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GLC STUDY AND BIOLOGICAL ACTIVITIES OF THE VOLATILE OIL COMPONENTS OF SOME *IN VIVO* AND *IN VITRO* TISSUES OF *FOENICULUM VULGARE* MILL. VAR. *VULGARE*

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ABSTRACT

Foeniculum vulgare Mill. is a wild, annual herb native to the Mediterranean area. The plant is commonly known as the bitter fennel or the common fennel, known in Egypt as "shamar".[1] This plant is rich in important pharmacologically active secondary metabolites as volatile oils, resins, coumarins, terpenes, sesquiterpenes, triterpenoid, saponins and acetylenic compounds; while alkaloids are.[2] This study focused on comparing the volatile oil constituents of different plant parts of wild, cultivated plant as well as *in vitro* induced calluses and plantlets using gas chromatography-mass spectroscopy (GLC/MS). Also, biological study of the plant volatile oils was performed to screen different activities like analgesic, anti-inflammatory, antipyretic, antispasmodic and anxiolytic activities which were highly significant. Moreover, a comparative antimicrobial study was conducted to extracts of both the wild plant and the micro-propagated plants. The observations were highly valuable as extracts of the micro-propagated plants showed stronger activity than the extracts of the wild plant against the tested microorganisms and also showed activity against *Candida albicans* contrary from the wild plant extracts that showed no activity against it.

KEYWORDS: *Foeniculum vulgare*, Volatile oils, Callus, *In vitro* propagation, GLC-MS of extracts, Biological activity.

INTRODUCTION

Foeniculum vulgare Mill. is an aromatic perennial herb native to the Mediterranean region and growing as annual or perennial herb worldwide.[3] The plant is rich in medicinally valuable volatile oils componenets such as anethole, fenchone, limonene, α-pinene, β-pinene, phellandrene, *p*-cymene, anisaldehyde and many others.[4] These volatile oils constituents are characterized by their biological activities as analgesic, anti-inflammatory, C.N.S, spasmolytic, antibacterial, antifungal, antiviral, hepatoprotective, anticancer and antioxidant activities.[5] This study aimed to compare the constituents of different *in vitro* and *in vivo* tissues through GLC/MS prior to test their pharmacological and antimicrobial activities.

MATERIAL

1. Plant material

The plant material used in this work, *Foeniculum vulgare* Mill. var. *vulgare* (wild), family Apiaceae, was collected on April 2013 from Sidi Barrani, north coast, Egypt and *Foeniculum vulgare* Mill. var. *vulgare* (cultivated), family Apiaceae, was collected on April 2013 from a farm in Shirbin, between Tanta and El-Mansoura, Egypt. The identification was verified by Assistant Prof. Dr. Eman Shams, Assistant Professor of plant taxonomy, Faculty of science, Cairo university. Voucher specimens are deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Zagazig University, Egypt.

2. Material for plant tissue culture

Seeds (Fruits) of *Foeniculum vulgare* Mill. var. *vulgare* (wild), family Apiaceae were used for establishment of plant tissue cultures.

Commercially available **Murashige and Skoog** (MS) powdered medium (Duchefa); Plant **growth regulator** such as Gibberellic acid (GA3), 6-Benzylaminopurine (BAP), Kinetin (K), Thidiazuron (TDZ), Naphthalene acetic acid (NAA) and 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma Chemical Co, U.S.A); **Sucrose** (Adwic, A.R.E.); **Agar** (purified agar for plant tissue culture, Bioworld, USA) and Commercial **hypochlorite solution** (Clorox®, which contains 5% sodium or calcium hypochlorite).

3. Material for GLC analysis

GLC method was used in the comparative study of the volatile oil components of the following materials isolated from *in vivo* and *in vitro* tissues of *Foeniculum vulgare* Mill.

- The *in vivo* tissues include

Fresh collected aerial parts of the wild plant, shade dried ripe fruits of the wild plant as well as the dried ripe fruits of the cultivated plant were ground separately by electric mill to moderately fine powder and extracted by Clevenger's apparatus for collection of the hydro-distilled volatile oils .

- The *in vitro* tissues include

Twelve weeks old calluses obtained from leaf, root and stem explants cultured on different hormonal combinations including (2,4-D 0.5+K 0.5), (2,4-D 1+K 1), (2,4-D 2+BAP 0.25 and (TDZ 0.5+ 2,4-D 1+BAP 0.1). Plantlets derived from indirect organogenesis of calluses of the hormonal combination NAA 1+BAP 0.1 from leaf, stem and root explants. *In vitro* propagated plantlets via somatic embryogenesis derived from (TDZ 0.5+2,4-D 1+BAP 0.1) of stem explants callus after 8 weeks from transferring on hormonal free medium.

4. Materials for biological activities

4.1. Analgesic activity

The hydro-distilled volatile oil of the fruits of the wild fennel. Eighteen adult male mice weighing 25 – 35 gm were provided by the Faculty of Veterinary Medicine, Zagazig University, Egypt. Acetic acid (0.8%), saline, tween 80 (1%) and paracetamol (Sigma drug company, A.R.E).

4.2. Anti-inflammatory activity

The hydro-distilled volatile oil of the fruits of the wild fennel. Fifteen male albino rats weighing 140-160 gm were provided by the Faculty of Veterinary Medicine, Zagazig University, Egypt. Diclofenac sodium (Farco drug company, Cairo, A.R.E), tween 80 (1%) and carrageenan (1% suspension in saline).

4.3. Antipyretic activity

The hydro-distilled volatile oil of the fruits of the wild fennel. Eighteen adult male mice weighing 25–35 gm were provided by the Faculty of Veterinary Medicine, Zagazig University, Egypt.Brewer's yeast (20%) and paracetamol (Sigma drug company, A.R.E).

4.4. Anxiolytic activity

The hydro-distilled volatile oil of the fruits of the wild fennel. Twenty adult male mice weighing 25 – 35 gm were provided by the Faculty of Veterinary Medicine, Zagazig University, Egypt and diazepam (Amoun Pharmaceutical Company, Cairo, A.R.E).

