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Determination of secondary metabolites in callus and different tissues of *Physalis angulate* L.

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ABSTRACT

Physalis angulata L. is an important herbal plant used in the food industry or in folk medicine in most countries around the world. The present study was carried out in order to determine the secondary metabolites in the samples of callus and seedlings growing *in vitro* of this plant using GC/MS technique. Results showed that the MS medium supplemented with the concentration of 9.04µM 2,4-D is the best in the growth of callus by giving the highest biomass weight (1241 mg) of callus induced from seeds. The GC/MS analysis of chloroform extract of callus and seedlings growing *in vitro* revealed the presence of 25 compounds in callus, 27 in seedlings and 18 in roots. Most of the compounds diagnosed in the callus and seedlings samples with pharmaceutical and economic benefits. This indicates the importance of *in vitro* cultures in the production of new compounds that are medically and economically effective.

Key words : *In vitro* cultures, medicinal plants , *Physalis angulate*, secondary metabolites

INTRODUCTION

The genus *Physalis* belongs to the Solanaceae family, comprising 120 species distributed throughout temperate, tropical and subtropical regions around the world. Its origins are from the Americas, from which it has introduced to Africa, Asia, Pacific and Europe (Nurtit Silva and Agra, 2005; Raju *et al.*, 2007; Am and Nidavani, 2014; Maiti and Singh,2019). *Physalis angulata* L.is one of the most important species of this genus. It 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-coloured balloon calyx which turns pale when fruits ripen. Fruits have a cherry-shape and colour ranging from yellow to bright orange with sweet and delicious test containing many seeds inside, and they are eaten fresh or used in the manufacture of juices and jams, or added to the salads, sweets and ice cream (Mejia and Rengifo, 2000). The plant is known by several names around the world, including the wild tomatillo in Mexico or camapu in Brazil and winter cherry or harankash in Arabic regions. The 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 anti-oxidants and anti-inflammatory (Zubair *et al.*, 2014; Zhang and Tong,2016).

Since the pharmaceutical industry does 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 plant tissue culture techniques in the manufacture and production of these compounds and convert them into therapeutic drugs. The callus cultures and cellular suspensions are important techniques that have created a new

source and substitute for the production of secondary metabolites and more efficient 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).

It has been noted through previous studies that 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; Lystvan *et al.*, 2018; Mastuti and Rosyidah, 2019, Souza *et al.*, 2019). However, there have been few studies on the method of comparison between secondary metabolites produced from seedlings and callus tissues induced from different explants. Additionally, there are no studies on *P. angulata* plant in Iraq. Therefore, the present study aimed at investigate secondary metabolites in the callus induced from seeds, and *in vitro* growing seedlings with their roots.

MATERIALS AND METHODS

Collection of Plant Material and Initiation of *In vitro* Cultures

Seeds were collected from mature fruits of the *P. angulata* L. plants growing in the vegetables field and washed with tap water to remove the viscous material from them and dried in the air where they were used as explants. In the tissue culture laboratory, seeds were sterilized with 96% ethanol for 90 sec, then rinsed with sterile distilled water for 30 seconds and cultured on the basal MS medium (Murashige and Skoog, 1962), which prepared by dissolving of 4.43 g of powdered MS medium (manufactured by HI Media, India) in 1000 ml of distilled water and enriched with sucrose (3%, w/v) and agar (0.6%, w/v). Different concentrations of 2,4-D (0.0, 4.52, 9.04. 13.57 or 18.09 µM) were added also. The pH of the medium was adjusted to 5.7 ± 0.1 with 0.1 N from NaOH or HCl. All media were 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 callus induction and fresh biomass of callus were calculated after eight weeks of culture. *In vitro* seedlings were obtained by cultured seeds on 1/2 strength hormone-free MS medium.

Extraction of Secondary Metabolites from Different Plant Tissues

The required samples were collected from air dried callus and *in vitro* growing seedlings and crushed into fine powder. 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 6h. Samples were then filtered using filter paper (Wattman No. 1) and operation was repeated twice. The filter samples were collected and dried on a water bath for using in the subsequent experiment.

Gas Chromatography-Mass Spectrometer (GC/MS) Analysis Conditions

All samples extracts of *P. angulata* 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:290 °C; temperature of column:4°C held to 2 min, rising 4°C.min^{-1}, then rising to 290 °C 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 callus experiment were statistically analyzed according to the Completely Randomized Design (CRD) and data were assessed using ANOVA analysis. The means were compared using the Duncan's test for significance at the probability level of P ≤ 0.05 (SAS, 2001).

