using Power- Sonic 410) for 6 hours. Samples were then filtered using filter paper (Wattman No. 1). The operation was repeated twice. The filtered samples were collected and dried on a water bath for using in the subsequent experiment. All samples of extracts were analyzed using GC/MS apparatus (Agilent 19091S- 33UI) equipped with National Institute of Standard and Technology (NIST) Library; column HP-5MS capillary column (cross bond 5% diphenyl-95% dimethyl polysiloxane); 30m×250µm with a 0.25µm film thickness; temperature of injection:290ºC; temperature of column:4ºC held to 2 min, rising 4ºC.min^{-1}, then rising to 290ºC and held for 5 min; mode of injection, split: split at ratio 1:20; injected volume: 5µl. Carrier gas was Helium (99.99%); acquisition mass range: 40-600 $m.z^{-1}$. The phytochemicals of the extract were identified by comparing their retention indices with NIST Library.

Statistical analysis: The results of *in vitro* germination of seeds experiment were statistically analyzed using the Duncan's test for significance (SAS 2001).

RESULTS AND DISCUSSION

***In vitro* growth conditions of seedlings:** The germination (98.4%), fresh weight (1053.6 mg) and dry weight (63.2 mg) were significantly higher as compared to non-soaked seeds (74.1%, 873.7 and 32.8 mg, respectively). Earlier studies indicated that seeds of *P. angulata* plant responded well to *in vitro* germination on the medium of MS with a reduced salts strength (Oliveira et al 2014, Souza et al 2019). Vasconcellos et al (2003), observed that the storage of seeds for 10 months at 4°C and cultured them on full strength MS resulted in of 83% germination in white light conditions and 25% in dark conditions.

Evaluation of free-radical scavenging activity: The standard compound, seedlings and fruits extracts had the

DPPH free radical scavenging activity in a way that is directly proportional to the increase in concentration (Table 1). The highest free radical scavenging activity mean (100%) was for ascorbic acid (standard compound), seedlings and fruits extracts at the concentrations of 500 and 1000µg.ml^{-1}. The *in vitro* growing seedlings and fruits extracts contain secondary compounds possess the antioxidant activity and have a significant role in sustaining living cells and tissues from damage by oxidative stress.

There was a significant superiority of the seedlings concentrations compared to the fruits extract with the exception of the concentrations of 500 and 1000 µg.l^{-1} (Table 2). This indicate that the seedlings contains more antioxidant compounds during the germination phase than the fruits. Besides, the organic and inorganic components of culture medium with appropriate conditions of temperature and light which was not only for the germination and growth of seedlings but also as practical elicitors for the biosynthesis of active secondary compounds in seedlings tissues (Khan et al 2018, Ali et al 2019).

Table 1. Effect of different concentrations of ascorbic acid, *in vitro* seedlings and fruits extracts on the percentage of DPPH free radical scavenging activity

Concentration (µg ml^{-1})	Ascorbic acid (%)	*In vitro* seedlings extract (%)	Fruits extract (%)
62.5	90.76^{b}	81.42^{c}	77.14^{d}
125	100^{a}	95.19^{b}	84.38^{c}
250	100^{a}	100^{a}	96.86^{b}
500	100^{a}	100^{a}	100^{a}
1000	100^{a}	100^{a}	100^{a}

Means followed by different letters in the same column are differ significantly at p≤0.05

Table 2. Effect of extract type on DPPH free radical scavenging activity

Extract type	Concentrations (µg ml^{-1})				
	62.5	125	250	500	1000
In vitro seedlings (%)	81.42^{a}	95.19^{a}	100^{a}	100^{a}	100^{a}
Fruits (%)	77.14^{b}	84.38^{b}	96.86^{b}	100^{a}	100^{a}

Means in the same column followed by different letters are differ significantly at p≤0.05

Analysis of Gas Chromatography Mass Spectrometry (GC/MS)

The results of Figure 1 and Table 3, referred to the

Table 3. Secondary metabolites determined in *in vitro* germinating seedlings of *P. angulata* using GC/MS analysis