4.5. Antispasmodic activity

Two cm. rabbit intestine muscle was isolated and suspended in organ bath. The preparation was supplied by Tyrode's solution and oxygen; and temperature of the Tyrode's was kept at 37˚c. The hydro-distilled volatile oil of the fruits of the wild fennel, Acetyl choline (0.001% solution), Aropine (0.0003% solution) and Bacl2 (3%).

4.6. Antibacterial and antifungal activities

The hydro-distilled volatile oil of the fruits of the wild fennel as well as the hexane extract of the micro-propagated plantlets of the hormonal combination (TDZ 0.5+ 2,4-D 1+BAP 0.1) of the stem explants of the wild fennel were used. *Aspergillus fumigatus* (RCMB 02569) and *Candida albicans* (RCMB 05039) as fungi (Regional Centre for Mycology and Biotechnology (RCMB) at Antimicrobial Unit to test microorganisms, (Al-Azhar University). *Streptococcus pneumoniae* (RCMB 010016) and *Bacillus subtilis* (RCMB 010068) as Gram positive bacteria (RCMB at Antimicrobial Unit to test microorganisms, Al-Azhar University). *Pseudomonas aeruginosa* (RCMB 010048) and *Escherichia coli* (RCMB 010052), as Gram negative bacteria (RCMB at Antimicrobial Unit to test microorganisms, Al-Azhar University). Dimethyl sulphoxide (DMSO), nutrient agar medium (Oxoid laboratories, UK), Saboroud dextrose agar (Oxoid laboratories, UK), Amphotericin B, ampicillin and gentamicin.

INSTRUMENTS

1. GLC

The GLC was carried out on Agilent 6890 gas chromatograph with fused silica capillary column PAS-5 ms (30 mm × 0.25 um film thickness). The carrier gas was Helium with1 ml/ min flow rate. The sample injection size was 1 µl. Oven temperature Program started at 60^0C then elevated to 280^0C at rate of 8^0C/ min. The Injector temperature was adjusted at 250^0C while the detector temperature was at 280^0C. The detector used was Mass spectrophotometric, scanning from m/z 50 to 500, EI 70 ev.

2. Equipments for biological study

2.1. Anti-inflammatory activity

- Micrometer (made in Germany).

2.2. Antipyretic activity

- Electro-couple thermometer.

2.3. Anxiolytic activity

- OFT box (24 cm x 32 cm; 12 squares).

METHODS

1. Methods for tissue culture.[6]

1.1. Sterilization of the seeds

Seeds of *Foeniculum vulgare* Mill. var. *vulgare* (wild) were sterilized by mixing with 70 % ethyl alcohol for 3 minutes then shaking with 5% commercial hypochlorite solution (**Clorox®)** containing 2 drops of 1% tween 20 for 25 minutes. Under the laminar flow hood, sodium hypochlorite was poured away from seeds then seeds were rinsed three times with sterile double distilled water before applying to media for germination.

1.2 Seed germination

Sterilized seeds were transferred to sterile solid media consists of 4.4 gm/l MS media, 30 gm/l sucrose and 8 gm/l agar. The pH was adjusted at 5.7-5.8 and the jars

were kept in the dark for 3 days then incubated at 25°C under white fluorescent lamp with a 16 hours photoperiod.

1.3 Induction of callus from the *in vitro* germinated seedlings

Uniformly sized explants (0.5-1 cm) were dissected from the different organs of the 4 weeks old seedlings under sterile conditions to produce three types of explants which are leaf, stem and root. The different explants were cultured in jars containing MS media 0.44% with 3% sucrose 0.8% agar and supplemented with different plant growth regulators (**Medium I**: 0.5mg/l 2, 4 D + 0.5 mg/l Kinetin, **Medium II** 1mg/l 2, 4 D + 1 mg/l Kinetin, **Medium III**: 1mg/l NAA + 0.1mg/l BAP, **Medium IV**: 0.5 mg/l TDZ + 1 mg/l 2, 4 D + 0.1 mg/l BAP and **Medium V**: 2,4-D 2+BAP 0.25). In each media the pH was adjusted to 5.7-5.8 and the cultures were incubated at 25±1°C under white fluorescent lamp with a 16 hours photoperiod for 4 weeks. The calli was transferred to fresh media of the same composition every 4 weeks.

1.4 *In vitro* regeneration of *Foeniculum vulgare* callus

After callus was successfully produced, representing stage I of indirect micropropagation, stage II began and involved trials to make shoots, roots or somatic embryos from callus cells. Different conditions were applied on different callus cells produced in order to induce organogenesis or embryogenesis. Phytohormones were used solitary and in combinations with different concentrations and hormonal free media was also used with high light intensity to induce photosynthesis in the callus cells and thus encourage the cells to differentiate into shooting cells. Media with auxins only were used to induce rooting, media with cytokinins only were used to induce shooting and also some callus were transferred to hormonal free media. In each trial, cells from callus were tested under microscope to detect the production of somatic embryos.

2. Methods of GLC/MS

Volatile oil extraction:

-Fruits of cultivated fennel (150 gm) and wild fennel (500 gm) as well as aerial parts of the wild fennel (300 gm) were separately hydro-distilled in Clevenger's apparatus. Volatile oils of each were collected after 3 hrs and dried using anhydrous sodium sulphate. The oils were kept in refrigerator at 4˚ C till analysis.

- Extracts of calluses obtained from leaf, root and stem explants cultured on medium I, II, III and IV were prepared by the following procedures.

-Fresh calluses (40 gm of each 12 weeks old callus) were separately crushed in a mortar with double distilled n-hexane coupled with ultra sonic waves at 40˚c for 15 minutes, maintained in well closed jars in a shaker at low speed over night, then filtrated, concentrated to 1 ml and passed to GC-MS analysis.

-10 gm of *in vitro* regenerated plantlets derived from indirect organogenesis of medium III from leaf, stem and root explants calluses as well as 10 gm of *in vitro* regenerated plantlets derived from indirect somatic embryogenesis of medium IV from stem explants callus were separately extracted as previously mentioned in callus after 8 weeks from transferring on hormonal free medium.

The GC/MS analysis was carried out at The Central Agricultural Pesticide Laboratory (CAPL), Cairo, Egypt. Identification of the components was based on matching the fragmentation pattern in the resulted mass spectra with the published data[7] and using Wiley and Nist 05 mass spectral data base.

