Egypt. J. Chem. **Vol. 65**, No. 9 pp. 155 - 169 (2022)

Egyptian Journal of Chemistry

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

Phytochemical Profiling by GC-MS Analysis and Antimicrobial Activity Potential of *In vitro* Derived Shoot Cultures of Some Egyptian Herbal Medicinal Plants

Nermeen Mohammad Arafa[1]*, Nancy Danial Girgis[1], Mona Mohamed Ibrahim[1], Sahar Saleh Mohamed[2] and Mohamed Kamal El-Bahr[1]

[1]*Department of Plant Biotechnology, National Research Center, El□Buhouth St., (P.O. Box 12622), Dokki, Cairo, Egypt.*

[2]*Department of Microbial Biotechnology, National Research Center, El-Buhouth St., (P.O. Box 12622), Dokki, Cairo, Egypt.*

Abstract

The Egyptian climate possesses many herbal medicinal plants, three species namely carnation (*Dianthus caryophyllus*), thyme (*Thymus vulgaris* L) and basil (*Ocimum basilicum*L) were selected to study in this research. The current study was designed to optimize a protocol of *in vitro* propagation for progeny uniformity mass production of the selected species to avoid their genetic and biochemical heterogeneity. Nodal segments of carnation, thyme and basil seedlings cultured on medium contained benzyl amino purine (BAP) at different concentrations with or without naphthalene acetic acid (NAA) at 0.5 mg/l. The presence of NAA combined with a high level of BAP was more efficient for shootlets mass production of all studied plant species. Shootlets were extracted using diethyl ether, most of their substantial phytocomponents (fatty acid derivatives, essential oils, , cyclohexane carboxylic acid, hydrocarbons, etc.,) were detected in different proportions by GC-MS analysis. Shootlets extracts were evaluated antimicrobial *in vitro*, and exhibited differentially good performance against *Bacillus subtilis*, *Staphylococcus aureus, Pseudomonas floureceans*, Escherichia *coli* and *Candida albicans*. Diameters of inhibition zones ranged from 16.133±0.696 to 23.466±0.837mm for all extracts. In conclusion, *in vitro* normal healthy shootlets of carnation, thyme and basil were obtained, and their extracts were rich in bioactive phytochemicals which in turns play a potent role for microbial growth inhibition.

Keywords: Type your keywords here, separated by semicolons ;

Introduction

Many herbs are commonly named medicinal plants and used for several purposes in the traditional medicine and pharmaceutical preparations around the Mediterranean area suchlike other regions of Egypt. Scientists are interested in researches relative to the medicinal plants field which are derived from folklore or anecdotal knowledge [1]. Natural products are ongoing to more increment scientific attention lately [2], since a lot of indigenous rights from plant resources and basic scientific investigations dependent medicinal plants and indigenous medical systems in the developing countries have been raised [3].

Among the herbal plants under study are thyme (*Thymus vulgaris* L), basil (*Ocimum basilicum* L) belonging to Lamiaceae family and carnation (*Dianthus caryophyllus)* belonging to Caryophyllaceae family. Thyme, basil and carnation are annual or perennial herbaceous and aromatic medicinal plants cultivated all over the world [4].

These herbs are most valuable medicinal plants which possess several pharmacological potential, aromatic and biological properties [5, 6]. The active substances in the thyme essential oil are thymol, carvacrol, p-cymene, β - pinene, γ- terpinene, β-caryophyllene, 1-borneol, 1, 8-cineole, etc [7]. Thyme used for cosmetic and pharmaceutical products as it contains large amounts of essential oils and rosmarinic acid [8]. Dried leaves of basil and its essential oil are used in the food industry as aromatic and flavoring ingredients. *Ocimum basilicum* contains linalool, eugenol, methyl chavicol, methyl cinnamate, ferulate, methyl eugenol, triterpenoids and steroidal glycoside known to exhibit antioxidant activities [9 , 10].

*Corresponding author e-mail: nermeenfouad78@yahoo.com

Received 08 January 2022; revised 21 January 2022; accepted 24 January 2022

DOI: 10.21608/EJCHEM.2022.115045.5230

Phytochemical analysis of *Dianthus caryophyllus* showed that it contained triterpenes, alkaloids, coumaruns, cyanogenic glycoside, cyanidin, pelargonidin, the yellow isosalipurposide, essential oil, volatile oil and many other chemical contents. Carnation, thyme and basil plants are source of aroma compounds and essential oils containing several biologically active constituents that possess insecticidal [11], nematicidal [12], fungistatic[13] and antimicrobial properties [14 , 15]. The potent antimicrobial ability is related to their essential oils content of high concentrations of eugenol, carvacrol and thymol [16].

The sexual propagation *via* seeds is obtainable in thyme, basil and carnation, but the recovered seedlings show variability due to cross-pollination [17]. Therefore, various studies have used plant tissue culture techniques to regenerate carnation, thyme and basil *in vitro* using different explants. The best multiple shoot proliferation of carnation nodal explants was observed in MS medium supplemented with 4.4µM BAP [18].The highest growth rate of shoot proliferation was obtained using semi-solid MS medium supplemented with 1 $mgdm^{-3}$ GA3 [19]. Maximum percent of *Ocimum basilicum* L shoot regeneration and average number of shoots were observed on MS medium containing 11 µM BAP + 0 µM IAA [20]. Likewise, many *in vitro* studies have been done to regenerate shoots induction using different explants, like shoot tips [21], node with pairs of axillry buds [22], nodal segments [23], leaf explants [24], young inflorescence [25] and through callus phase [26]. In vitro propagation of the medicinal and aromatic plants has been significantly increased [17], because of the promising role of plant tissue culture techniques as influential procedure for quick multiplication with high progeny uniformity in the valuable herbal medicinal plants.

Recently, herbal medicinal plants used as alternative natural drugs of chemo drugs in pharmaceutical preparations field due to their lesser cytotoxicity with less harmful side effects. As well as the continuous necessity to find new components for pharmaceutical industries, particularly under the condition of antibiotic resistance incidence, one of the most important safe and available source, is the use of plant extracts [27], this is needs more studies to reach the best results. Consequently, the aim of our current study was to describe and performance a successful protocol of plant tissue culture to induce multiple shoots via in vitro propagation and multiplication of three most beneficial medicinal plants (thyme, basil and carnation) which were selected for their valuable content of bio-active compounds. Screening of Phytochemical constituents and antimicrobial activity of the multiple shoots have been studied.

Experimental technique and materials

1-Materials

The chemicals utilized right now were purchased from Aldrich with degree of purification and without extra filtration, and the solvents utilized were had a perfect degree of unpolluted

2- Plant material preparation of carnation, thyme and basil.

The seeds of carnation (*Dianthus caryophyllus*), thyme (*Thymus vulgaris*) and basil (*Ocimum basilicum*) were obtained from Horticulture Research Institute, Agricultural Research Centre, Ministry of Agriculture, Dokki Giza, Egypt. This study was conducted at Department of Plant Biotechnology, Biotechnology institute, National Research Center, Egypt.

a. Seeds germination.

Seeds of carnation, thyme and basil were surface sterilized with a solution of 70% alcohol for 1min and rinsed 3 times with sterile distilled water, followed by soaking in a solution of 10% sodium hypochlorite (NaOCl) for 15min before washing 3 times with sterile distilled water. Seeds were blotted dry on sterile filter paper and then were put into jar filled with 50ml of MS medium including mineral concentrations as specified by Murashige and Skoog [28]. MS-medium supplemented with 30 g/l sucrose, 7g/l agar and then the pH of the medium was adjusted to 5.8 with0.1 M NaOH or HCl solutions. All jars were autoclaved at 121 °C for 20min following the potential addition of plant growth regulators to the culture medium. The cultures were incubated in a plant growth chamber at 25 ± 2 °C and 16/8h photoperiod.

b. In vitro shoots multiplication.