Seq.	RT*	Compounds	*In vitro* germinating seedlings (%)
1	2.277	1,2-Ethanediol,monoformate	3.49
2	2.343	Acetic acid	2.23
3	4.984	2(3H)-Furanone ,dihydro-	0.88
4	8.578	Cyclohexanone,4-(1,1-dimethypropyl)-	1.12
5	8.973	Oxirane,2,3-dimethyl-	11.33
6	9.095	cis-Terpineol	0.89
7	9.412	Bicyclo[3.1.1]heptane,2,6,6-trimethyl-	1.59
8	9.779	1-Pentanol,2-methyl-	2.65
9	10.369	3-Isopropylbenzaldehyde	0.40
10	11.051	Estragole	2.60
11	12.471	Dodecane,1-chloro-	0.90
12	14.090	trans-.alpha.-Bergamotene	2.98
13	14.836	alpha.-D-Glucose	0.82
14	15.127	3-Tetradecane,(Z)-	1.04
15	15.315	Benzene,(1-methoxylnonyl)-	1.36
16	16.163	2,6-Nonadien-1-ol	0.49
17	16.552	Tridecane,2-phenyl-	2.05
18	17.210	Cyclohexane carboxylic acid, octyl ester	1.77
19	17.492	E-11,13-Tetradecadien-1-ol	0.92
20	18.298	Octane,1-fluoro-	0.67
21	19.022	Cholesta-8,24-dien-3-ol,4-methyl-,(3.beta.,4.alpha.)	0.60
22	19.428	Nonadecane, 1-chloro-	25.01
23	20.874	1,14-Tetradecanediol	0.60
24	21.280	Oleic acid	25.01
25	21.382	Octadecanoic acid	5.94

*RT: Retention time (min)

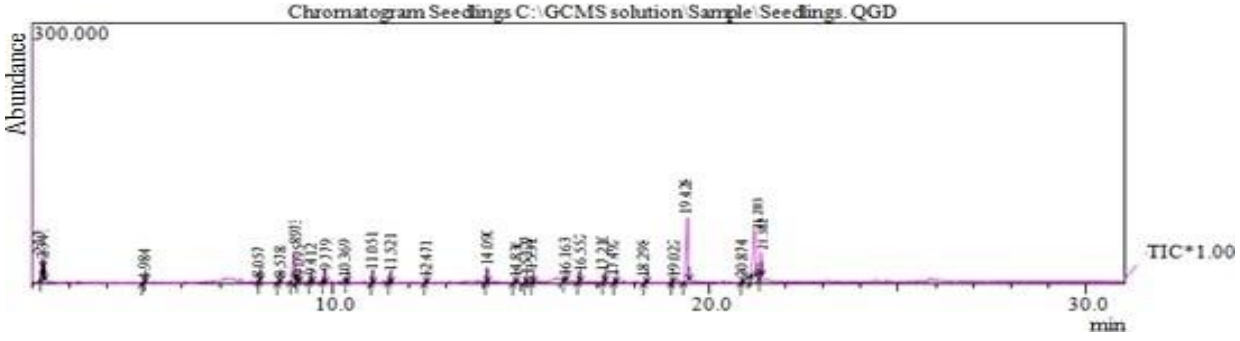

Fig. 1. GC/MS Chromatogram of *P. angulata in vitro* germinating seedlings

presence of 25 compounds resulted from GC/MS analysis of chloroform extract of *P. angulata* seedlings growing *in vitro*, that differed in their quality, retention time and relative content. Most of these compounds with therapeutics and nutritional benefits.

The results of GC/MS analysis of chloroform extract of natural plant samples of *P. angulata* are shown in, revealed the presence of 141 compounds where fruits gave the highest number of compounds (46 compounds) followed by leaves (43 compounds) and seeds (27 compounds), while the samples of the stems gave 25 compounds where these compounds differed among them in the retention times and their relative content. Most of these diagnostic compounds possess pharmaceutical and nutritional merits and this may explain the medicinal and economic importance of this plant (Fig. 2 and Table 4). The 8 compounds were diagnosed in all