3. Methods of screening biological activities

3.1. The analgesic activity

The analgesic activity of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* was determined using the acetic acid induced writhing technique.[8] Acetic acid of 0.1 ml/10 g body weight of 0.8% solution in normal saline was used as an inducer for writhing and paracetamol was used as reference standard.

Eighteen adult male mice weighing 25 – 35 gm were used in this part of the study. A sensitivity test for acetic acid was carried out one day before experiment as follows: each mouse was injected intraperitoneally by 0.1 ml/10 g body weight of 0.8% of the acetic acid. Mice were observed for 15 min.; the response in animals manifested as a contraction of the abdominal muscles and stretching of hind limbs, the mouse that did writhing was considered as positive. After 24 hours of the sensitivity test, acetic acid sensitive mice were divided into three groups (n= 6). The first group was given tween 80 (1%) in saline solution orally and served as control. The second group received the hydro-distilled volatile oil of the fruits of the wild fennel, emulsified in 1% tween 80, in a dose of 400 µl/kg orally. The third group received paracetamol, in a dose of 45 mg/kg orally. After one hour, acetic acid was injected and the number of writhes during the following 30 min period was counted.

3.2. The anti-inflammatory activity

The anti-inflammatory activity of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* on the rat paw edema induced by carrageenan was studied using the hind paw edema method.[9] Diclofenac sodium was used as a reference standard.

Fifteen male albino rats weighing 140-160 gm were used in this study. The rats were divided into three groups, five rats in each. The first group served as control and was given tween 80 (1%). The second group received diclofenac sodium at a dose of 7 mg/kg. The third group was given the hydro-distilled volatile oil of the fruits of the wild fennel (400 µl/kg) emulsified in 1% tween 80.

All treatments were given by means of oral administration.

Thirty minutes later, paw edema was induced by subcutaneous injection of 0.1 ml carrageenan (1% suspension in saline) into the sub-plantar surface of the right hind paw of all animals. The left legs of hind paw were injected by 0.1 ml normal saline. The hind paw diameter was measured, using a micrometer, just before the injection of carrgeenan and 1, 2, 3, 18 and 21 hrs after the injection. The hind paw diameter was measured for each rat at each time interval and the mean thickness of edema was calculated.

Since the time course of the effect was followed, it was possible to use the cumulative anti-inflammatory effect during the whole observation period as the area under the curve (AUC). Because the AUC curve represents the integrated anti-inflammatory effect (variation of paw diameter) during the observation period, it then includes both the maximal response and the duration of action.

The AUC relating variation of edema to time was obtained using the trapezoidal rule.[10] Total inhibition (TI, %) was obtained for each group and at each record, using the following ratio.

TI (%) = [AUC control −AUC treat] ×100/AUC control.
Data were expressed as mean ± standard error of mean (SEM) of five animals.

3.3. The antipyretic activity
Eighteen male and female mice, weighing 25-35 g were used in this study. The animals were fed standard diet and allowed free access to water, The mice were rendered hyperthermic using an adaptation of the method of.[11] In which the aqueous suspension of brewer's yeast 20% (w/v) was injected subcutaneous (s.c.) in the back of the animals in a dose of 20 mg/kg. The body temperature was measured using an electric thermocouple inserted into the rectum. The rectal temperature of the mice were recorded before the injection of the yeast suspension and 18 hours after its injection. The animals were divided into 3 groups, each consisting of 6 animals and the tested extracts were adminstered orally after 18 hrs from yeast injection, according to the following.
Group I: received tween 80(1%) in saline solution and served as control.
Group II: Recevied the the hydrodistilled volatile oil of the fruits of the wild fennel (400 µl/kg) emulsified in 1% tween 80.
Group III: received paracetamol 45 mg/k.g b. w. as standard antipyretic.

The rectal temperature of animals was recorded every hour in three hours.

Since the time course of the effect was followed, it was possible to use the cumulative antipyretic effect during the whole observation period as the area under the curve (AUC). Because the AUC curve represents the integrated antipyretic effect during the observation period, it then includes both the maximal response and the duration of action.

The AUC relating variation of rectal temperature to time was obtained using the trapezoidal rule.[10] Total inhibition (TI, %) was obtained for each group and at each record, using the following ratio.
TI (%) = [AUC control −AUC treat] ×100/AUC control.

3.4. The anxiolytic activity
Adult Swiss albino male mice weighting 25-35 g were housed under standard environmental conditions and were allowed free access to tap water and standard laboratory *ad libitum*. The mice were randomly divided into 4 groups (n = 5). The first group received the vehicle (1% Tween 80 in distilled water), the second group received standard drug, diazepam (0.5 mg/kg, I.p). Groups III and IV using oral gavage received 200 and 400µl/kg doses of the essential oil respectively.

Open field test (OFT)[12]
The present work has evaluated the anxiolytic activity of two doses of the hydrodistilled volatile oil of the fruits of the wild fennel (*Foeniculum vulgare* Mill. var.*vulgare)* in mice employing non-conditioned behavioral animal model of anxiety (OFT). This test is classic and standard model for screening central nervous system actions providing information about anxiety and psychomotor performance.[13] Further, this model can create an anxiety state in normal rodents in a reproducible paradigm while minimizing some of the confounding factors of other conditioned assays.[14]

The test was conducted as described by.[15] After 30 min of diazepam treatment or 60 min essential oil/vehicle pretreatment, the mice were individually placed in animal models. All the tests were carried out at early morning with a minimal amount of background noise. After each test, the OFT model was cleaned with ethyl alcohol to eliminate any olfactory cues to the next animal. Briefly, the mice were placed individually in a corner square of the OFT model and the total number of squares crossed at the periphery, number of central squares crossed, total number of squares traveled, and the total number of rearings were video recorded for 5 min.

3.5. The antispasmodic activity
The antispasmodic activity of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* was studied using the isolated rabbit intestine method.[16]

A suitable Dose Response Curve (DRC) of acetyl choline 0.001% solution (as agonist) was performed and the submaximal dose was selected.

Equal volumes of both of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* (1%) and 1% tween 80 were emulsified with each other. Different doses were injected and the inhibition in the muscle response was examined and compared with the submaximal dose of acetyl choline (Ach).

