Egypt. J. Chem. **Vol. 65**, No. 12, pp. 327 – 335 (2022)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

Induced Biosynthesis of Acephenanthrylene in Callus Culture of Pimpinella anisum L., by Yeast and Phenylalanine Application

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Abstract

Anise (*Pimpinella anisum* L.), is a traditional famed plant belonging to Apiaceae family with eminent therapeutic potentials throughout the world. In this study, the effects of an elicitor such as yeast extract (YE), and a precursor such as phenylalanine on the growth and accumulation of bioactive compounds in Anise shoot tip cultures were identified. Shoot tip callus growing well on Murashige and Skoog (MS) medium supplemented with 1mg/l indole acetic acid (IAA) and 1mg/l 2,4-dichlorophenoxy acetic acid (2.4-D) as auxins in addition to 2mg/l of kinetin (kin). Fresh weight and increase values were progressively increased and reached the maximum (26.9 g and 52.8; 13.7 g and 26.5 respectively) at 5.46 mM/l yeast and 2mM phenylalanine concentrations compared to control treatment (12.9 g, and 24.7). Total volatile constituents content were enhanced to 43.51% at 6 mM/l phenylalanine and 45.45% at 1.82 mM/l yeast correspondingly, compared to control treatment (16.83%). Gas chromatography-mass spectrometry (GC-MS) analysis of cultures calli of *Pimpinella anisum* showed the presence of 20 different compound which account for the 99.9% of the total amount. 1,2-Diphenyl-5-(t-butyl) acephenanthrylene was scrutinized in all treatments. The results obtained may support the use of acephenanthrylene in traditional medicine for the treatment of various diseases and in antitumor drug developments.

Keywords: Pimpinella anisum; Callus Culture; GC-MS; Acephenanthrylene; Elicitation.

Introduction

Anise (*Pimpinella anisum* L.) is an ancient Egyptian annual herb from Apiaceae family, with white flowers and small green to yellow seeds. Anise was first cultivated in Egypt and the Middle East, and was brought to Europe for its therapeutic effect [1]. *P. anisum* is thought as a natural plant and used for pharmaceutics, perfume, cooking and makeup manufacturing [2,3]. Nowadays, a wide range of biological effects as anti-microbial, anti-fungal and insecticidal are deeply considered [4-8]. The essential oil is characterized by a lot of curing outcomes as approved in many drug industries [9].

Biotechnological approaches, specifically, plant tissue cultures were regarded as a potential alternative for production of desirable bioactive plant metabolites [10]. Many studies were conducted to evaluate the *in vitro* production of medicinal plants [11]. In medicinal plants, the majority of the bioactive compounds were belonging to secondary metabolites. As the increment of plant secondary metabolites is due to different stresses, so, biotic and abiotic elicitors can stimulate their concentration. Thus, plant *in vitro* cultures elicitation, is an effective approach for production of secondary metabolite [12, 13].

As the intact plant and the undifferentiated callus cells have identical genetic setting, the active metabolites could be similar in both. At this point, our callus cultures were established and studying the effect of yeast and phenylalanine to improve the production of anise important constituents using callus cultures.

The precursor of the phenyl propanoide pathway (Phenylalanine) is an amino acid that playing an important role in flavonoids and phenolic compounds formation. Application of phenylalanine has been effectively used to boost the plant metabolite production in diverse *in vitro* cultures [14].

Several studies had described different extraction methods of *Pimpinella anisum* such as steam distillation [15-17], Hydro distillation [18,19] and Supercritical fluid extraction using carbon dioxide [20,21]. Moreover, solvent extraction with ethanol, methanol and water [4,6], petroleum ether,

*Corresponding author e-mail: usamajp@yahoo.com
Receive Date: 27 February 2022, Revise Date: 16 March 2022, Accept Date: 24 March 2022
DOI: 10.21608/EJCHEM.2022.124157.5541
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chloroform, ethyl acetate, acetone and hexane [22-25] were also reviewed.

As one of the biotic elicitors, scientists are using yeast extract for long time. It was revealed that, ethylene biosynthesis was elevated by yeast extracts in tomato [26]; bacterial resistance was stimulated in bean (*Phaseolus vulgaris*) [27] and also tanshinone production in the root culture of *Perovskia abrotanoides* [28].