RESULTS AND DISCUSSION

Callus Induction and Biomass

The results obtained in Table 1 showed the difference in seed susceptibility (explants) in the formation of callus by different concentrations of 2,4-D in the culture medium, where no callogenesis was induced in the auxin-free MS medium (control), while the best response(100%) for callus induction was at concentrations 9.04 and 13.57µM of 2,4-D (Fig. 1). As for the biomass callus (Table 1), the results showed that MS medium supplemented with9.04µM 2,4-D was significantly stimulated the production of the highest biomass rate which was 1241 mg as compared with other concentrations of auxin. Also, the morphological features of callus were different among auxin concentrations, i.e., friable creamy, friable creamy to greenish and friable brownish. Previous studies have indicated the strong efficacy of 2,4-D in stimulating the production of highest proliferation of callus from different explants such as ovaries of *Linum usitatissimum* (Burbulis *et al.*, 2007); seeds of *Boehaavia paniculata* (Souza *et al.*, 2014) and internodes, leaves and petioles of *Terminalia arjuna* (Salim, 2018).Lashin and Elhaw (2016) referred in their study to the possibility of callus induction from seeds, stems and leaves of the plant *Physalis peruviana* in the presence of a combination of growth regulators BAP and NAA. Accordingly, we have adopted the concentration 9.04µM of 2,4-D in the production of callus to obtain the required quantity for subsequent experiments.

Analysis of Gas Chromatography Mass Spectrometry (GC/MS)

Results of GC/MS analysis of chloroform extract of *in vitro* cultures of *P. angulata* are shown in Table 2 and Figs. 2 and 3 revealed the presence of 25(35%)compounds in callus, 27 (38%) in seedlings and 18 (27%) in roots, where these compounds differed among them in the retention times and their relative content. Most of these diagnostic compounds possess pharmaceutical and

Table 1. Effect of 2,4-D concentrations on percentage of induction(%), fresh biomass(mg) and morphological features of callus induced from seeds of *Physalis angulata* L. after eight weeks of culture.

2,4-D (µM)	Percentage of callus induction (%)	Callus fresh biomass (mg)	Morphological features
0.0	0.0[c]	0.0[c]	-
4.52	83[ab]	973[b]	Friable creamy
9.04	100[a]	1241[a]	Friable and creamy to greenish
13.57	100[a]	1062[b]	Friable and creamy to greenish
18.09	75[b]	918[b]	Friable brownish

Figures in a column followed by different superscript are significantly different at P≤0.05.

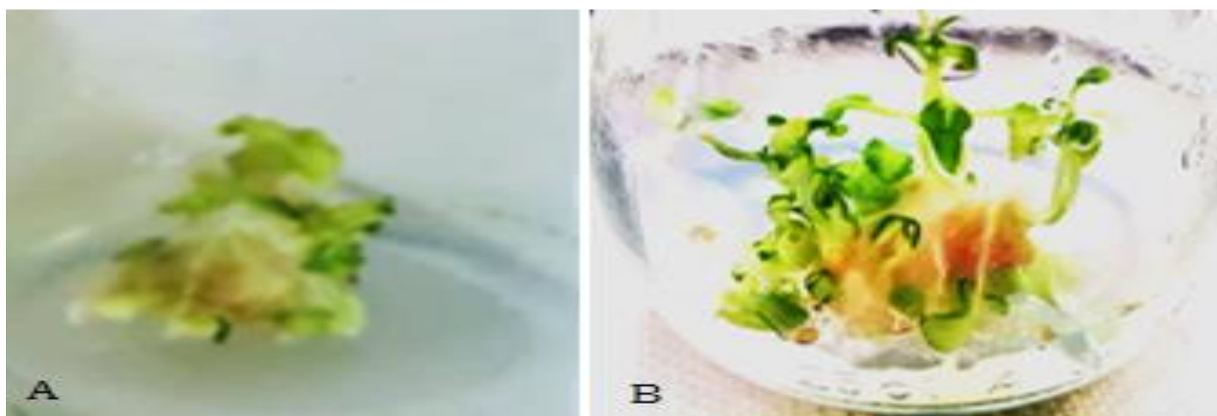

Fig 1. In vitro cultures of P. angulata : A. Callus and B. Seedlings.