Barium chloride solution (3%) was injected as direct smooth muscle stimulant.

In the same experiment, indirect assay was performed using matching technique.

The test was carried out by comparing the dose of the hydro-distilled volatile oil of the fruits (1% emulsion) which causes 50% reduction to the concentration produced by acetyl choline 0.001% solution with that of the standard atropine (0.0003% solution) which causes the same response (50% reduction).

3.6. The antimicrobial activity

Antibacterial and antifungal activities of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* and the hexane extract of the micro-propagated plants were determined using the well diffusion method.[17] The tested extracts were dissolved in dimethyl sulfoxide (DMSO) at concentration of 1mg/ml. The tested organisms were subcultured on nutrient agar medium for bacteria and Saboroud dextrose agar for fungi. Ampicillin and Gentamicin were used as a positive control against Gram positive and Gram negative bacteria, respectively. Amphotericin B was used as a positive control for fungi. The plates were done in triplicate. Bacterial cultures were incubated at 37°C for 24 hrs, while the fungal cultures were incubated at 37°C for 2-7 days. Antibacterial and antifungal activities were determined by measuring the diameter of the inhibition zone formed around the well (mm). Mean zone of inhibition and standard deviations were calculated. Results were expressed in mean zone of inhibition in mm ± standard deviation (SD) beyond well diameter (6mm) produced on a range of environmental and clinically pathogenic microorganisms.

Preparation of the oil for the biological screening

Oil was mixed with tween 80 (1%) to be easily emulsified with water and adjusted to the selected concentrations for each experiment.

Animals used in the study

Rats and mice used in this study were held under standard laboratory conditions in the animal house of the Faculty of Pharmacy, Zagazig University at 27°C with 12/12 light-dark cycle. They were fed laboratory diet and water *ad libitum*.

Statistical analysis

The data are presented as mean ± S.E.M (standard error of mean). Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by Tukey posthoc test. Significant differences were set at P values lower than 0.05.

RESULTS AND DISCUSSION

1. Tissue culture of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare*.[6]

The optimum growth of seeds was obtained after 28 days culture on full strength solid MS media at 25 ± 1˚c and 16 hrs photoperiod.

The growth of callus was observed on all previously mentioned hormonal combinations but the best growth was achieved using medium IV and V.

Micropopagation has occurred either through indirect organogenesis on medium III or via indirect somatic embryogenesis on medium IV.

Micropropagated plantlets obtained from medium IV continued their growth and differentiation up on transfer to hormonal free media as an initial step of acclimatization.

Plantlets were transferred to pots containing autoclaved soil mixture to proceed in acclimatization but plantlets started to wilt within 10 days due to weakness of root system.

Hardening of root system has been done by culturing plantlets in liquid media containing NAA 1 mg/l and this step extended the plantlets survival in the soil to 20 days old but they started to wilt later.

2. GLC study of the volatile contents of some *in vivo* and *in vitro* tissues of *Foeniculum vulgare* Mill.

This study is concerned with the analysis of essential oils of the fruits and leaves of the cultivated and the wild plants, as well as *in vitro* induced calluses and *in vitro* regenerated plantlets.

The hydro-distillation of 500 gm of the air dried powdered fruit of the wild fennel resulted in the isolation of 6 ml of pale yellow oil with characteristic pleasant odour in a percentage of 1.2 % of dry weight. The GLC/MS of the essential oil of the fruit revealed the presence of 62 components, 39 of them were identified representing 97.1 % of oil composition (Table1).

The hydro-distillation of 300 gm of the air dried powdered aerial parts resulted in the isolation of 2.7 ml of yellow oil which is lighter than water in a percentage of 0.9 % of dry weight. The GC/MS of the essential oil of the fruit reveal the presence of 53 components, 37 of them were identified representing 97.31% of oil composition (Table1).

The hydro-distillation of 150 gm of the air dried powdered fruits of the cultivated fennel resulted in the isolation of 1.5 ml of yellow oil which is lighter than water in a percentage of 1 % of dry weight. The GC/MS of the essential oil of the fruit revealed the presence of 53 components, 29 of them were identified representing 80.16% of oil composition (Table1).

The analysis showed different medicinally valuable volatile oils components as shown in Table (1) such as *trans*-anethole, *l*-limonene, *beta*-myrcene, *alpha*-phellandrene, *alpha*-fenchone, *cis* and *trans* -thujone.

The most interesting point was that estragole which is a constituent responsible for many harmful effects as hepatocelluar carcinoma and genotoxcicity[18-19] was present in lower percentages in the fruits of the wild plant (**12.36%**) as well as the aerial parts (**38.61%**) in comparison with the fruits of the cultivated plant (**57.12%**).

It was obviously clear that there is an inverse relationship between the percentage of estragole and *trans* anethole.

Table (1): Characterization and identification of different volatile oils components in fruits of cultivated and wild fennel as well as aerial parts of wild fennel.