GC-MS studies have been progressively applied for the examination of plant active products as volatile oils, alkaloids, flavonoids, fatty acids and lipids [29].

In this paper, the volatile constituents from shoot tip calli cultures of *Pimpinella anisum* have been extracted and the active components have been identified by GC-MS. This report planed to appraise the effects of various yeast and phenylalanine concentrations on cell growth and the production of acephenanthrylene from callus cultures of *Pimpinella anisum* L.

Materials and Methods

Plant material and tissue culture experiment

To provide the necessary plant materials for callus induction, seeds of anise were obtained from the Agricultural Research Center, Ministry of Agriculture and Land Reclamation, Egypt. The seeds were surface sterilized with 5% sodium hypochlorite (NaOCl) and 0.1% Tween-20 for 20min and washed thrice with sterilized, distilled water. Aseptic seeds were inoculated on Murashige and Skoog [30] solid medium without growth hormones, and allowed to germinate under laboratory**.**The medium was adjusted to pH 5.7 and supplemented with 0.7% agar and 3% sucrose. The cultures were incubated at 25±2°C under 16/8 h (light/dark) photoperiod. Shoot-apices were dissected from 4-weeks-old anise seedlings. For callus induction, the Shoot-tip explants were aseptically inoculated on MS-medium (Murashige and Skoog, 1962) supplemented with

1 mg/l NAA + 1 mg/l 2, 4-D + 2 mg/l KIN

1 mg/l KIN + 1.5 mg/l 2, 4-D

Callus maintenance and growth measurements

The callus was maintained on MS medium containing 1 mg/l NAA + 1 mg/l 2, 4-D + 2 mg/l KIN, and incubated under the same culture conditions with subcultures at 4 weeks interval in fresh medium. Increase Value (IV) of callus tissue or increasing value of callus fresh weight was calculated as below formula:

IV = (W1-W0)/W0

Where W0 was the weight of callus tissue before treatment and W1 the final weight of callus after culture period. Fresh callus tissues (g) were harvested and washed with distilled water three times to remove any residual medium.

The growth of calli cultures was monitored by weekly measurement of average fresh weight (mg). Growth rate (mgfw/day) was determined according to [31] as follows:

Growth rate = Ge - G s / 7

Where: Ge: Callus mass (mg) at the end of every week and Gs: The starting mass (mg) of callus.

Precursor and elicitor preparation and administration

This research was conducted to appraise the effect of a precursor and biotic elicitor, each at different concentrations on callus growth and active ingredient content. Pieces of callus about (0.5 g) were cultured on MS medium supplemented with 1 mg/l NAA + 1 mg/l 2, 4-D + 2 mg/l KIN. Precursor and elicitor preparation were done by dissolving the required amount into the sterile distilled water; filter sterilized and added before solidification of the autoclaved media. Each of elicitor and precursor were added to the *Pimpinella anisum* callus cultures in triplicate. Culture medium without elicitor and precursor were also included as control set.

Yeast elicitor

Ten grams of yeast extract was dissolved in 100 ml of double distilled water (10 g/100 ml) and then ethanol was added up to 80% (v/v) and was kept at 4 °C for 3 days for precipitation. The supernatant was decanted and the precipitate was re-dissolved in 100 ml double distilled water then autoclaved and was used as elicitor [32].Tested concentrations were 0; 1.82; 3.64 and 5.46 mM/l. free yeast medium was used as control medium.

Phenylalanine precursor

A stock solution of 10 mM concentration was prepared by dissolving phenylalanine in distilled water, sterilized using 0.2µm micro filters and concentrations of 0, 2, 4 and 6 mM/l were prepared. Sterile filtered concentrations of Phenylalanine were added before solidification of the autoclaved media. Free Phenylalanine medium was used as control medium.