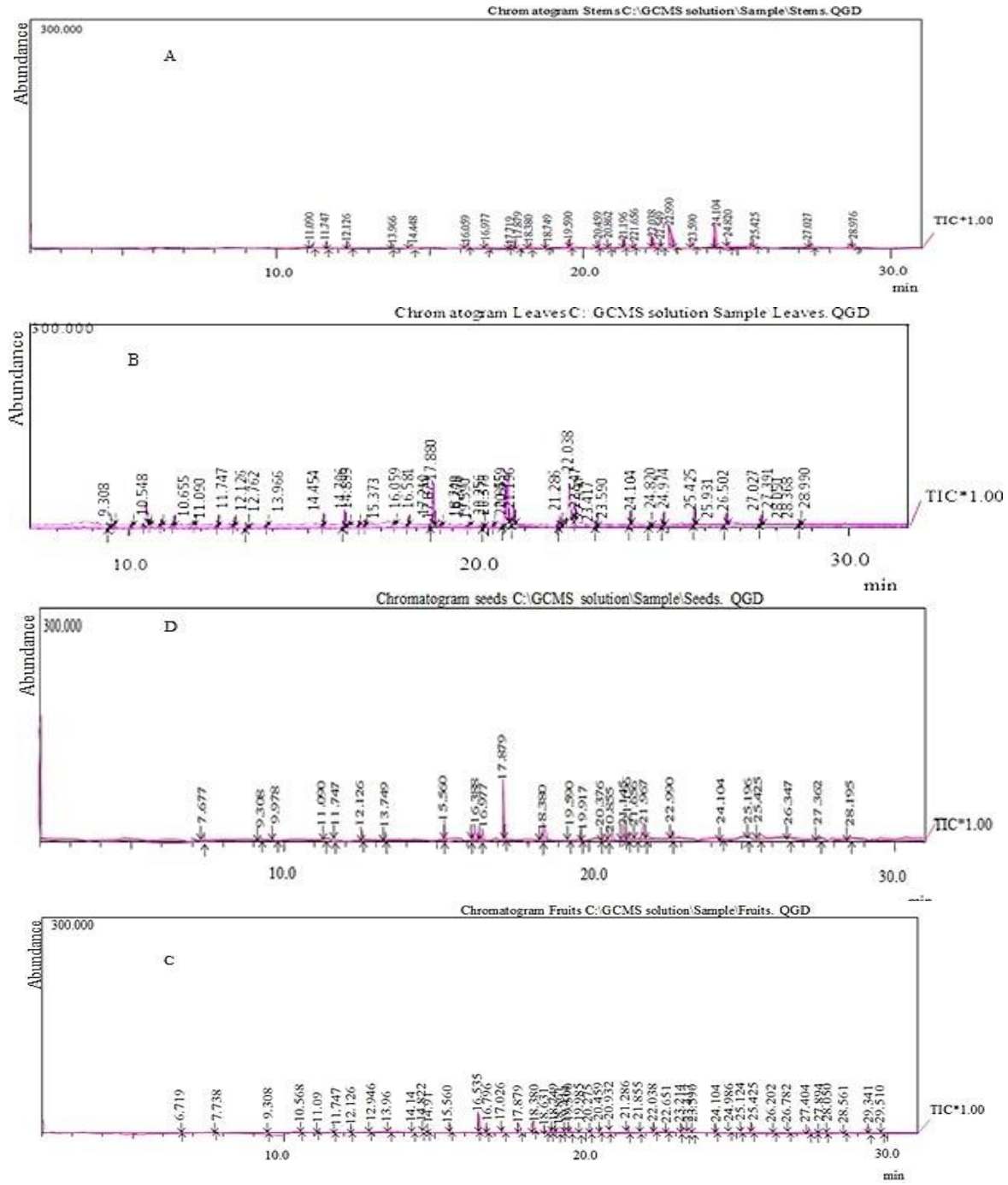

Fig. 2. GC/MS Chromatogram of *P. angulata* natural plant samples: (A) Stems, (B) Leaves, (C) Fruits and (D) Seeds

Table 4. Secondary metabolites determined in natural plant organs using GC/MS analysis