Nodal segments about 0.5-1 cm in length were excised under sterilized conditions from carnation, thyme and basil aseptic 6- week-old seedlings cultured on MS medium without growth regulators. Nodal segments were cultured on MS medium containing different concentrations of Plant Growth Regulators (PGR) such as BAP (cytokinin) combined with or without NAA (auxin) as mentioned in Table (1). Each jar containing 50 ml of different supplementations medium was inoculated with sterilized four nodal segments under aseptic conditions. After four weeks of cultivation on culture medium, percentage of segments produced shoots, number of new shoots per explant and their shoot lengths were calculated.

Table (1) MS-medium supplemented with BAP and NAA at different combinations for each plant type.

Plant type	Plant Growth Regulators (PGR) mg/l	
	NAA	BAP
Carnation	0	2.0
	0	3.0
	0.5	2.0
	0.5	3.0
Thyme	0	2.0
	0	4.0
	0.5	2.0
	0.5	4.0
Basil	0	2.5
	0	5.0
	0.5	2.5
	0.5	5.0

3- Bioactive phytochemicals extraction and GC-MS analysis.

a. Extraction procedure

Extraction of phytochemical constituents from carnation, thyme and basil multiple shoots (shootlets) at age 30 days was performed using soxhlet extractor procedure according to Miller [29]. Twenty grams of fresh in vitro shoot cultures for each plant were put in soxhlet apparatus with 100 ml of diethyl ether solvent for continuous extraction overnight at respective solvent temperature. Each crude was filtered using a 0.45-µm filter before subjection to centrifugation at 13 000 rpm three times. The supernatant of each sample was evaporated to be concentrated by vacuum evaporator using rotary evaporator device (Buchi, USA, R-210/215; Switzerland). The dried crude extract yields were resolved in the least volume of diethyl ether and then all samples were kept in the refrigerator at 4°C for GC-MS analysis and biological study.

b. Gas chromatography mass spectrometry (GC-MS) analysis.

Samples prepared previously were analyzed by GC-MS to identify and determine their metabolites. Overall, 2 ml of diethyl ether extract was evaporated till dryness and re-solubilized in 5 µl of the adequate solvent for injection in GC-MS apparatus under the convenient conditions. GC-MS analysis was occurred using a Thermo Scientific, USA, Trace GC Ultra/ISQ Single Quadrupole MS, TG-5MS detection, with an electron ionization system with ionization energy of 70 eV. Helium gas was used as the carrier gas at a constant flow rate of 1 ml/min. The injector and MS transfer line temperature was set at 280°C. The oven temperature was programmed at an initial temperature 40°C (hold 3 min) to 280°C as the final temperature at an increasing rate of 5°C/min (hold 5 min). The quantification of all the identified components was investigated using a percent relative peak area. A tentative identification of the compounds was done based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.

4- In vitro antimicrobial assay.

Antimicrobial capacity of all preserved extracts was screened in vitro using conventional agar diffusion technique according to Greenwood [30]. The provided extracts as described above were tested against cultures of Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*), Gram - negative bacteria (*Psedumonas floureceans, Escherichia coli*) and yeast (*Candida albicans*). Bacterial strains were cultured on nutrient medium and yeast strain was cultured on yeast media. Broth media included the same contents except for agar, yeast and bacteria strains that were incubated for 24h. For preparation of inoculated plates, 50 ml of agar media at 50˚C was infected by 1 ml of inoculant with mixing by simple inversion. The agar was poured into 12 Cm Petri dishes with allowing to cooling till room temperature. Microorganism plates were undisturbed for 30 min to allow distribution of the examined extracts into the agar medium. The inhibition zone of the microbial growth was calculated after incubation at 30˚C, the semblance of lucid microbial free inhibition zones, beginning during 24-48 h for bacteria strains and 24 h for the yeast.

4- Statistical analysis.

All experiments were set up in a factorial experiment based on completely randomized design and data were statistically analyzed by one-way analysis of variance (ANOVA) for testing the differences among treatments, using MATATC

software and the protected least-significant-difference (LSD) test at $p<0.05$ ($LSD_{0.05}$), applying Duncan's Multiple Range Test (DMRT) according to Gomez and Gomez [31].$LSD_{0.05}$ test was used to compose the significant differences between means of treatment [32].

Data pertaining to percentage of shoots induction, average number of shoots and average shoot lengths were subjected to mean separation, standard deviation and standard error. The results were expressed using analysis of variance from which mean standard error values (mean ± SE) included superscript letters, were computed for comparison between treatments with indicating the presence of significant difference at the 0.05 probability level. All the experiments were repeated three times, three replicates per treatment with 5 explants for each replicate were used.

Results and Discussion

1- In vitro seedlings formation of carnation, thyme and basil seeds.

Seeds of carnation, thyme and basil plants were cultured on MS-basal medium after protocol of seeds disinfection in agriculture cabinet to avoid the symptoms of fungal and bacterial contamination for healthy *in vitro* seedlings recovery. Seeds were culture as described above under the optimum sterilized conditions allowed to establish a sterile primary seedlings to prevent most of contaminants that are often associated with the ex vitro which cause poor germination rate and infected plants. This sterilization procedure applied in this experiment confirmed to be efficient in the next studies of *in vitro* propagation, was agree with those of Aicha *et al.* [33]

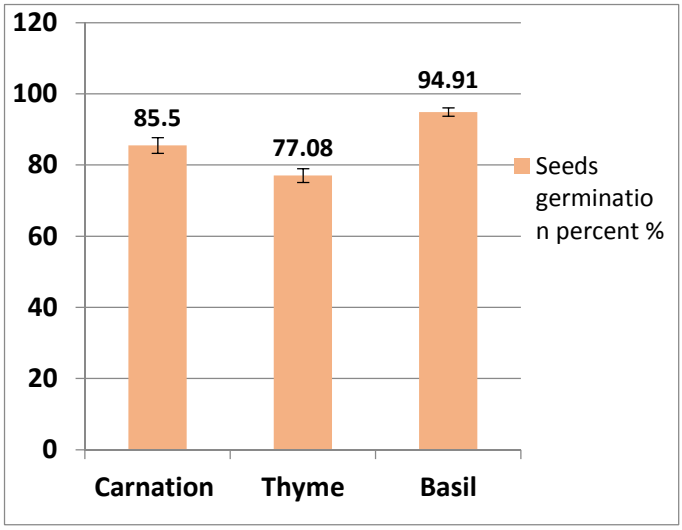

Fig. (1) Germination percentage of carnation, thyme and basil seeds

who used 70% ethanol solution before submerging in 10% NaOCl, 10 min in seeds decontamination process to avoid most of the contaminants such bacteria and fungi. Seeds of carnation and thyme started to germinate successfully after 7 days of inoculation on MS- medium free growth regulators against 14 days for basil seeds culture using the same medium.

The obtained results are close to what have been achieved by Aich et al. and Asghari et al. [33, 34] who reported that the germination of thyme seeds was investigated on MS medium without any auxin or cytokinin after 3 of subcultures. The germination percentage of carnation, thyme and basil seeds was 85.5 ± 2.179 %, 77.08±1.938% and 94.91±1.175%, respectively as illustrated in Fig. (1).

As shown in Fig (2), in vitro seedlings formation of carnation, thyme and basil look in a normal and healthy appearance at age 30 days.