***Extraction*:**

In vitro shoot tip derived calli of *Pimpinella anisum* L. (20 g) **which** grown on **control** MS- medium **(elicitor free)** and MS-medium fortified with different concentrations of either phenylalanine (2, 4, 6 mM/l) or yeast extract (1.82, 3.64, 5.46 mM/l) were extracted separately using a soxhlet **apparatus** with 250 ml acetone solvent for 3 hours. Crude extracts for each treatment were filtered using 0.45 µm filter before subjected to centrifugation at 12 000 rpm three

times. The obtained supernatants were evaporated and concentrated by vacuum distillation using rotary evaporator device (Buchi, USA, R-210/215; Switzerland). The extracts were separated from the solvent using a rotary evaporator at 30–40 °C and stored at 4 °C for GC-MS analysis and biological studies.

Gas chromatographic-mass spectrometric

The extracts were analyzed by GC-MS to determine the total content of volatile constituents. The analysis was performed using a Thermo Scientific, USA, Trace GC Ultra/ISQ Single Quadrupole MS, TG-5MS detection The ionization voltage was 70 eV, mass range m/z 39-400 a.m.u. The quantification of volatile constituents was investigated using a percent relative peak area. The identity of the components in the extract was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library NIST [33], WILEY [34] and also with published literatures.

Statistical analysis

All the experiments were conducted in a completely randomized design with four replicates. The data was subjected to statistical analyses following standard procedure.

Results and Discussion

Three types of growth regulators (IAA, 2,4-D and KIN) included in MS medium were attempted for callus induction using excised shoot tip explants derived from growing anise seedlings cultured on basal Murashige and Skoog medium (Fig. 1A). Noticeable calli were produced at the basal ends of shoot tip explants after three weeks of inoculation (Fig. 1B). Depending on the plant growth regulators treatments used, no calli were induced in the media without the plant growth regulators, indicating that PGRs are required for callus induction. Karam et al. [35] also recorded the same results and emphasized the importance of exogenous plant growth regulators for dividing cells and thus callus formation in *Salvia fruticosa*. Our results revealed that the highest percentage of callus induction was achieved from the MS medium addend with 1 mg/l NAA + 1 mg/l 2, 4-D + 2 mg/l KIN.

Optimum concentration of these growth regulators may depend on many factors, such as a genotype of original plant, explants origin and etc. Considering the callus morphology, the calli obtained were relatively compact and greenish in color.

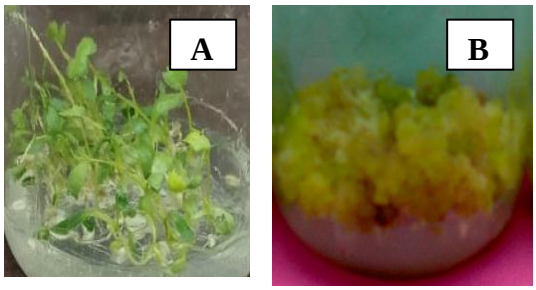

Fig.1. Seedlings growing basal on Murashige and Skoog medium (A) and shoot tip produced calli (B), of anise plant cultured on MS-medium supplemented with1 mg/l NAA + 1 mg/l 2, 4-D + 2 mg/l KIN after four weeks cultivation.

In this study, callus fresh weight was progressively increased (3.1; 6.3; 14.6 and 18.3 g), during four weeks cultivation (Fig.2). In this case, the maximum growth rate (2.41) and maximum increase value (11.24) were obtained from the callus cultured on MS medium addend with 1 mg/l NAA + 1 mg/l 2, 4-D + 2 mg/l KIN (Fig.2).

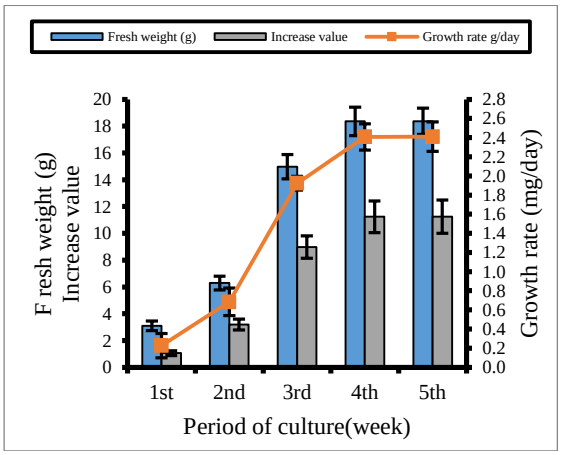

Fig.2. Growth dynamics of anise shoot tip calli during 5 weeks of cultivation.