Table 2. Secondary metabolites determined in callus, seedlings and root *in vitro* using GC/MS analysis

Seq.	RT	Compounds	Callus (%)	Seedlings (%)	Roots (%)
1	2.028	Heptane,3,4-dimethyl-	-	-	13.93
2	2.037	1,2,5-Oxadiazole	10.40	-	-
3	2.059	Nonane,2,6-dimethyl-	-	-	17.84
4	2.066	Cyclopentane,1,2-dimethyl-	16.02	-	-
5	2.106	cis-1,3-Dimethylcyclopentane	-	-	13.67
6	2.113	Pentane,2,3-dimethyl	26.73	-	-
7	2.184	Dipropylmethane	-	-	16.06
8	2.277	1,2-Ethanediol,monoformate	-	3.49	-
9	2.327	Cyclohexane, methyl-	3.30	-	-
10	2.343	Acetic acid	-	2.23	-
11	2.360	Hydroxylamine, O-(2-methylpropyl)-	-	-	16.06
12	3.092	Propanoic acid, 2-hydroxy-,ethyl ester	0.40	-	-
13	3.914	4-Penten-1-ol,2-methyl-	-	-	0.59
14	4.984	2(3H)-Furanone ,dihydro-	-	0.88	-
15	6.286	Oxalic acid, isobutyl pentyl ester	0.25	-	-
16	6.292	Hexane,2,4-dimethyl-	-	-	0.23
17	7.867	2-Norbornanone,1,3,3-trimethyl-	0.34	-	-
18	8.165	2,5-Octadiene	1.73	-	-
19	8.578	Cyclohexanone,4-(1,1-dimethypropyl)-	-	1.12	-
20	8.973	Oxirane,2,3-dimethyl-	-	11.33	-
21	9.095	cis-Terpineol	-	0.89	-
22	9.316	2-Oxo-n-valeric acid	0.38	-	-
23	9.412	Bicyclo[3.1.1]heptane,2,6,6-trimethyl-	-	1.59	-
24	9.779	1-Pentanol,2-methyl-	-	2.65	-
25	10.369	3-Isopropylbenzaldehyde	-	0.40	-
26	11.051	Estragole	-	2.60	-
27	11.444	2,4-Decadienal	0.38	-	-
28	12.471	Dodecane,1-chloro-	-	0.90	-
29	14.090	trans-.alpha.-Bergamotene	-	2.98	-
30	14.824	n-Hexadecanoic acid	0.34	-	-
31	14.836	.alpha.-D-Glucose	-	0.82	
32	15.127	3-Tetradecane,(Z)-	-	1.04	-
33	15.315	Benzene,(1-methoxylnonyl)-	-	1.36	-
34	16.163	2,6-Nonadien-1-ol	-	0.49	-
35	16.552	Tridecane,2-phenyl-	-	2.05	-
36	17.210	Cyclohexane carboxylic acid, octyl ester	-	1.77	-
37	17.225	Pentanoic acid,2-hydroxy-,methyl ester	3.03	-	-
38	17.492	E-11,13-Tetradecadien-1-ol	-	0.92	-
39	18.298	Octane,1-fluoro-	-	0.67	-
40	19.022	Cholesta-8,24-dien-3-ol,4- methyl,(3.beta.,4.alpha.)	-	0.60	-
41	19.404	Decanoic acid	9.14	-	-
42	19.428	Nonadecane, 1-chloro-	-	25.01	-
43	19.507	Pentadecanoic acid	-	-	6.31
44	19.635	Docosanoic acid. ethyl ester	-	-	0.33
45	20.632	Octadecanoic acid,2(2-hydroxy) ethyl-	0.46	-	-
46	20.874	1,14-Tetradecanediol	-	0.60	-
47	21.181	5-Methyl-1-heptanol	11.55	-	-
48	21.280	Oleic acid	3.53	25.01	6.08
49	21.382	Octadecanoic acid	-	5.94	-
50	21.442	Octadecanoic acid,2-(2-hydroxyethoxy)ethyl ester	-	-	1.22
51	22.506	Eicosanoic acid	1.78	-	-
52	22.543	Eicosane,2-methyl-	-	-	0.48
53	23.400	Heptafluorobutyric acid, n-pentadecyl ester	0.87	-	-
54	23.414	Heptadecane,2,6,10,14-tetramethyl-	-	-	0.45
55	24.234	1-Iodo-2-methylundecane	1.60	-	3.70
56	24.267	Docosane,7-butyl-	-	-	-
57	24.707	Undecane,2,10-dimethyl-	0.50	-	-
58	25.034	Phthalic acid. ditridecyl ester	1.59	-	-
59	25.052	Heptadecane.9-octyl-	-	-	0.84
60	25.808	Benzenedicarboxylic acid, ditridecyl ester	2.08	-	-
61	25.839	Tetradecane	-	-	2.82
62	26.583	Tridecane	1.65	-	-
63	26.600	Tridecane,2-methyl-	-	-	0.85
64	27.435	Tricosane,2-methyl-	1.37	-	-
65	27.459	Pentadecane	-	-	1.05
66	28.394	1-Decanol,2-methyl-	0.57	-	-

RT: Retention Time (min).