Seq.	RT*	Compounds	Stems (%)	Leaves (%)	Fruits (%)	Seeds (%)
1	6.719	1-Fluorononane	-	-	0.19	-
2	7.677	Trichloromethane	-	-	-	0.04
3	7.738	Methane,bromodichloro-	-	-	0.06	-
4	9.308	1-Tetradecene	-	0.31	0.38	0.07
5	9.978	1H-Cyclopropa[a]naphthalene,1a,2,3,5,6,7, 7a,7b-octahydro-1,1,7,7a-tetramethyl,[1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]-	-	-	-	0.15
6	10.548	Ethane,1,1,2,2-tetrachloro-	-	0.28	-	-
7	10.568	10-Methylnonadecane	-	-	0.08	-
8	10.655	Trichloromethane	-	0.09	-	-
9	11.090	Phenol,2,4-bis(1,1-dimethylethyl)	0.73	0.40	0.48	0.49
10	11.747	Cetene	0.75	0.54	0.75	0.63
11	12.126	Dodecanoic acid	1.23	1.42	1.86	1.14
12	12.762	2-Tridecenal,(E)-	-	0.41	-	-
13	12.946	Oxalic acid, 3,5-difluorophenyl tetradecyl ester	-	-	0.11	-
14	13.749	Octadec-9-enoic acid	-	-	-	1.81
15	13.966	E-15-Heptadecenal	0.77	0.46	0.40	-
16	14.145	Tetradecanoic acid	-	-	0.73	-
17	14.448	11,13-Dimethyl-12-tetradecen-1-ol	0.37	-	-	-
18	14.454	Bicyclo[3.1.1]heptane,2,6,6-trimethyl-	-	2.09	-	-
19	14.706	9,12-Octadecadienoic acid(Z,Z)-	-	0.57	-	-
20	14.822	Heptafluorobutyric acid, pentadecyl ester	-	-	0.28	-
21	14.899	Hexadecanal	-	1.04	-	-
22	14.918	Phthalic acid,2,7-dimethyloct-7-en-5-yn-4-yl isobutyl ester	-	-	0.10	-
23	15.373	(2E)-1-Methoxy-3,7-dimethlocta-2,6-diene	-	0.68	-	-
24	15.560	cis-Vaccinic acid	-	-	2.21	6.49
25	16.059	n-Hexadecanoic acid	4.23	3.70	-	-
26	16.388	6-Octadecenoic acid	-	-	-	5.69
27	16.535	n-Hexadecanoic acid		-	11.74	-
28	16.581	Cyclopentadecanone,2-hydroxy-	-	1.83	-	-
29	16.796	Ether,dodecyl isopropyl	-	-	2.81	-
30	16.977	trans-13-Octadecanoic acid	0.26	-	-	4.92
31	17.026	2-Bromo dodecane	-	-	2.64	-
32	17.210	Oxirane,tridecyl-	-	1.36	-	-
33	17.719	Octadecane,2,6,10,14-tetramethyl	4.52	-		-
34	17.879	Oleic acid	5.40	2.91	6.43	38.67
35	17.880	Pentadecane,8-heptyl-	-	4.97	-	-
36	18.380	9-Octadecenoic acid,(E)-	1.02	1.98	10.18	3.82
37	18.631	9,17-Octadecadienal, (Z)-	-	-	1.49	-
38	18.749	Nonadecane,9-methyl-	1.89	2.50	2.52	-
39	18.861	Hentriacontane	-	-	2.22	-
40	19.376	trans-13-Octadecenoic acid	-	1.10	-	-
41	19.414	cis-11,14-Eicosadienoic acid, methyl ester	-	-	1.00	-
42	19.590	Tetracosane	6.00	5.61	3.64	1.94

Cont...

Table 4. Cont....