Numerous factors as explants and growth regulating substances are usually influence growth of calli cultures. For callus initiation, auxins and cytokinins were commonly applied [36-39]. Gamborg and Shyluk [40] showed that callus formation is a consequence of cell division and cell enlargement as well as auxins increase cell growth, while cytokinins stimulate cell division. Literature revealed that the 2,4-D has an induction effect on callus cells number as well as increase in cells volume due to increase of water content in cells[41-43]. Depending on the plant species, 2,4- D, was used in tissue culture [44,45]. The most potent auxin, 2,4- D, may be used in the range of 0.1- 10.0 mg/l [46]. Callus production from kidney bean by 2mg/l2,4- D plus 0.64mg/l Kin was reported [44] and was indispensable in faba bean cultures [47].

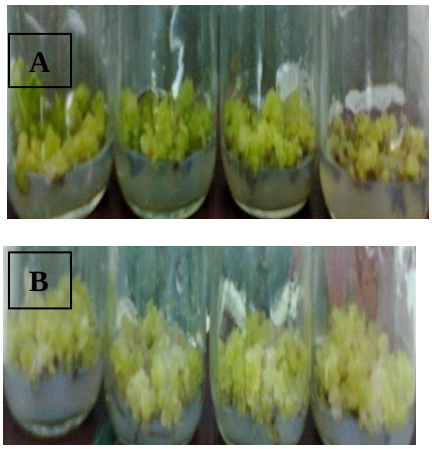

Fig.3. Calli of anise shoot tip explants grown on MS-medium enhanced by 2, 4 and 6 mM /l phenylalanine (A) and 1.82, 3.64 and 5.46 mM/l yeast (B).

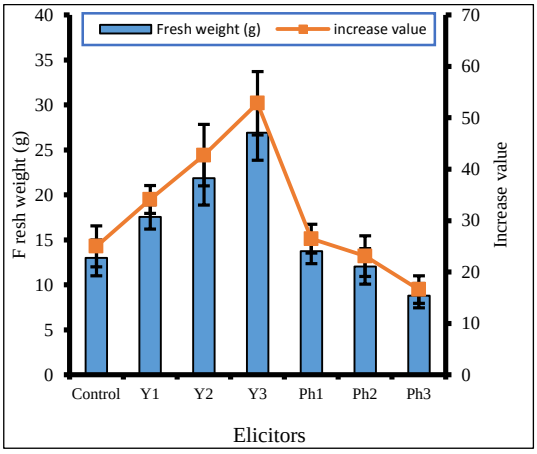

Fig.4. Fresh weight and increase value of anise shoot tip calli grown on medium fortified with Yeast (Y) and phenylalanine (Ph) with different concentrations for 4 weeks of culture; Y1: 1.82mM/l;Y2: 3.64mM/l ;Y3: 5.46mM/l; Ph1: 2mM/l;Ph2: 4mM/l;Ph3: 6mM/l; Free elicitor medium, (Control).

To improve the callus productivity, we tested the effects of a biotic elicitor (yeast) and phenylalanine as precursors after four weeks cultivation compared to control treatment (medium free from yeast and phenylalanine). Data presented in Figure 3 indicated that calli fresh weights and increase values of *Pimpinella anisum* were gradually increased in case of yeast elicitor till the end of experiment. Both fresh weight and increase values were progressively increased and reached the maximum (26.9 g, and 52.8 respectively) at 5.46 mM/l yeast concentrations compared to control treatment (12.9 g, and 24.7; Fig.3).

On the other hand, adding phenylalanine to the medium at 2mM concentration caused slight rising in fresh weight and increase value (13.7 g and 26.5 respectively) compared to control treatment (12.9 g, and 24.7; Fig.3). Contradictory, using 4 and 6 mM phenylalanine concentration shows the lowest mean of fresh weights and increase values compared to yeast and control treatments (Fig.3)

Elicitation of plant tissue culture recently has opened a novel possibility for secondary metabolite synthesis through stimulating stress responses in plants.