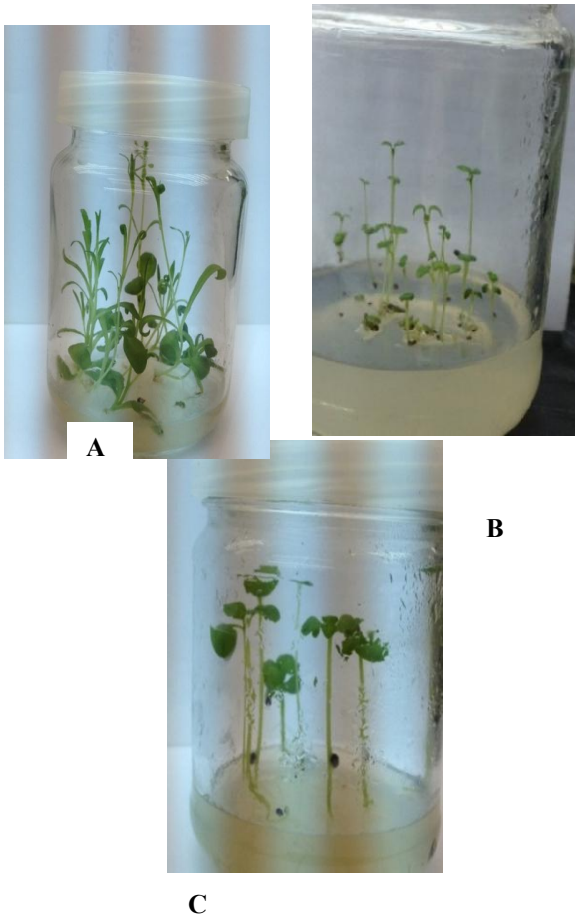

Fig. (2) In vitro seedlings of carnation (A), thyme (B) and basil (C) seeds germinated on MS basal medium after 30 days of culture

2- Optimization of an efficient micropropagation protocol of carnation, thyme and basil.

In vitro seedlings of carnation, thyme and basil plants have been established and prepared under sterilized conditions for micropropagation completion. In this experiment, protocol of in vitro propagation for carnation, thyme and basil plants have been performed starting with nodal segments of the in vitro derived seedlings passing by culturing on MS medium fortified with Plant Growth Regulators (PGR) such NAA as a type of auxin and BAP as a type of cytokinin for more progress proliferation of the primary shoot cultures to recovery mass production of an aseptic in vitro shoot cultures forming healthy multiple shoots.

2-1-Effect of NAA and BAP on multiplication rate of carnation.

Nodal segments about 1 cm length with a pair of axillary buds were excised from uniform carnation in vitro seedlings, then transferred to MS-medium supplemented with BAP at concentrations of 2.0 and 3.0 mg/l with or without NAA at 0.5 mg/l concentration. After 10-15 days of cultured nodal explants on those multiplication media, many stem cutting explants began to proliferate and their axillary buds continued to develop forming microshoots (Fig.3).

Fig. (3) Carnation microshoots from nodal explants after 15 days of culture on MS-medium containing 0.5mg/l NAA + 3mg/l BAP.

Stage of shoots multiplication from nodal segments on MS-proliferation media were evaluated after 4 weeks of culture when the micro shoots became about 2.0 cm in length by calculation the percentage of shoots induction; average shoots number formation and shoot lengths as reported in Table 2. Addition of 0.5 mg/l NAA to BAP at 2.0 or 3.0 mg/l enhanced strongly the morphogenic potential of the segments, comparable of NAA free media which gave less response of the multiplication recovery. In term of regeneration percent, no statistical differences were observed between 2.0 mg/l BAP (99.166^{a}%) or 3.0 mg/l BAP (99.566^{a}%) in combination with 0.5 mg/l NAA, while using BAP alone in shoot culture medium at concentrations 2.0 or 3.0 mg/l gave less values which were limited to 90.333^{c} % and 92.066^{b} %, respectively with clear significant between them in favor of 3.0 mg/l BAP.

Data in Table (2) show a significant variability for average number of shoots per explant without any significance for their shoot lengths among all treatments. The highest value of average shoots number was achieved using 3 mg/l BA + 0.5 mg/l NAA (11.33 ± 1.201^{a}) as the best selected treatment for shoot cultures induction (Fig. 4)with high significant difference compared to the treatment of 2 mg/l BA + 0.5 mg/l NAA (8.66 ± 1.01^{b}). Taking into account that the treatments of BAP (2 and 3 mg/l) without NAA decreased significantly the average number of shoots (3.5 ± 0.577^{c} and 2.43 ± 0.554^{c}, respectively) without any significance between them, maximum value of shoot lengths (3.3 ± 0.416^{a}cm) was verified on MS-medium fortified with 0.5 mg/l NAA + 3 mg/l BAP. Following those results, the presence of NAA in shoot regeneration medium proved to be superior for shoots proliferation.

Table (2) Effect of different BAP levels alone or combined with 0.5 mg/l NAA on carnation shoots proliferation after 4 weeks of cultivation.

Plant Growth Regulators (PGR)		Percentage of shoots induction %	Average number of shoots per explant	Average shoot lengths per explant (Cm)
NAA mg/l	BAP mg/l			
0.0	2.0	90.333^{c}	3.5 ± 0.577^{c}	2.16 ± 0.600^{a}
0.0	3.0	92.066^{b}	2.43 ± 0.554^{c}	2.8 ± 0.577^{a}
0.5	2.0	99.166^{a}	8.66 ± 1.01^{b}	2.16 ± 0.440^{a}
0.5	3.0	99.566^{a}	11.33 ± 1.201^{a}	3.3 ± 0.416^{a}
$LSD_{0.05}$		1.645	2.3206	1.3555

Means (±SE) in the same column followed by values with the same superscript letters are not significantly different from each other whilst, values with different superscript letters are significantly different, LSD test at the 5 % probability level (P<0.05) according to DMRT

Fig.(4) Multiple shoots of carnation (30 day old) grown on MS-medium containing 3.0 mg /l BAP+0.5 mg/l NAA.

2-2-Effect of NAA and BAP on multiplication rate of thyme.

Healthy nodal segments (1cm) were aseptically excised from the *in vitro* seedlings of thyme before culturing in jars (300 ml) containing 50 ml of MS-medium including either BAP individual at concentrations 2.0 and 4.0 mg/l or combined with NAA (0.5 mg/l) for shoots proliferation and multiplication stage. Data tabulated in Table (3) exhibited multiple shoots at all treatments of MS shoots proliferation media after 4 weeks by calculation these parameters, percentage of shoots induction %, average shoots number per explants and the average shoot lengths. The favorable media for shoots induction were 2.0or 4.0 mg/l BAP combined with0.5 mg/l NAA, recording 98.833^{a} % and 99.666^{a} %, respectively of each without any significance between them. Whilst, the individual concentrations of BAP (2.0 and 4.0 mg/l) decreased significantly the percentage of shoots induction (93.5^{c} % and 96.833^{b}%, respectively).

Maximum value with high significant difference was appeared in shoots number (12.66 ± 0.333^{a}) at the treatment of 4.0 mg/l BAP +0.5 mg/l NAA, compared to the treatment of 2.0 mg/l BAP +0.5 mg/l NAA (8 ± 0.577^{b}). Meanwhile, using 2.0 or 4.0 mg/l of BAP individual decreased significantly the shoots number (3.66 ± 0.881^{c} and 4.33 ± 0.881^{c}, respectively). For shoot lengths, it is not observed clear significance difference among all treatments except between the treatment of 4.0 mg/l BAP + 0.5 mg/l NAA (4.36 ± 0.384^{a} cm) and 2.0 mg/l BAP (1.93 ± 0.987^{b} cm). A reduction response of thyme nodal explants for shoots proliferation in terms of shoots number and shoot lengths were using BAP alone particularly its low concentration in culture medium. So, the combination of BAP with NAA for mass production of shoots proliferation has been recommended, particularly the higher concentration of BAP as a distinct treatment for recovery more and more of multiple shoot cultures as shown in Fig. (5).

Table (3) Effect of different BAP levels alone or combined with 0.5 mg/l NAA on thyme shoots proliferation after 4 weeks of cultivation.