No	M^+	BP	Fragments	RT	Identification	% in fruits of cultivated plant	% in fruits of wild plant	% in aerial parts of wild plant
1	136	93	121,105,77,67,55	5.36	Thujene	0.19	0.02	
2	136	93	121,105,77,67,53	5.48	*alpha*-Pinene	**1.25**	**3.14**	**1.18**
3	136	93	121,105,77,67,53	6.11	Fenchene	0.19	0.11	
4	136	93	121,105,77,69,57	6.13	Camphene	0.50	0.57	
5	136	93	121,105,77,69,57	6.42	Sabinene	0.12	0.08	0.04
6	136	93	121,105,79,69,55	6.54	*beta*-Pinene	0.42		0.11
7	136	93	121,105,79,69,57	6.70	Myrecene	0.14	0.57	0.38
8	136	93	121,105,77,65,51	7.31	*alpha*-Phellandrene	**1.59**	0.67	
9	136	93	121,107,79,68,53	7.34	*l*-limonene	**6.4**	**13.48**	**25.29**
10	136	68	121,105,79,68,53,39	7.48	*dl*-limonene	**7.10**	**1.03**	0.38
11	136	68	121,105,79,68,53,39	7.99	*beta*-Ocimene	0.83	0.11	0.90
12	136	93	121,105,77,67,57,50	8.09	*gamma*-Terpinene	0.49	0.20	0.07
13	152	81	137,123,109,95, 67,53	8.48	*alpha*-Fenchone	**2.67**	**5.86**	
14	152	81	137,121,109,91, 69,53,41	8.56	*cis*-Thujone	**1.02**		
15	154	93	136,126,111,105, 86,81,71,65,55	8.65	*trans*-Sabinene hydrate		0.11	
16	152	81	137 ,109,91,69,53	9.08	*trans*-Thujone	**1.28**	0.09	
17	152	109	137,121,115,103, 94,84,79,67,55,50	10.00	*p*-mentha-*trans*-2,8-dien-1-ol			**1.09**
18	152	95	137,121,109,81, 67,55	10.56	Camphor		0.36	0.06
19	152	69	137,123,109, 97,91,84,79,55	10.85	*E*-Citral			0.11
20	148	148	133,121,105,91, 77,63,51	11.18	**Estragole**	**57.12**	**12.36**	**38.61**
21	196	81	154, 136, 121,114, 107,100,93,69,55	11.91	Fenchyl acetate			**1.97**
22	148	148	133,121,105, 91,77,63,51,41	12.21	Isoestragole		**4.20**	
23	150	82	135,122,108,95, 67,54	12.41	Carvone	**1.43**	0.05	0.84
24	148	135	119,107,92,77, 63,51	12.48	4-anisaldehyde	0.72	0.02	0.60
25	148	148	133,117,105, 93,77,63,51	12.81	***trans*-Anethole**	**9.07**	**53.57**	**1.22**
26	150	82	148,135,119,107,91, 77,71,65,57,51	12.84	*p*-mentha-1,8-dien-3-one		0.01	

No	M^+	BP	Fragments	RT	Identification	% in fruits of cultivated plant	% in fruits of wild plant	% in aerial parts of wild plant
27	154	121	148,136,107, 93,85,79,67,55	13.55	*p*-menth-4(8)-en-9-ol			0.09
28	164	164	149,135,121,103,91,77,69,55	13.56	Eugenol	0.05		
29	212	108	194,179,164, 148,126,85,70,55	13.64	Exo-2-hydroxy cineole-acetate	0.07	0.01	
30	164	164	149,131,115, 103,91,77,55	13.75	Isoeugenol	0.13		
31	204	161	189,147,133,119,105, 98,91,84,77,69,62,55	13.90	*alpha*-Copaene	0.13		0.33
32	178	178	163,156,147,135,122, 115,103,91,77,65,58, 51	14.16	Methyl eugenol	0.57	0.06	
33	204	133	189,175,168,161,154, 147,120,105,93,86,79, 69,62,55	14.56	*trans*- (*beta*) Caryophellene	0.28	0.06	0.63
34	204	81	161,148,133, 115,105,91,69,55	14.67	*beta*-Burbonene	0.06	0.01	0.28
35	204	161	192,148,133,121,105, 91,79,71,57	14.72	*beta*-Gurjunen		0.02	
36	164	135	148,120,107, 92,77,64,50	14.92	Thymol methyl ether	0.49		
37	204	119	161,148,133,107,93, 79,69,55	15.40	*trans*-alpha-bergamotene		0.01	
38	204	161	191,177,147,133, 119,105,91,77,55	15.44	Germacrene D	0.43	0.10	0.37
39	204	119	189,178,170,161, 148,133,105,93,77,69, 57	15.54	Zingiberene	0.15		
40	204	69	189,161,148,133, 120,107,93,81,57	15.67	*Trans*-beta-farnesene		0.03	
41	204	93	191,175,161,147, 133,119,109,102,79,69,62,53	15.73	*beta*-Bisabolene	0.10	0.02	
42	202	132	187,159,145,119,105, 91,77,69,55	15.81	Cuparene	0.03		
43	192	192	177,161,147,133,119, 105,91,69,53	15.96	Myristicine	0.65	0.10	
44	220	123	205,194,187,177,159, 146,138,131,115,105, 98,91,79,69,55	16.46	Aristolene epoxide	0.10		0.43
45	178	178	163,147,135,115, 107,91,77,65,55	16.52	*cis*-Methyl isoeugenol		**0.03**	**0.50**
46	162	162	147,131,119,108,91, 77,63,51	16.66	*Para*-methoxycinnama-ldehyde	0.23	0.02	**1.17**
47	220	205	196,187,177,162, 147,131,119,105,91,79,69,55	16.84	(+)spathulenol	0.06		0.41
48	208	208	193,177,165,148, 133,121,105,91,77,57	17.22	*cis*-Asarone		0.01	
49	220	91	205,187,177,159,145, 121,105,79,67,55	17.26	Caryophyllene oxide	0.16	0.03	1.85
50	220	159	102,187,177,149,141, 131,121,109,98,91,79, 67,55	18.19	Vulgarol B	0.11		0.39

No	M^+	BP	Fragments	RT	Identification	% in fruits of cultivated plant	% in fruits of wild plant	% in aerial parts of wild plant
51	220	109	202,177,159,147,135, 121,99,91,81,67,55	18.20	Aromadendrene Epoxide		0.02	0.42
52	222	222	207,191,177,161,149, 134,121,106,91,77,65, 63	18.37	**Apiole**	**0.97**	**0.17**	**0.44**
Total % of the identified volatile oils components						**97.30**	**97.31**	**80.16**

As shown in Table (2), analysis of extracts of micropropagated plants obtained by organogenesis from **Medium III** also showed different valuable volatile oils components such as *trans*-*p*-Menthane, *cis*-*p*-Menthane, *m*-Menthane, 6,6-dimethyl-Menth-2-ene and apiole. The most interesting point was that apiole which is a valuable antimicrobial, colon anti-proliferative[20] and insecticidal[21] volatile oil component which is also a major constituent of parsley (23%) and dill (16.8%), was formed in the different extracts in highly different concentrations which may be higher than parsley or dill in some extracts.

Apiole percentage in extracts of micropropagated plants obtained by organogenesis varied between those obtained from leaf, root and stem calluses on **Medium III**.

Table (2): Different volatile oils components identified by GC-MS analysis of plantlets derived from indirect organogenesis of NAA 1+BAP 0.1 hormonal combination from leaf, stem and root explants calluses.