The precursor of the phenyl propanoide pathway (Phenylalanine) is an amino acid that playing an important role in flavonoids and phenolic compounds formation. Application of phenylalanine has been effectively used to boost the plant metabolite production in diverse in vitro cultures [14].

Masoumian et al. [48] discussed the effect of Phenylalanine as precursor on callus culture enlargement in many plant species. They revealed that influence of Phenylalanine concentration and culture medium on calli cultures. The investigators demonstrated that in *H. bonariensis* cultures, the addition of Phenylalanine has no affect callus growth, while in *Artemisia* spp. low Phenylalanine concentrations were leading to decreased callus growth.

Yeast extract (YE) is composed of variety of compounds, apart from amino acids, vitamins and minerals [49, 50].Yeast addition was used in callus cultures as growth nutrients [51, 52]. To promote plant growth, it was recommended to use yeast extract, as a result of its high content of amino acid [38]. Elicitors can cause quick commencement of key enzymes in biosynthetic pathway [53]. Similar observations were found [54, 55] in *Panax ginseng* and *Salvia miltiorrhiza* hairy root cultures. Alternatively, in *Morinda elliptica* cultures, yeast treatment raised the dry weight about 10–20% more than controls [12]. Also, in lupine cell cultures, Ibrahim et al. [56] found an increased callus growth that as results of yeast application.

Plants have an almost infinite aptitude to manufacture active metabolites which considered as plant defence mechanisms.

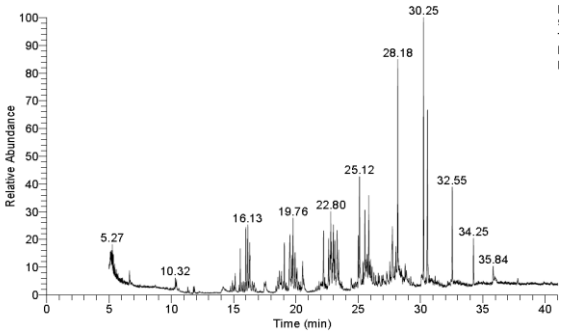

Fig.5. GC-MS chromatogram of *Pimpinella anisum* oil, shows 1,2-Diphenyl-5-(t-butyl)-acephenanthrylene peak with RT 30.24

TABLE 1. Percentage of various volatile oil constituents of *Pimpinella anisum* calli cultured on MS-basal medium analyzed by GC-MS.

Compound Name	Retention Time (RT)	Molecular Weight	Molecular Formula	Area%
Dodecane,2,6,10-trimethyl	15.53	212	$C_{15}H_{32}$	2.78
Octadecene	15.99	252	$C_{18}H_{36}$	2.95
1-Nonene,4,6,8-trimethyl	16.13	168	$C_{12}H_{24}$	3.08
Dodecane,2,6,11-trimethyl	16.32	212	$C_{15}H_{32}$	3.73
Eicosane	19.08	282	$C_{20}H_{42}$	2.76
5-Isopropyl-4-(trifluoromethyl)-1-Hpyrimidin-2-one	19.49	206	$C_8H_9F_3N_2O$	5.58
Nonadecane	19.76	268	$C_{19}H_{40}$	4.51
Hexadecane,2,6,11,15-tetramethyl	22.21	282	$C_{20}H_{42}$	3.44
Pentacosane	22.80	352	$C_{25}H_{52}$	6.64
1-Dodecanol, 2-hexyl	23.00	270	$C_{18}H_{38}O$	4.28
1-Nonadecene	23.32	266	$C_{19}H_{38}$	2.96
Heneicosane	25.01	296	$C_{21}H_{44}$	2.95
7,9-Di-tert-butyl-1-oxas Piro-(4,5)-deca-6,9-diene 2,8-dione	25.11	276	$C_{17}H_{24}O_3$	3.29
Pentatriacontane	25.53	492	$C_{35}H_{72}$	4.90
1-Docosene	25.84	308	$C_{22}H_{44}$	2.43
6,6-Dimethyl-9-hydroxy-10-(1-oxopropyl)-4-propylpyran(2,3-f)chromene-2-one	27.74	342	$C_{20}H_{22}O_5$	6.82
Behenic alcohol	28.17	326	$C_{22}H_{46}O$	6.89
1,2-Diphenyl-5-(t-butyl)-acephenanthrylene	30.24	410	$C_{32}H_{26}$	20.87
n-Tetracosanol-1	30.55	354	$C_{24}H_{50}O$	5.94
9-Hexacosene	32.55	364	$C_{26}H_{52}$	3.21
				100.01