Plant Growth Regulators (PGR)		Percentage of shoots induction %	Average number of shoots per explant	Average shoot lengths per explant (Cm)
NAA mg/l	BAP mg/l			
0.0	**2.0**	93.5^{c}	3.66 ± 0.881^{c}	1.93 ± 0.987^{b}
0.0	**4.0**	96.833^{b}	4.33 ± 0.881^{c}	3.16 ± 0.333^{ab}
0.5	**2.0**	98.833^{a}	8 ± 0.577^{b}	3.33 ± 0.600^{ab}
0.5	**4.0**	99.666^{a}	12.66 ± 0.333^{a}	4.36 ± 0.384^{a}
$LSD_{0.05}$		1.004	1.86	1.6607

Means (±SE) in the same column followed by values with the same superscript letters are not significantly different from each other whilst, values with different superscript letters are significantly different, LSD test at the 5 % probability level (P<0.05) according to DMRT

Fig. (5) Multiple shoots of thyme (30 day old) grown on MS-medium containing4.0 mg /l BAP+0.5 mg/l NAA.

2-3-Effect of NAA and BAP on multiplication rate of basil.

Aseptic nodal segments about 1cm length with a pair of axillary buds were cut from the in vitro seedlings of basil plants then recultured on MS-medium supported with 2.5 and 5.0 mg/l with or without NAA at 0.5 mg/l concentration. Mathematical parameters such as

shoots induction percentage%, average number of shoots per explants and average shoot lengths were calculated as tabulated in Table (4). High concentration of individual BAP (5.0 mg/l) decreased significantly the percentage of shoots induction recording the lowest value (91.3^{c} %) compared to its low concentration (2.5 mg/l) which recorded high significant value (98.666^{a} %).Taking into account that, addition of 0.5 mg/l NAA to 2.5 mg/l BAP gave intermediate response (94.266^{b}%) of shoots induction, while addition of 0.5 mg/l NAA to 5.0 mg/l BAP gave significantly the highest value (99.166^{a}%) of shoots induction. The highest values of average number of shoots (3.73 ± 0.560^{a}) and shoots length (4.23 ± 0.240^{a} cm) were recorded in distinct significant differences using 5.0 mg/l BAP + 0.5 mg/l NAA compared to 5.0 mg/l BAP alone which gave the least values(1.5 ± 0.577^{b} and 1.66 ± 0.333^{b} cm, respectively). BAP at 2.5 mg/l individual and with 0.5 mg/l NAA gave slightly significant values in both of average shoots number (2.9 ± 0.737^{ab}and 2.33 ± 0.726^{ab}, respectively) and shoots length (2.43 ± 0.811^{b}cm and 2.2 ± 0.513^{b} cm, respectively).

Table (4) Effect of different BAP levels alone or combined with 0.5 mg/l NAA on basil shoots proliferation after 4 weeks of cultivation.

Plant Growth Regulators (PGR)		Percentage of shoots induction %	Average number of shoots per explant	Average shoot lengths per explant (Cm)
NAA mg/l	BAP mg/l			
0.0	**2.5**	98.666^{a}	2.9±0.737^{ab}	2.43 ± 0.811^{b}
0.0	**5.0**	91.3^{c}	1.5± 0.577^{b}	1.66 ± 0.333^{b}
0.5	**2.5**	94.266^{b}	2.33±0.726^{ab}	2.2 ± 0.513^{b}
0.5	**5.0**	99.166^{a}	3.73± 0.560^{a}	4.23 ± 0.240^{a}
$LSD_{0.05}$		1.6072	1.7243	1.3731

Means (±SE) in the same column followed by values with the same superscript letters are not significantly different from each other whilst, values with different superscript letters are significantly different, LSD test at the 5 % probability level (P<0.05) according to DMRT

Fig. (6) Multiple shoots of basil (30 day old) grown on MS-medium containing 5.0 mg /l BAP+0.5 mg/l NAA

From the previous results, it could be concluded that the highest productivity of shoots proliferation as the basic stage of multiplication processes was successfully promoted using high concentration of BAP (5.0 mg/l) in presence of low concentration of NAA (0.5 mg/l) (Fig. 6).

Data in Tables (2, 3, and 4) indicate to successful protocol of *in vitro* multiple shoots production by culture nodal segments derived from carnation, thyme and basil in vitro seedlings on MS medium involving BAP combined with NAA for multiplication rate optimization. On basis our results, it could be highlighted a positive relationship between BAP concentrations and the multiplication rate of in vitro shoot cultures. This point of view leads to this investigation, increasing the multiple shoots directly proportional with altitude of BAP concentration in shoots proliferation medium particularly with a little concentration of NAA regardless the plant species.

Furthermore, authors in this part of study intended to optimize the highest productivity of multiple shoots stock using protocol of *in vitro* propagation dependent BAP presence at certain concentration combined with NAA in the culture medium. Therefore, this work was objected to confirm the essential role of BAP to be an efficient cytokinin and NAA as influential auxin for satisfied progression of multiplication rate of carnation, thyme and basil plants. Our previous investigations referred to the improvement of multiplication rate by adding BAP in culture medium which is, contrary to the observation of Ozudogru [19] who showed that KIN is the most favorable cytokinin in culture medium for regeneration of *Thymus vulgaris*.

Although that our investigations are in accordance with those obtained by Saez *et al*. and Coelho *et al*. [35, 36] who reported that the most efficiency of BAP as cytokinin for micropropagation was performed in *Thymus Pepirella* and *Thymus lotocephalus,* respectively, as well Zarooshan *et al*. [37] who obtained seven times number of shoots in medium including 1 mg/l BAP more than the non – BAP for *Thymus daenensis* and Shetty *et al.* [38] who induced shooting in medium containing 1 mg/l BAP for *thymus vulgaris* L.

Likewise, our findings about regard BAP as potent influent in shoots proliferation medium are in line with the reports of Asghari *et al*. [34] who proved that BAP dosage influenced the shoots formation percentage of *Ocimum basilicum* L., in both of their number of produced shoots and shoot regeneration which increased with increasing BAP concentration in the culture medium, whilst, BAP free medium did not respond. Thereby, authors in this paper deduced the importance of BAP at specific concentration with 0.5 mg/l NAA in culture medium

for optimal shoots mass production of all studied species, due to BAP is one of the synthetic cytokinins which play an essential role in shoots proliferation in plant tissue culture technique.

Several literatures confirmed our view regarding BAP role in shoots regeneration and multiplication, BAP enters in the direction of various development forms in the plants [39 , 40], BAP promoted axillary buds proliferation for obtaining multiple shoots [41] and bud formation [42]. Not only that but also, BAP influenced the accumulation of chlorophylls, total soluble sugar, free amino acids [43] and chloroplast development [44] which in turn lead to distinct shoot cultures establishment. For obtaining normal in vitro shoot cultures system of carnation, thyme and basil, BAP applied in shoots culture medium needs to be carefully monitored and combined with little concentration of auxin such NAA, this in accordance of Dhed'a *et al.* [45] who revealed that the treatment of BAP with auxinin culture medium of bananas and plantains was functional for *in vitro* multiplication.

3- Phytochemical composition of carnation, thyme and basil of in vitro multiple shoots by GC-MS.

Phytochemical constituents for *in vitro* multiple shoots (shootlets) at age 30 days of the three determinant plant species (carnation, thyme and basil) were extracted using diethyl ether solvent and identified by GC-MS analysis.

3-1- Phytochemical constituents of carnation shootlets.

The present data of *Dianthus caryophyllus* shootlets showed 40 different compounds (Table 5). The most abundant compounds were 3-tertbutyl-2-hydroxy-5-vinylbenzaldehyde represents 10.34%,docosane and heptacosane are present by 4.76%, 4.5% respectively, also cadinol which found with 4.10%, the other detected compounds were found in lower concentrations. Among these detected compounds, the majority of them are hydrocarbons and fatty acids represent 32.66%, 14.69% respectively. The hydrocarbon compounds are dodecane,pentadecane, 6-Tetradecene, heptadecane, octadecane, 1-hexadecene,9-nonadecene, docosane, nonacosane,tricosane, octacosane and heptacosane. While fatty acids that detected were hexadecatrienoic acid methyl ester, octadecanoicacid,hexadecanoic acid trimethylsilylester, hexadecadienoic acid methyl ester, 10-octadecenoic acid methyl ester, ethyl 14-methyl hexadecanoate, 9-octadecenoic acid (Z) ethyl ester and Propyl oleate. Essential oils were also detected in diethyl ether extract and found with considerable proportions which were 9.68%as methyl eugenol (2.10%), cadinol (4.10%), α-eudesmol (2.17%) and farnesyl acetate (1.31%).