N0.	M^+	BP	Fragments	RT	Identification	% in ROOT	% in STEM	% in LEAF
1	140	97	123,112,81, 71,55	9.19	*trans*-*p*-Menthane	0.02	-	-
2	140	97	123,112,81, 71,55	9.29	*cis*-*p*-Menthane	-	-	0.15
3	140	97	121,111,81, 69,55	9.74	*m*-Menthane	0.43	-	0.16
4	168	97	151,137,125, 111,83,69,55	13.9	*6,6*-dimethyl-menth-2-ene	0.31	-	-
5	222	222	195,177,147, 119,91,65	21.79	**Apiole**	**13.72**	**39.1**	**41.55**

As illustrated in Table (3), analysis of calluses extracts showed that only calluses extracts of the hormonal combinations (TDZ 0.5+2,4-D 1+BAP 0.1) and (2,4-D 2+BAP 0.25) were able to form different volatile oils components such as *m*-Menthane, *p*-Menthane, *p*-Menth-2-ene, *dl*-Limonene, cis-Methyl-isoeugenol, *beta*-Pinene, *gamma*-Terpinene, *alpha*-Terpinolene and propiovanillone. But the notable point was that these volatile oils components were formed in very low concentrations in different analyzed calluses. This has given the green light for proceeding towards micropropagation to optimize formation of different valuable volatile oils.

Table (3): Different volatile oils components identified by GC-MS analysis of different explants calluses of (TDZ 0.5+2,4-D 1+BAP 0.1) and (2,4-D 2+BAP 0.25) hormonal combinations.

NO	M^+	BP.	Fragments	R.T	Identification	% in TDZ root	% in TDZ leaf	% in TDZ stem	% in DBAP root	%in DBAP leaf	% in DBAP Stem
1	140	97	123,112,81, 71,55	9.19	*p*-Menthane	**0.25**	**0.32**		-	-	-
2	136	93	121,69,53	9.35	*beta*-Pinene	-	-		-	0.07	-
3	138	97	125,111,83, 69,55,41	9.56	*p*-Menth-2-ene	-	-		**0.14**	-	-
4	140	97	121,111,,81, 69,55	9.74	*m*-Menthane	**0.17**	**0.20**	0.01	**0.20**	-	-
5	136	68	119,93,51	10.55	*dl*-Limonene	-	-	0.05	-	**0.34**	-

6	136	93	121,105,77, 55	11.23	*gamma*-Terpinene	-	-		-	0.07	-
7	136	121	105,93,79, 67	11.88	*alpha*-Terpinolene	-	-		-	0.05	-
8	178	178	163,147, 135,115,107,91, 77,65,55	19.33	*cis*-Methyl-Isoeugenol	-	-	**0.42**	-	-	-
9	180	151	123,91,57	21.11	Propio-vanillone	-	-		-	-	**0.37**

As shown in Table(4), apiole that was previously obtained by the micropropagated plants formed via organogenesis by the hormonal combination NAA 1+BAP 0.1, but here, its percentage reached about 53% which is a very considerable percentage that gave us the green light for proceeding towards transplantation.

Table (4): Different volatile oils components identified by GC-MS analysis of plantlets of somatic embryogenesis derived from (TDZ 0.5+2,4-D 1+BAP 0.1) of stem explants callus.

NO	M^+	BP	Fragments	RT	Identification	% in TDZ stem plantlets
1	208	208	193,177,165,148,133,121,105, 91,77,57	17.22	*cis*-Asarone	2.93
2	222	222	207,191,177,161,149,134,121, 106,91,77,65,63	20.8	**Apiole**	**53.02**

3. Results of biological activities

3.1 Analgesic activity

The results recorded in Table (5) and illustrated by Figure (1), showed that the hydro-distilled volatile oil of the fruits of the wild fennel in a dose of 400 µl/kg excreted **96.875%** protection against writhing compared with control while paracetamol in a dose of 45 mg/kg excreted **46.875%** protection against writhing compared with control. In conclusion, the hydro-distilled volatile oil of the fruits of the wild fennel showed significant analgesic activity nearly **twice** the paracetamol in the selected doses.

Table(5): Evaluation of the analgesic activity the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* on acetic acid-induced writhing model in mice.

Time interval (minutes)	Control	Paracetamol 45 mg/kg	Hydro-distilled volatile oil of the fruits (FVoil) 400 µl/kg
Total number of writhes	32	17	1
% protection	---------	46.875	96.875

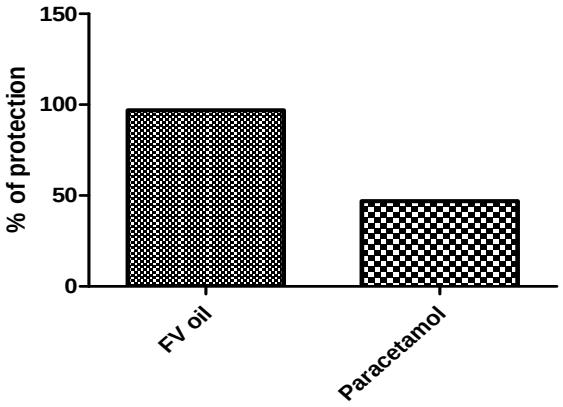

Figure (1): Evaluation of the analgesic activity of the hydrodistilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* on acetic acid-induced writhing model in mice.

3.2 Anti-inflammatory activity

As shown in Tables (6) and (7) and Figure (2) and (3); the intradermal injection of 0.1 ml carrageenan (1%) in the rat hind paw significantly increased the paw thickness in all specified time points. On the other hand, oral pretreatment with the hydro-distilled volatile oil of the fruits of the wild fennel (400 µl/kg) significantly decreased rats hind paw edema thickness compared to control group. In conclusion, the hydro-distilled volatile oil of the fruits of the wild fennel (400 µl/kg) showed a strong significant anti-inflammatory activity. In addition the results obtained from AUC calculation showed that the potency of the anti-inflammatory activity of the hydro-distilled volatile oil of the fruits of the wild fennel (400 µl/kg) is biologically nearly **twice** equivalent to that of diclofenac sodium (7 mg/kg).