The GC-MS analysis of the acetone extract of shoot tip calli of *Pimpinella anisum* L. cultured on MS-basal medium (precursor and elicitor free medium) confirmed the occurrence of twenty bio-active constituents of therapeutic importance. GC-MS running time was 37.50 minutes. Figure 5 and Table1; correspond to GC-MS spectrum and volatile oil (%) constituents of *P. anisum* callus cultures on control medium analysed by GC-MS respectively.

The most prevailing major compounds were 1,2-Diphenyl-5-(t-butyl)- acephenanthrylene (20.87%), Behenic alcohol (6.89%), 6,6-Dimethyl-9-hydroxy-10-(1-oxopropyl)-4-propylpyran (2,3-f)chromene-2-one (6.82%), Pentacosane (6.64%), n-Tetracosanol-1 (5.94%),5-Isopropyl-4-(trifluoromethyl)-1-Hpyrimidin-2-one (5.58%), Pentatriacontane (4.90%), Nonadecane (4.51%), 1-Dodecanol, 2-hexyl (4.28%), Dodecane,2,6,11-trimethyl (3.73%), Hexadecane,2,6,11,15-tetramethyl (3.44%), 7,9-Di-tert-butyl-1-oxas Piro-(4,5)-deca-6,9-diene 2,8-dione (3.29%),9-Hexacosene (3.21%),1-Nonene,4,6,8-trimethyl (3.08%), 1-Nonadecene (2.96%), Heneicosane (2.95%), Octadecene (2.95%), Dodecane,2,6,10-trimethyl (2.78%), Eicosane (2.76%), 1-Docosene (2.43%).The above detected ingredients have not been reported from *Pimpinella anisum* callus cultures to date. Accordingly, the newly detected constituents can be studied for possible therapeutic abilities.

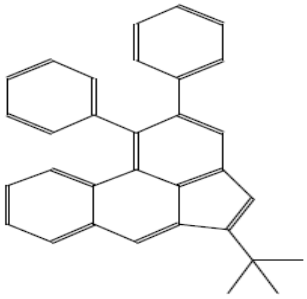

Fig.6. Chemical structure of 1,2-Diphenyl-5-(t-butyl)-acephenanthrylene

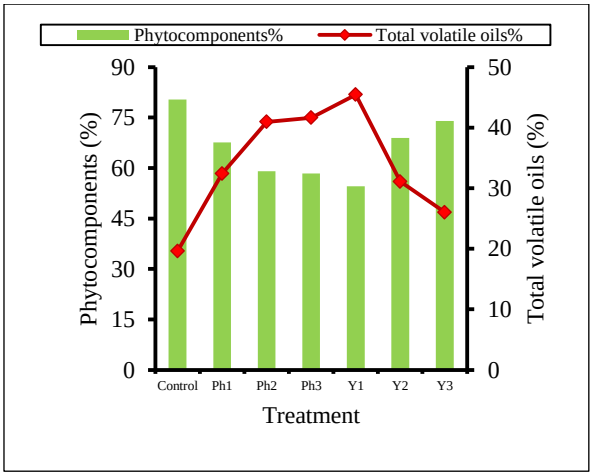

Fig.7. Total volatile oil and phytocomponents percentage (%) of *Pimpinella anisum* shoot tip calli grown on medium fortified with Yeast (Y) and phenylalanine (Ph) with different concentrations for 4 weeks of culture; Ph1: 2mM/l; Ph2: 4mM/l; Ph3: 6mM/l; Y1: 1.82mM/l; Y2: 3.64mM/L; Y3: 5.46mM/l; Free elicitor medium, (Control).