Chemical constituents of carnation focused in several studies for instance, Zuker *et al.* [46] studied the chemical constituents of *Dianthus caryophyllus* which contains various compounds such as triterpenes, alkaloids, coumaruns, cyanogenic glycoside and volatile oil.Aroma of *D. caryophyllus*is principally due to the existence of eugenol, β-caryophyllene and benzoic acid derivatives [47]. Twelve volatile constituents were identified by Ibrahim [48] using GC-MS in carnation flower oil, the major components was phenyl ethyl alcohol, eugenol and benzoate derivative compounds. Another study performed on the oil of Egyptian *Dianthus caryophyllus* by Galeotti *et al.* [49] and detected various compounds mainly consist of four major chemical groups which are monoterpene hydrocarbons, oxygenated monoterpene, sesquterpenes hydrocarbons and benzoate derivatives. Phenolic and flavonoids biological activities were also studied and isolated in *Dianthus caryophyllus* [50, 51].

Table (5) Phytochemical constituents of in vitro carnation shootlets extracted with diethyl ether.

Compound	Peak Area (%)	Molecular Formula
Serotonin	3.45	C10H12N2O
Heptane, 2,4dimethyl	1.09	C9H20
Dodecane	2.48	C12H26
2-Decenal	1.38	C10H18O
Pentadecane	3.74	C15H32
2-Undecenal	2.29	C11H20O
6-Tetradecene	1.11	C14H28
Methyl eugenol	2.10	C11H14O2
Heptadecane	2.61	C17H36
Di-isopropyl-N-t-butylphosphonicdiamide	1.42	C10H25N2OP
Octadecane	2.94	C18H38
Hexadecatrienoicacid,methyl ester	0.85	C17H28O2
1-Hexadecene	1.36	C16H32
Cadinol	4.10	C15H26O
a-Eudesmol	2.17	C15H26O

3-tert-Butyl-2-hydroxy-5-vinylbenzaldehyde	10.34	C13H16O2
Docosane, 11decyl	2.82	
Benzoic acid-3,4,5-trimethoxy, methyl ester	3.37	C11H14O5
Hexadecane-2,6,10,14-tetramethyl	2.09	C20H42
9-Nonadecene	1.06	C19H38
Farnesyl acetate	1.31	C17H28O2
Phthalic acid, hept-3-ylisobutyl ester	0.93	C19H28O4
Docosane, 11decyl	2.12	C32H66
7,9-Ditert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	2.91	C17H24O3
Docosane	4.76	C22H46
1,2-Benzenedicarboxylicacid, dibutyl ester	1.16	C16H22O4
Octadecanoic acid	2.87	C18H36O2
Ethylene brassylate	1.32	C15H26O4
Hexadecanoicacid,trimethylsilyl ester	0.83	C19H40O2Si
13-Hexyloxacyclotridec-10-en-2-one	0.69	C18H32O2
Hexadecadienoicacid,methyl ester	2.0	C17H30O2
10-Octadecenoicacid, methyl ester	2.16	C19H36O2
Ethyl-14-methylhexadecanoate	1.01	C19H38O2
Nonacosane	2.35	C29H60
9-Octadecenoicacid (Z), ethyl ester	3.8	C20H38O2
Propyl oleate	1.17	C21H40O2
Tricosane	2.29	C23H48
Octacosane	3.46	C28H58
1,2-Benzenedicarboxylicacid-3-nitro	3.35	C8H5NO6
Heptacosane	4.5	C27H56

3-2- Phytochemical constituents of in vitro thyme shootlets.

Table (6) shows different detected compounds that found in diethyl ether extract of *thymus vulgaris* shootlets which represent 30 compounds, the most *plentiful* compounds were nonacosane, docosane and 2-undecenal represent 21.7%, 13.45% and 6.30% respectively, obviously that hydrocarbons and fatty acids were the most abundant constituents which represent 37.54% and 20.46% respectively. Four hydrocarbon compounds that detected are docosane,8- heptadecene, nonadecane and nonacosane. As regard to fatty acid compounds, nine compounds were detected such as 9-octadecenoicAcid, methyl-6,9,12,15-docostetraenoate, hexadecadienoic acid methyl ester, methyl-9,9,10,10-octadecanoate, hexadecanoicacidtrimethylsilylester,ricinoleic acid, 10-octadecenoic acid methyl ester, cis-6-octadecenoicacid trimethylsilyl ester and Propyl oleate. In addition to a plausible percentage of essential oils were also detected represent 6.06%, found as three compounds which are trans-geranylgeraniol (3.33%), hexahydrofarnesol (1.86%) and dihydrocarvyl acetate (0.87%).The chemical composition of any extract principally depends on the type of solvent and this is the main factor of extract effectiveness. In the study of Samein and Kamel [51], volatile oils were separated using diethyl ether. Four major components (carvacrol, p-Cymene, Camphor and thymol) alongside other minor compounds have been detected. Chemical constituents were also investigated in *in vitro* produced plant of *Thymus vulgaris* L. 54 different compounds in total were identified [52]. The major constituents were thymol (33.37%), γ-terpinene (11.62%), p-cymene (9.81%) and carvacrol (5.63%). Also Soomro *et al.* [53] examined different extracts (*n*-hexane, chloroform, ethyl acetate, and *n*-butanol) as immunomodulation and anti-bacterial properties and reported that, all extracts except butanol have properties as immunomodulatory agents. Ethyl acetate and *n*-butanol fractions displayed significant activity especially against *E. coli*. Phytochemical analysis exhibited positive results for alkaloids, glycosides, tannins, flavonoids, triterpenes and sterols.

Table (6) Phytochemical constituents of in vitro thyme shootlets extracted with diethyl ether.

Compound	Peak Area (%)	Molecular Formula
Sonapax	3.38	C21H26N2S2
Dihydrocarvyl acetate	0.87	C12H20O2
Docosane	13.45	C22H46
11-Octadecenal	0.84	C18H34O
9-Octadecenal	3.76	C18H34O
2-Undecenal	6.30	C11H20O

9-Octadecenoic acid	1.01	C18H34O2
Methyl-6,9,12,15-docosatetranoate	1.12	C23H38O2
Cholestan-3-ol,2-methylen	1.46	C28H48O
Hexadecadienoicacid,methyl ester	4.14	C17H30O2
8-Heptadecene	1.36	C17H34
Benzoic acid-3,4,5-trimethoxy,methylester	2.74	C11H14O5
Isobutyl phthalate	0.92	C16H22O4
Nonadecane	1.03	C19H40
Methyl-9,9,10,10-octadecanoate	2.28	C19H34D4O2
1,2-Benzenedicarboxylicacid, dibutyl ester	1.34	C16H22O4
Hexahydrofarnesol	1.86	C15H32O
Ethylene brassylate	1.84	C15H26O4
Hexadecanoicacid,trimethylsilyl ester	4.66	C19H40O2Si
Ricinoleic acid	1.70	C18H34O3
Aspidofractinin-3-one	1.87	C19H22N2O
10-Octadecenoicacid,methyl ester	2.15	C19H36O2
Trans-Geranylgeraniol	3.33	C20H34O
Cis-6-Octadecenoicacid,trimethylsilyl ester	1.17	C21H42O2Si
Propyl oleate	2.23	C21H40O2
Nonacosane	21.7	C29H60
5-Cyano-3,19-dioxoandrostan-17-yl acetate	1.16	C22H29NO4
1,2-Benzenedicarboxylicacid, dioctyl ester	3.67	C24H38O4
1,3-Dithiane-2-phenyl	1.49	C10H12S2
Squalene	3.60	C30H50

3-3- Phytochemical constituents of in vitro basil shootlets.