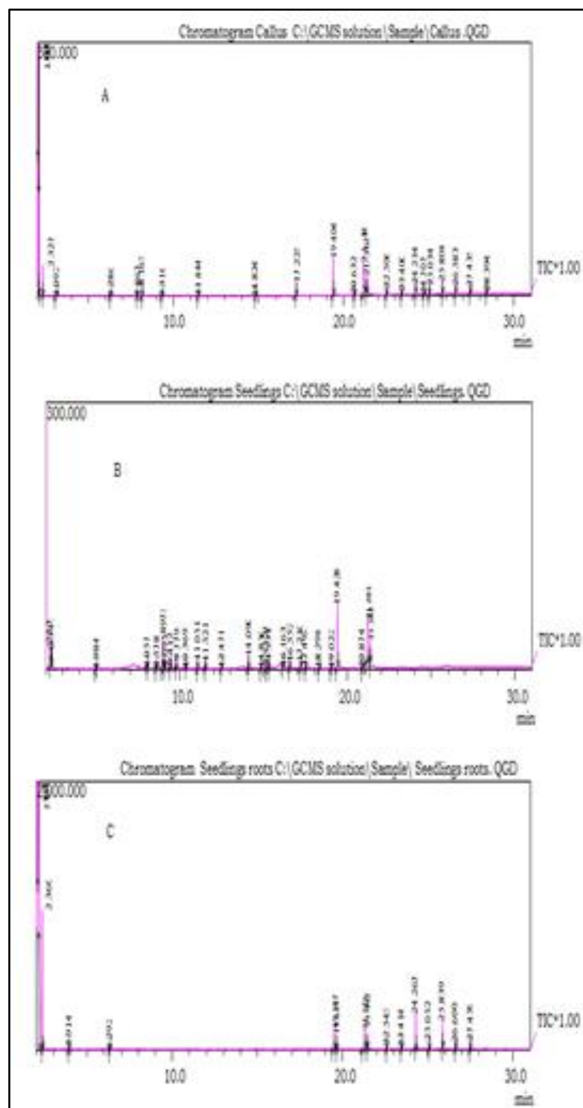

Fig. 2. GC/MS Chromatogram of *P. angulata* in vitro cultures : A. Callus, B. Seedlings and C. Roots.

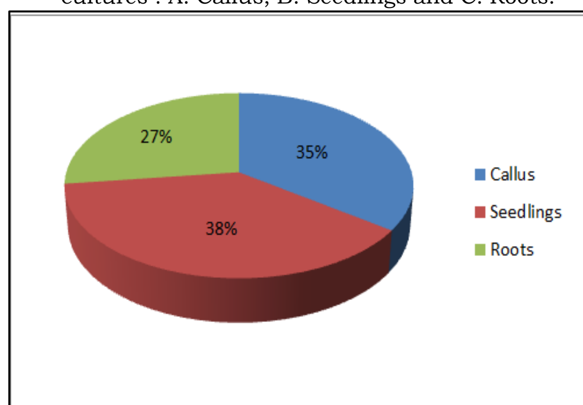

Fig. 3. Percentages of secondary metabolites in different parts of *P. angulata* analyzed by GC/MS.

nutritional merits and this may explain the medicinal and economic importance of this plant. It was noticed that only Oleic acid found in all samples.

This is the first study about *P. angulata* plant in Iraq. We have recorded the presence of various effective compounds, which have been diagnosed quality and quantity using the analysis of GC/MS. These compounds have many antioxidant and disease-fighting activities as well as the nutritional value of some of them. This makes the tissue culture technology an important and promising source for the production of many secondary compounds of pharmaceutical and nutritional importance, 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 supplements 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 (Lystvan *et al.*, 2018; Mastuti and Rosyidah, 2019, Souza *et al.*, 2019).

CONCLUSION

This study has described the establishment of callus culture from *P. angulata* seeds and the adopted of the concentration 9.04 µM of 2,4-D in the callus production, as well as the growing of seedlings *in vitro*. The GC/MS analysis confirmed the presence of several secondary compounds with medically and economically important in all samples of *in vitro* cultures. This enhances the medical and economic importance of this 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 in order to isolate and produce active compounds individually and activate them commercially.

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