Table (6): Effect of oral administration of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* and diclofenac sodium on hind paw thickness at different time intervals after induction of edema using carrageenan.

Thickness of hind paw ± SE	Time (h)	Treatment		
		Control	Diclofenac sodium (7 mg/kg)	Volatile oil (400µl/kg)
	1	4.932±0.2334	5.252±0.06719	5.060±0.04000
	2	5.396±0.08256	5.348±0.04554	5.198±0.05970
	3	6.388±0.1001	5.494±0.06531^{***a}	5.300±0.05805^{***a}
	18	4.932±0.1871	4.752±0.1337	4.352±0.03121^{*a}
	21	4.812±0.1998	4.394±0.09558	4.270±0.05621^{*b}

- ^{*a}= significantly different from control
- ^{*b}= significantly different from diclofenac

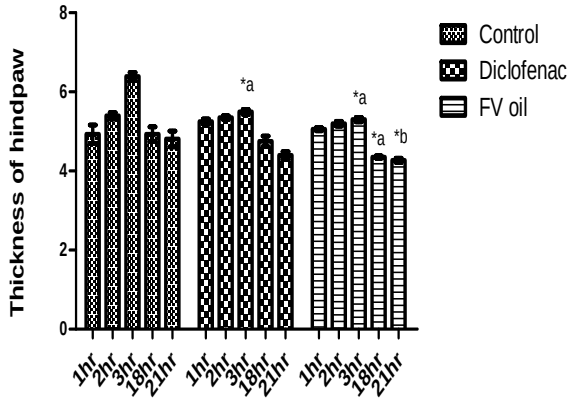

Figure (2): Effect of oral administration of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* and diclofenac sodium on hind paw thickness at different time intervals after induction of edema using carrageenan.

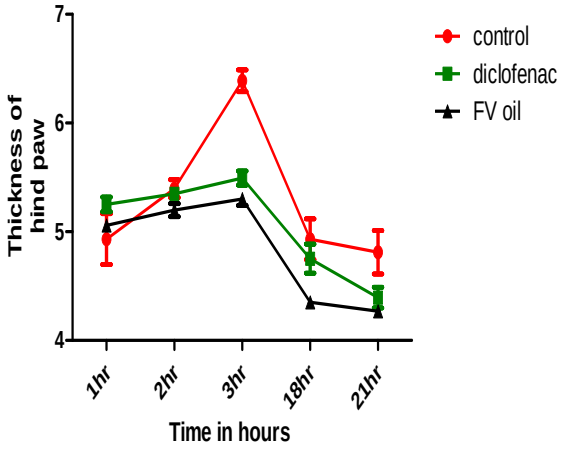

Figure (3): Total cumulative effect of oral administration of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* and diclofenac sodium on hind paw thickness at different time intervals after induction of edema using carrageenan.

Table (7): Total cumulative effect of oral administration of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* and diclofenac sodium on hind paw thickness at different time intervals after induction of edema using carrageenan.

Group	AUC	Total inhibition
Control	21.59	0%
Diclofenac	20.42	5.42%
Volatile oil	19.52	9.58%

3.3 The antipyretic activity

As shown in Tables (8) and (9) and Figures (4) and (5); oral administration of the hydro-distilled volatile oil of the fruits of the wild fennel in dose (400µl/kg) has significantly decreased the temperature of yeast induced hyperthermic mice compared to the control and paracetamol (45 mg/kg).

Table (8): Effect of oral administration of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* and paracetamol on yeast induced hyperthermic mice.

Group	Rectal temperature ±S.E.		
	1 hour	2 hours	3 hours
Control	38.78± 0.2023	38.32± 0.1014	38.06± 0.08411
Paracetamol	37.52± 0.1797^{***a}	37.18± 0.1493^{***a}	36.63± 0.1116^{***a}
Oil	37.23± 0.2246^{***a}	35.95± 0.1628^{***a***b}	34.80± 0.1949^{***a***b}

- ^{*a}= significantly different from control
- ^{*b}= significantly different from paracetamol

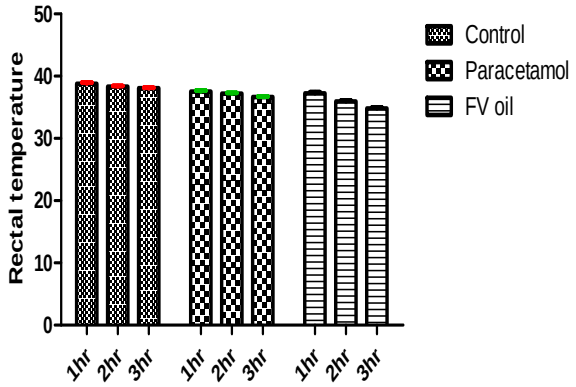

Figure (4): Effect of oral administration of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* and paracetamol on yeast induced hyperthermic mice.

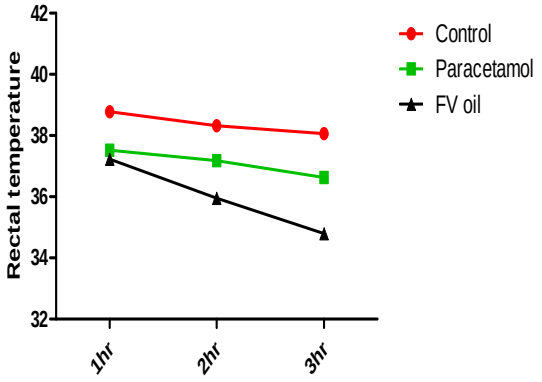

Figure (5): Effect of cumulative effect oral administration of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* and paracetamol on yeast induced hyperthermic mice.

Table (9): Effect of the cumulative effect oral administration of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* and paracetamol on yeast induced hyperthermic mice.

Group	AUC	Total inhbition
Control	76.74	0%
Diclofenac	74.26	3.23%
Volatile oil	71.97	6.22%

3.4 The anxiolytic activity

The decrease in ambulation and rearing was seen at the chosen doses of the oil but was more remarked at the highest dose (400µl/kg) of the oil. This might be due to the sedative property of the oil. Suppression of exploratory behavior is an indication of central nervous system depressant activity and potential anxiolytic activity. Results are recorded in Table (10) and illustrated in Figure (6).