Table (7) shows the most important constituents *of Ocimum basilicum* shootlets which contains 20 different components. Among these compounds, dimethyl cyclohexene-1, 4-dicarboxylate, benzoyl isocyanate and 10-octadecenoic acid methyl ester displayed the maximum percentages which were 39.02%, 10.44% and 7.98% respectively. It is also noticeable that, there are fatty acids, glycosides, benzoic acid derivatives, cyanide compounds and other constituents which found as minor compounds. Between these minor components, fatty acids represent the highest amount about 17.86% including 2,3-dihydroxy-2-methyl pentanoic acid,9,12-octadecadienoic acid methyl ester, 10-octadecenoic acid methyl ester and heptadecanoic acid-14-methyl ester. Chemical composition in various extracts (petroleum ether, chloroform, ethyl acetate and methanol) of *Ocimum basilicum* leaves previously studied by Purushothaman *et al.* [54], among these extracts petroleum ether and ethyl acetate displayed different class of secondary metabolites including Alkaloids, flavonoids, steroids, cardiac glycosides and phenolics. In another investigation, the chemical constituent of *Ocimum minimum* L. diethyl ether extract was characterized by GC-MS [55]. Thirty-two compounds were detected, the major of them were linalool (24.7%), eugenol (20.5%), 1,8-cineole (9.6%), β-elemene (6.6%) and *trans*-α-cubebene (6.3%).Other fractions isolated from *Ocimum basilicum* such as ethanol and hexane extracts, some phytochemicals were detected in common, which were diterpenes, flavonoids, glycosides, phenols, saponins, steroids and tannins, both extracts displayed Anti-Inflammatory, Antioxidant, and Anthelmintic activities [56].

Table (7) Phytochemical constituents of in vitro basil shootlets extracted with diethyl ether.

Compound	Peak Area (%)	Molecular Formula
dimethyl cyclohexene-1,4-dicarboxylate	39.02	C10H14O4
Methyl-2cyano4methyl-4-phenylcrotonate	0.77	C13H13NO2
Thiocoumarin	1.11	C9H6OS
α-D-Glucopyranoside, methyl--2,3,4,6-tetra-O-methyl	3.46	C11H22O6
2,3-Dihydroxy-2-methylpentanoic acid	0.71	C6H12O4
A-D-Fructofuranoside, methyl-1,3,4,6-tetra-O-methyl	1.6	C11H22O6
2,3-Dihydroxy-2-methylpentanoic acid	1.50	C6H12O4
Benzenepropanoic acid, α-methyl, ethyl ester	0.90	C7H15NO5
2-Allyl-2,4-dihydro-7-methoxy1methyl-3-Hpyrido [3,4b] indole	2.36	C16H18N2O
Benzoyl isocyanate	10.44	C14H14N2O
5-Cyano-2--dimethylamino4-methoxy-6-H-1,3-oxazinone	1.13	C8H9N3O3
Dimethyl-2-Ferrocenophane	0.93	C14H16Fe
Valtrate	0.57	C22H30O8
Trideuteriomethoxycyclohexane	4.03	C7H11D3O
(2R,5R)2(Methoxymethyl)-5-methyl-2-nonyltetrahydropyran	3.82	C18H36O3
9,12-Octadecadienoic acid, methyl ester	4.78	C19H34O2
10-Octadecenoic acid, methyl ester	7.98	C19H36O2
Heptadecanoic acid, 14-methyl, methyl ester	3.50	C19H38O2
Phthalic acid, bis(2-ethylhexyl) ester	2.71	C24H38O4
N-Benzyl dilactone	0.58	C30H27NO4
Benzoic acid-2(12-heptadecenyl) 6-methoxy, methyl ester	0.90	C26H42O3

Table (8) Inhibition zone of pathogenic microbial strains induced by extracts of carnation, thyme and basil multiple shootlets.

Microbial pathogenic strains	Mean halo Diameter of inhibition zone (mm) by extracts of in vitro shootlets		
	Carnation	Thyme	Basil
Bacillus subtlius	0	0	16.133±0.696
Staphylococcus aureus	0	16.2±0.757	20.366±0.856
Pseudomonas floureceans	0	0	18.1±0.493
Escherichia coli	0	17.3±0.862	0
Candida albicans	16.4±0.832	21.666±0.927	23.466±0.837

Obviously from data in Tables (5, 6 and 7) that diethyl etherextracts derived from multiplied shootlets of carnation, thyme and basil proved the presence of lots of diverse bioactive substances such fatty acid derivatives, essential oils, benzoic acid derivatives, cyclohexane carboxylic acid, volatile constituents and other essential hydrocarbons. Diethyl ether extracts of those bioactive compounds were assayed for *in vitro* antimicrobial activity in the following study.

4- Screening of antimicrobial efficiency of carnation, thyme and basil shootlets extracts.

Diethyl ether extracts of carnation, thyme and basil in vitro multiple shoots were screened their ability to inhibit some of pathogens such *Bacillus subtilis* and *Staphylococcus aureus* as Gram-positive bacteria*, Pseudomonas floureceans* and *Escheria coli* as Gram-negative bacteria and yeast such *Candida albicans* by using the diffusion method in agar. The antimicrobial activity of carnation, thyme and basil extracts against the determinant microorganisms was estimated in this work quantitatively by the absence or presence of repression zones and zone diameter, the results are given in Table (8).

The inhibition potency of all extracts against the pathogenic microbial strains exhibited different multi responses, carnation shootlets extract affected negatively on all examined microbial strains excluding *Candida albicans* (16.4±0.832), thyme shootlets extract inhibited the growth of one Gram-positive bacteria (*Staphylococcus aureus)* and one Gram- negative bacteria *(Escherechia coli)* by 16.2±0.757mm, 17.3±0.862mm, respectively, beside the inhibition zone by 21.666±0.927mm of *Candida albicans*. Except of *Escherichia coli*, basil shootlets extract suppressed all tested microbial strains giving inhibition zones with different halo diameter, the highest value of inhibition zone was 23.466±0.837mm of *Candida albicans*, followed by 20.366±0.856mm of *Staphylococcus aureus,* then18.1±0.493mm of *Pseudomonas floureceans,* whilst the least value was16.133±0.696mm of *Bacillus subtlius*.

Our results regarding the antimicrobial activity of carnation, thyme and basil multiple shoots proved their extracts more potent efficiency to inhibit the microbial growth, those are in agreement with the confirmed results by Mohsenipour and Hassanshahian [57] who revealed that the antimicrobial characteristics of thyme extract against *Staphylococcus* aureus, *Escherichia coli*, *Bacillus subtilis* and *Candida albicans*, beside [58 , 59] who indicated to the antimicrobial properties enhancement by essential oils of thyme plant.

As well our findings are mostly similar to the obtained results by Nascimento *et al.* [60] who detected the antimicrobial capacity of the extracts from thyme, basil, clove, rosemary, etc., against at least one of fourteen microbial species such as *Bacillus subtlius*, *Staphylococcus aureus*, *Pseudomonas floureceans*, *Escherichia coli* and *Candida albicans*, etc., Kaya *et al.* [61] who exhibited the antimicrobial activity of *Ocimum basilucum* extract against these microorganisms, *Pseudomonas aeruginosa, Staphylococcus aureus Shigella sp., Listeria monocytogenes* and *Escherichia coli*.

Based on the obtained results, authors indicated to presence of substantial relationship between carnation, thyme and basil shootlets content of bioactive compounds which have been detected previously (Tables, 5, 6 and 7) and their extracts capacity as antimicrobial activity agents (Table 8). Lastly, authors in this research recommended that most of valuable phytochemical constituents, extracted from carnation, thyme and basil shootlets using diethyl ether solvent and identified by GC-MS may be consider the responsible of antimicrobial activity as mainstay and durable potential in the extracts of carnation, thyme and basil shootlets against the microbial growth.