Total volatile oil and phytocomponents percentage of *Pimpinella anisum* calli derived from shoot tip was showed in figure 7. Regarding the phytocomponents (%), it was declared that control medium recorded the highest value followed by Y3; Y2and Ph1 media (80.39%; 73.99%; 68.91%; 67.62% respectively). On the other side, total volatile oils gave a different trend, as Y1 showed the highest value (45.45%) followed by Ph3 and Ph2 media (41.63%; 40.96% respectively).As shown in figure 7, control medium recorded the lowest value (19.61%), while Ph1,Y2

and Y3 took a comparable values (32.38%;31.09%;26.01% respectively).

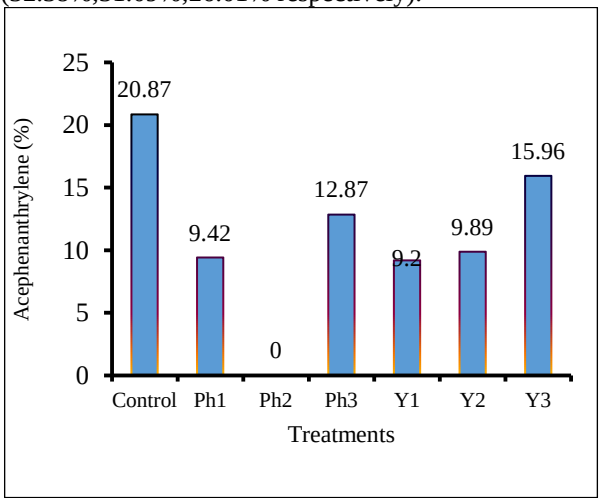

Fig.8. Acephenanthrylene percentage (%) of *Pimpinella anisum* shoot tip calli grown on medium fortified with Yeast (Y) and phenylalanine (Ph) with different concentrations for 4 weeks of culture; Ph1: 2mM/l; Ph2: 4mM/l; Ph3: 6mM/l; Y1: 1.82mM/l; Y2: 3.64mM/L; Y3: 5.46mM/l; Free elicitor medium, (Control).

P.anisum is well known to synthesis and accrue an assortment of volatile oils which used as antibacterial and antifungal [6, 23, 57]; anticancer [58]. Different experiments were performed to prove the antiviral [59]; antioxidant, analgesic, muscle relaxant and anticonvulsant activity. Aniseeds cause hypolipidemic and hypoglycemic effect and reduce lipid per-oxidation [60-63].

Plants considered being factories of different compounds of pharmaceutical importance in health-related applications.

With special thought to 1,2-Diphenyl-5-(t-butyl)-acephenanthrylene, as a major volatile oil constituents (**Fig.6**), it was determined in media supplemented with a biotic elicitor (yeast) and phenylalanine as precursors. Figure 8 revealed that the highest percentage of acephenanthrylene was recorded in control medium (20.87%), followed by Y3 medium (15.96%) and (12.87%) with Ph3 medium. while the lowest percentage (0%) was recorded at 4mM/l concentration of phenylalanine. Y2 (9.89%); Ph1 (9.42%) and Y1 (9.2%) media took a similar trend in acephenanthrylene accumulation (Fig. 8**)**.

1,2-Diphenyl-5-(t-butyl)- acephenanthrylene ($C_{32}H_{26}$) is an aromatic compounds (flavonoids) with many health benefits as Cancer-preventive, flavor analgesic, antinociceptive, anti-implantation [64].

Conclusions:

Plant extract are naturally derived phytochemicals that have a wide range of claims and used all over the world for treatment of many diseases. Plant volatile constituents and extracts could incline to be less harmful than analogous man-made drugs. GC-MS analysis of *Pimpinella anisum* L. callus cultures derived from shoot tip confirmed the presence of 1,2-Diphenyl-5-(t-butyl)-acephenanthrylene ($C_{32}H_{26}$). In the near future, the progress in purification of different constituents identified in this study may be of great inquisitiveness to scientists in the field of pharmaceutical industry to improve the existing drugs of antitumor activity and in the production of safe and healthy food supplements.

Acknowledgements:

The authors would like to thank the National Research Centre (NRC) for its facilities during this scientific work (Project ID: E121106).

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