Seq.	RT*	Compounds	Stems (%)	Leaves (%)	Fruits (%)	Seeds (%)
43	19.917	Cyclopropaneoctanal,2-octyl-	-	-	-	2.65
44	19.985	(R)-(-)-14-Methyl-8-hexadecyn-1-ol	-	-	0.94	-
45	20.256	cis-13-Octadecenoic acid	-	1.53	-	-
46	20.275	Z,E-3,13-octadecadien-1-ol	-	-	1.05	-
47	20.376	Nonadecane,1-chloro-	-	1.53	-	1.24
48	20.459	Pentacosane	5.78	5.22	4.54	-
49	20.855	Octadecane,1-chloro-	-	2.84	-	1.74
50	20.862	Oleyl alcohol,heptafluorobutyrate	0.23	-	-	-
51	20.932	Octacosanol	-	-	0.96	-
52	21.145	Pentacosane, 13-undecyl-	-	-	-	6.83
53	21.196	Octadecane	8.19	-	-	0.83
54	21.286	Heptadecane,9-octyl-	-	7.78	5.80	-
55	21.656	1-Eicosane	0.30	1.47	-	1.03
56	21.855	9-Tricosene, (Z)-	-	-	1.41	-
57	21.967	Hexadecane, 1-iodo-	-	-	-	6.75
58	22.038	Heptacosane	8.87	8.63	6.80	-
58	22.547	Pyridine-3-carboxamide,oxime,N-(2-trifluoromethylphenyl)-	-	1.41	-	-
59	22.549	Disulfide,di-tert-dodecyl	1.01	-	-	-
60	22.651	Octacosane	-	-	0.96	-
61	22.990	Hexacosane	15.46	7.84	-	2.47
62	23.214	Docosane,11-butyl-	-	-	5.96	-
63	23.417	Squalene	-	1.55	0.85	-
64	23.590	Eicosane	4.56	1.10	0.60	-
65	24.104	Nonacosane	15.08	7.56	5.79	1.86
66	24.820	1-Nonadecene	0.51	1.00	-	-
67	24.974	13-Methyl-Z-14-nonacosane	-	0.97	-	-
68	24.986	i-Propyl,11-octadecenoate	-	-	0.93	-
69	25.124	4,8-Dimethylheptacosane	-	-	0.34	-
70	25.196	2,3-Dihydroxypropylelaidate	-	-	-	2.71
71	25.425	Triacontane	5.84	5.31	4.12	1.37
72	25.931	3-Eicosene,(E)-	-	1.42	-	-
73	26.202	Cyclohexane,1-(1,5-dimethylhexyl)-4-(4-methylpentyl)-	-	-	0.58	-
74	26.347	beta-Tochopherol	-	-	-	0.32
75	26.502	Cyclotetracosane	-	1.54	-	-
76	26.782	Cyclohexane,1-(1-tetradecylpentadecyl)-	-	-	1.19	-
77	27.027	Heneicosane	4.47	3.58	-	-
78	27.362	Stigmastan-3,5-diene	-	-	-	0.64
79	27.391	Bicyclo[10.8.0]eicosane,(E)-	-	0.67	-	-
80	27.404	Octane,2-cyclohexyl-	-	-	3.63	-
81	27.894	2-Piperidinone,N-[4-bromo-n-butyl]-	-	-	0.23	-
82	28.050	Vitamin E	-	0.92	0.75	-
83	28.195	Pyridine-3-carboxamide,oxime,N-(2-trifluoromethylphenyl)-	-	-	-	0.16
84	28.368	Tetratriacontane	-	0.35	-	-
85	28.561	Cyclotriacontane	-	-	0.21	-
86	28.976	Hexatriacontane	2.54	-	-	-
87	28.990	Dotriacontane	-	1.81	-	-
88	29.341	Heneicosane	-	-	1.75	-
89	29.510	Undecane, 2-cyclohexyl-	-	-	0.09	-

*RT: Retention time (min)

four samples with a difference in their relative content, these are Phenol,2,4-bis(1,1- dimethylethyl), Cetene, Dodecanoic acid, Oleic acid, Tetracosane, Nonacosane, 9- Octadecenoic acid,(E)- and Triacontane.

This is the first study about *P. angulata* plant in Iraq. The presence of many effective compounds, which have been diagnosed quality and quantity using the analysis of GC/MS was recorded. These compounds have many antioxidant and disease-fighting activities as well as the nutritional value of some of them. Most of the compounds diagnosed in *in vitro* seedlings were not present in natural plant samples, and this indicates the importance of *in vitro* cultures in the production of new secondary compounds as well as the possibility of increasing these compounds in these cultures using biotic and abiotic elicitors. This is useful for pharmaceutical and food companies in increasing the production and manufacture of natural medicinal drugs or increase the production of food additives and flavorings desired. Additionally, productivity is carried out throughout the year without adherence to the plant growth season with the possibility of using genetic engineering and biotechnology methods to produce highly productive cell lines for desired compounds (Hussain et al 2017, Lystvan et al 2018, Mastuti and Rosyidah 2019, Souza et al 2019).

CONCLUSION

This study has described the establishment of *in vitro* seedlings culture from *P. angulata* seeds as well as the seedlings extract has significantly proved to be effective in antioxidant activity through scavenging of free radicals compared with fruits extract. The GC/MS analysis confirmed the presence of several secondary compounds with medically and economically important in all samples of *in vitro* seedlings and natural plant (stems, leaves, fruits and seeds). This enhances the medical and economic importance of this plant. Additionally, some compounds were present in the seedlings samples only without their presence in the samples of natural plant. Accordingly, *in vitro* cultures can be used to produce secondary compounds important and effective at the medical and economic levels. Future studies on tissue cultures of this plant can be carried out where seedlings parts can be used to induce callus and cellular suspensions and expose them to various elicitors in order to produce and isolate active compounds individually and activate them commercially.

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