Conclusion

Briefly, this study highlights three Egyptian herbal medicinal plants (carnation, thyme and basil) which act as safety and effectiveness promising herbal drugs. On the basis of our investigations, it was reached to an efficient protocol for optimization the in vitro propagation and multiplication of the three plant species. This protocol documented a stable supplier as natural provider of these valuable plants in limited area and time for commercial utilization in personal care, nutritional supplements and pharmaceutical preparations for future medical applications. Our current study achieved a reliable and influential protocol of multiple shoot cultures for the three species, their extracts investigated an impressive performance for microbial suppression against some determinant pathogenic strains due to their content of worthy phytocomponents which have been extracted by diethyl ether solvent and identified by GC-MS. In conclusion, such extracts ought to consider as promising natural materials for chemo drug replacements used widely in developing regions for prevention any infectious attack of microbes and treatment the chronic dangerous diseases.

Disclosure of conflict of interest: The authors declare that there is no competing interest.

References

1. Helton, L.R., Folk medicine and health beliefs: An Appalachian perspective. *J. Cult. Divers*. 3: 123-128. (1996).
2. Vickers, A., Botanical medicines for the treatment of cancer: Rationale, overview of current data, and methodological considerations

for phase I and II trials. *Canc. Investig*. 20: 1069-1079. (2002).

3. El-Shemy, H.A.,. Aboul-Enein, A.M., Aboul-Enein,K.M. and Fujita,K., Willow leaves' extracts contain anti-Tumor agents effective against three cell types. *Plos One*. 2(1): 178. (2007).
4. Makri, O. and Kintzios, S., *Ocimum* sp. (basil): botany, cultivation, pharmaceutical properties, and biotechnology. *J. Herbs Spices med. Plants* 13: 123-150. (2007).
5. Al-Snafi, A.E. Chemical contents and medical importance of *Dianthus caryophyllus*- A review. *IOSR Journal of Pharmacy*. 7: 61-71.(2013)
6. Dauqan, E. M. A. and A.Abdullah. Medicinal and Functional Values of Thyme (*Thymus vulgaris* L.) Herb. Journal of Applied Biology & Biotechnology. 5 (2): 17-22. (2017).
7. Juven, B.J., Kanner,J., Schveol,F., and Weisslowicz,H., Factors that interact with the antibacterial action of thyme essential oils and its active constituents. *The Journal of Applied Bacteriology*. 76 (6): 626-631.(1994)
8. Juliani, H.R., Koroch,A.R. and. Simon,J.E., Basil: A new source of rosmarinic acid - In: Ho, C.T., Simon, J.E., Shahidi, F.,Shao, Y. (ed.): Dietary Supplements. Pp. 129-143. *American Chemical Society, Washington*.(2008)
9. Siddiqui, B.S., Aslam,H., Ali,S.T., Begum,S. and Khatoon,N., Two new triterpenoids and a steroidal glycoside from the aerial parts of *Ocimum basilicum*. *Chemical and Pharmaceutical Bulletin (Tokyo)*. 55: 516-519.(2007)
10. Zheljazkov, V.D., Callahan,A. and Cantrell,C.L., Yield and oil composition of 38 basil (*Ocimum basilicum* L.) accessions grown in Mississippi. *J. Agric. Food Chem.* 56: 241–245.(2008)
11. Deshpande, R.S. and Tipnis,H.P., Insecticidal activity of *Ocimum basilicum* L. *Pesticides*. 12: 21-28. (1997)
12. Chatterjee, A., Sukul,N.C., Laskar,S. and Ghoshma,J.S., Nematicidal principles from two species of Lamiaceae. *Journal of Nematology*. 14: 118–20. (1982).
13. Reuveni, R., Fleischerand,A. ,Putievsky,E., Fungistatic Activity of Essential Oils from *Ocimum basilicum* Chemotypes. *Journal of phytopathology*. 110 (1): 20-22. .,(1984).
14. Mirzaei-Aghsaghali A., Alireza Syadati,S. and Fathi,H., Some of thyme (Thymus vulgaris) properties in ruminant's nutrition. *Annals of Biology Researches*. 3(2):1191-1195. (2012).
15. Nicolič M., J.Glamočlia,J., I.C.F.R.Ferreria,I.C.F., C.R.Calhelha,C.R., A. Fernandes,A., Markovič,D., Markovič,T., Giweli,A. and Sokovič,M., Chemical composition, antimicrobial, antioxidant and antitumor activity of*Thymus serpyllum* L., Thymus *algeriensis*Boiss. And Reut and *Thymus vulgaris* L. essential oils. *Industrial Crop Products*. 52:183-190. (2014).
16. Tzima, K., Makris,D., Nikiforidis,C.V. and Mourtzinos,I., Potential use of Rosemary, Propolis and Thyme as Natural Food Preservatives. *J Nutri Health.* 1(1): 6. (2015).
17. Sahoo, Y., Pattnaik,S. and Chand,P., In vitro clonal propagation of an aromatic medicinal herb *Ocimum basilicum* L. (sweet basil) by axillary shoot proliferation. *In Vitro Cell Dev Biol Plant.* 33:293–296. (1997).
18. Jaggi, D. , A Micropropagation System for Carnation (Dianthus caryophyllus L.)-an Important Ornamental Plant. M.Sc. Thesis. Department of Biotechnology and Environmental Sciences, Thapar University, Patiala.78p. (1997).
19. Ozudogru, E.A., Kaya,E., Kirdok,E. and Issever- Ozturk,S., *In vitro* propagation from young and mature explants of thyme (*Thymusvulgaris* and *T. longicaulis*) resulting in genetically stable shoots. *In Vitro Cellular and Developmental Biology – Plant.* 47(2):309–320. (2011).
20. Begum, F., Amin,M.N. and Azad,M.A.K.,. *In vitro* rapid clonal propagation of *Ocimum basilicum* L. *Plant Cell Tissue Organ Cult.* 12: 27-35. (2002).
21. Siddique, I. and Anis,M., Rapid micropropagation of *Ocimum basilicum* using shoot tip explants pre-cultured in thidiazuron supplemented liquid medium. *Biologia Plantarum*. 51:787–790. (2008).
22. Kasem, M. M. (2006). In vitro culture of basil plants. M.Sc. Thesis Fac. of Agric., Mansoura Univ., Egypt.
23. Sharzad, A. and S.A.Siddiqui,S.A., *In vitro* organogenesis in *Ocimum sanctum* L. – A multipurpose heb. *Phytomorphology,* Delhi.50(1): 27-35. (2000).
24. Phippen,W.B. and Simon,J.E., Shoot regeneration of young leaf explants from basil (*Ocimum basilicum L.*). *In Vitro Cell Dev Biol Plant.* 36: 250–254. (2000).
25. Singh, N.K. and Sehgal,C.B., Micropropagation of "Holy basil" (*Ocimum sanctum* L.) from young inflorescens of mature plants. *Plant Growth Regul.,* (1999) .
26. Wankhede, M., Patil,S. and Lakshmi,K., *In vitro* propagation of carnation cv. Supergreen. Journal of Soilsand Crops.16: 165–169. (2006).
27. Zaid,H., Silbermann,M., Amash,A., Gincel,D., Abdel-Sattar,E. and Sarikahya,N.B. Medicinal Plants and Natural Active Compounds for Cancer Chemoprevention/Chemotherapy. Evidence-Based Complementary and Alternative Medicine Volume 2017, Article ID 7952417, 2 pages. (2017)

28. Murashige, T. and Skoog,F., A reversed medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantanum* 15: 473-479. (1962).
29. Miller, G.L. (1972). Biochemical methods for agricultural sciences. *Wiley Eastern Ltd*, New Delhi. 6–100.
30. Greenwood, D. (1983). Antimicrobial Chemotherapy. In: Greenwood, D. (Ed.), Laboratory Aspects of Antimicrobial Therapy. Part II, Bailliere, Tindall, London, p. 71.
31. Gomez, K.A. and Gomez,A.A., Statistical Procedures for Agricultural Research, John Wiley and sons, New York, USA. (1984).
32. Waller, R.A. and Duncan,D.B.,. A boys rule for symmetric multiple comparison problem. *An. State Assoc. J.* 65: 1485-1503. (1969).
33. Aicha, N., EL Meskaoui, M.H. and Abdelmalek,M., *In vitro* clonal propagation through direct shoot organogenesis of *thymus broussonetii* – a vulnerable aromatic and medicinal Plant species. *International journal of pharmaceutical research and bio-science*. 3(1): 425-439. (2014).
34. Asghari F., Hossieni,B., Hassani,A., and Shirzad,H., Effect of explants source and different hormonal combinations on direct regeneration of basil plants (*Ocimum basilicum* L.). *Australian Journal of Agricultural Engineering*. 3(1):12-17(2012).
35. Saez, F., Sknchez,P., and Piqueras,A., Micropropagation of *Thymus piperella*. *Plant Cell, Tissue and Organ Culture.* 39(3):269−272. (1994).
36. Coelho, N., Goncalves,S., Gonzalez-Benito,M.E. and Romano,A., Establishment of an *in vitro* propagation protocol for *Thymuslotocephalus*, a rare aromatic species of the Algarve (Portugal). *Plant Growth Regulation.* 66(1):69−74. (2012).
37. Zarooshan, M., Bernard,F. and Heydarian,Z., Effects of benzyl adenine and salisylic acid on hyperhydricity syndrome in micro propagated stem culture of *Thymus daenensis* Celak. *Iranian Journal of Medicinal and Aromatic Plants.* 29(1):130-143(Persian). (2013).
38. Shetty, K., Carpenter,T.L., Kwok,D., Curtis,O.F. and Potter,T., Selection of high phenolic-containing clones of Thyme (*Thymus vulgaris* L.) using *pseudomonas Sp*. *J. Agric. Food Chem*. 44 (10): 3408–3411.(1996).
39. Skoog, F.H., Hamzi,Q., Srey Kowswka,A.M., Leonard,N.J., Carraway,K.L., Fuji,T., Helgenson,J.P. and Leoppky,R.N., Cytokinin structure activity relationships. *Phytochemistry*. 6: 1109-1192. (1967).
40. Ibrahim, S.M.M., Taha,L.S. and Farahat,M.M., Vegetative growth and chemical constituents of croton plants as affected by foliar application of benzyl adenine and gibberellic acid. *J. Amer. Sci*. 6: 126-130. (2010).
41. George, E. (1993). Plant propagation by tissue culture. Part 1. The Technology Exegetics Ltd., Edington, Wilts BA 13.4 Qg, England.
42. Gangaprasad, A., Lakshmi,G.N., Radhakrishnan,K., Seeni,S., Nair,G.M. and Pushpangadan,P. , Micropropagation of Utleria Salicifolia, An endemic endangered ethanomedicinal plant of the Western Ghats. *J. Med Aromatic Plant Sci.* 25: 19–24. (2003).
43. Abdel-Motagaly, Sara, Yasmin,M., Abdellatif,M.R., Manaf,H.H. and Ibrahim,I.S., Effect of benzyladenine on micropropagation of banana shoots tip .*Arab Univ. J. Agric. Sci.* (AUJAS), Ain Shams Univ., Cairo, Egypt Special Issue. 26(2D): 2557-2564. (2019).
44. Caers, M. and Vendrig,J., Benzyladenine effects on the development of the photosynthetic apparatus in Zea mays: studies on photosynthetic activity, enzymes and (etio) chloroplast ultrastructure. *Physiologia Plantarum*, 66(4): 685-691. (1986).
45. Dhed'a, D., Dumortier,F., Panis,B., Vuylsteke,D. and De Langhe, E.,. Plant regeneration in cell suspension cultures of cooking banana 'Bluggoe' cultivar (Musa spp. ABB group). *Fruits*. 46: 125-135. (1991).
46. Zuker, A., Tzfira,T., Ben-Meir,H., Ovadis,M., Shklarman,E., Itzhaki,H., Forkmann,G., Martens,S., Neta-Sharir,I., Weiss,D. and Vainstein,A., (Modification of flowercolor and fragrance by antisense suppression of the flavanone 3-hydroxylase gene. *Mol Breed*. 9: 33-41. (2002).
47. El-Ghorab, A.H., Mahgoub,M.H., and Bekheta,M.,. Effect of Some Bioregulators on the Chemical Composition of Essential Oil and its Antioxidant Activity of Egyptian Carnation (*Dianthus caryophyllus* L.). *Journal of essential oil-bearing plants.* 9 (3): 214 – 222. (2006).
48. Ibrahim, M.E.. Agrochemical studies on *Dianthus caryophyllus* L. grown in Egypt. *Int J PharmTech Res.* 9(4): 113-117. (2016).
49. Galeotti, F., Barile,E., Curir,P., Dolci ,M. and Lanzotti,V., Flavonoids from carnation (*Dianthus caryophyllus*) and their antifungal activity. *Phytochemistry Letters*. 1: 44–48. (2006).
50. Curir, P., Dolci,M., Dolci,P., Lanzotti,V., and L.De Cooman,L., Fungitoxic phenols from carnation (*Dianthus caryophyllus*) effective against Fusarium oxysporum f. sp. dianthi. *Phytochem Anal.* 14(1):8-12. (2003).
51. Samein, N.M. and Kamel, F.H., Extraction of compounds from thyme leaves and their antimicrobial activity. Diyala *Journal of Agricultural Sciences.* 11(1): 18-24. (2019).

52. Kulpa, D., Wesołowska,A. and Jadczak,P., Micropropagation and composition of essentials oils in garden thyme (*Thymus vulgaris* L.). *Not Bot Horti Agrobo*. 46(2):525-532. (2018).
53. Soomro, S., Chidrawar,V.R., Imran,M. and Alshammari,H.O., Immunomodulating, anti-bacterial and anti-cancer potential of za'ater (*Thymus vulgaris*) and its combination with essential oil (olive and balsam oil). *International Journal of Pharmaceutical Science and Research*. 10(8): 3721-3726. (2019).
54.Purushothaman, B., Suganthi,N. and Shanmugam,K., Qualitative and quantitative determination of various extracts of *Ocimum basilicum L.* leaves. *Journal of natural remedies*. 20 (1): 53-60. (2019).
55. Skalicka-Woźniak, K., Ludwiczuk,A., Widelski,J., Filipe,J.J., Asakawa,Y. and Głowniak,K., Volatile constituents of *Ocimum minimum* herb cultivated in Portugal. *Natural Product Communications*, 4 (10): 1383 – 1386. (2009).
56. Akoto, C.O., Acheampong,A., Boakye,Y.D., Naazo,A.A. and Adomah,D.H., Anti-Inflammatory, Antioxidant, and Anthelmintic Activities of *Ocimum basilicum* (Sweet Basil) Fruits. *Journal of Chemistry*.1-9. (2020).
57. Mohsenipour, Z. and Hassanshahian,M., The inhibitory effect of Thymus vulgaris extracts on the planktonic form and biofilm structures of six human pathogenic bacteria, *Avicenna journal phytomedicine*. 5(4): 309–318. (2015).
58. Ismail M.M., EssamT.M., Mohamed,A.F. and Mourad,F.E., Screening for the antimicrobial activities of alcoholic and aqueous extracts of some common spices in Egypt. *Int J Microbiol Res*. 3:200-207. (2012).
59. Priti, V., Use of essential oils against gram negative pathogens. *J. Drug Deliv. Therap.* 2:134-137. (2012).
60 Nascimento, G.G.F., Locatelli,J., Freitas,P.C. and Silva,G.L., Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Braz. J. Microbiol*. 31: 247-256. (2000).
61. Kaya, I., Yiğit,N. and Benli,M., Antimicrobial activity of various extracts of *Ocimum basilicum*L. And observation of the inhibition effect on bacterial cells by use of scanning electron microscopy. Traditional, Complementary and Alternative Medicines. *Afr. J. Trad. CAM*. 5 (4): 363 – 369. (2008).