Table (10): Effect of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* and diazepam on the behavior of mice in the open field model.

Group	Number of squares crossed			No. of rearing
	Center	Periphery	Total	
Control	13.80±1.530	58.00±2.720	71.80±2.518	34.80±2.518
Diazepam	10.60±1.435	61.80±8.127	72.40±8.140	20.00±3.435***a
FV 200	4.800±1.594	47.60±7.840	52.40±9.309	19.60±7.420***b
FV 400	2.200±0.8602	26.40±6.055**a**b	28.60±6.462***a***b	4.800±2.396*a

- *a= significantly different from control
- *b= significantly different from diazepam

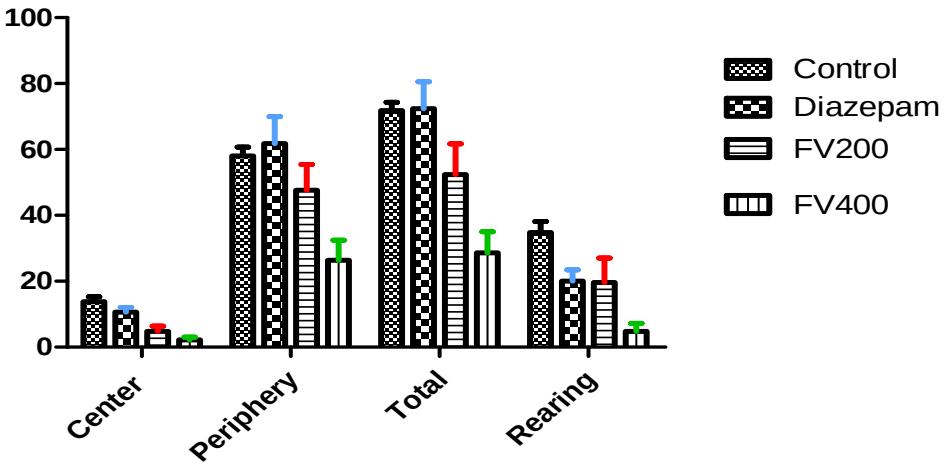

Fig.(6): Effect of hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare* and Diazepam on the behavior of mice in the open field model.

3.5 The antispasmodic activity

As shown in Figure (7), injection of 0.2 ml acetyl choline 0.001% solution in the Tyrode's solution showed an elevation in the muscle response. The addition of 0.05 ml of the wild fennel oil fruit extract (1%) resulted in a significant reduction in the elevation in the muscle response caused by 0.2 ml acetyl choline 0.001% solution. Thus the extract either blocks the muscarinic receptors or directly inhibits the intestine muscle contraction. When Barium chloride solution (3%) was added to the Tyrode's solution after the extract addition, an elevation in the muscle response was shown. So, it was suggested that the wild fennel oil extract has antimuscarinic activity.

As shown in Figure (7), 0.1ml of the standard atropine (0.0003% solution) caused 50% reduction of the contraction produced by0.1ml acetyl choline 0.001% solution (used as agonist). And 0.05 ml of the hydro-distilled volatile oil of the fruits (1% emulsion) caused the same response (50% reduction) produced by the standard atropine (0.0003% solution). These results suggested that the hydro-distilled volatile oil of the fruits (1% emulsion) was twice as potent as the standard atropine (0.0003% solution) as antimuscarinic activity.

$$\text{Relative potency} = \frac{\text{Atropine potency}}{\text{Oil potency}} = \frac{\text{Oil dose}}{\text{Atropine dose}} = \frac{0.05}{0.1}$$

Atropine: Oil = 1: 2

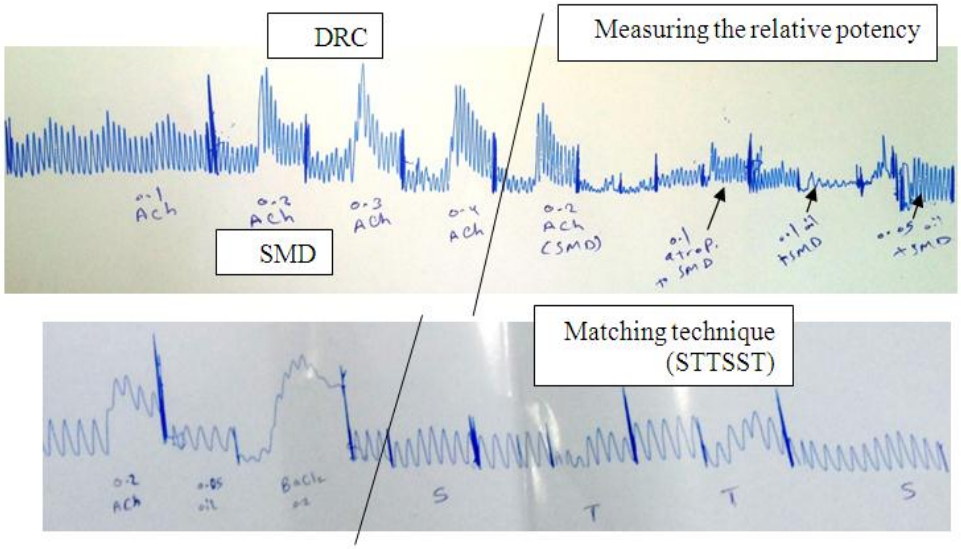

Figure (7): Screening and assay of the antimuscarinic activity of the hydro-distilled volatile oil of the fruits of the wild fennel *Foeniculum vulgare* Mill. var.*vulgare.*

3.6. A preliminary study of the antibacterial and antifungal activities

As shown in Table (11), in comparison with amphotericin B, ampicillin and gentamicin, the hydro-distilled volatile oil of the fruits of the wild fennel and the hexane extract of the micropropagated plant have a significant antifungal and antibacterial effect against the tested microorganisms except for *Pseudomonas aeruginosa* (RCMB 010048). It was clear that the hexane extract of the micropropagated plants has stronger activity than the fruits volatile oil against the tested microorganisms and also showed activity against *Candida albicans* in contrary with the fruits volatile oil that showed no activity against it

Table (11): Results of antimicrobial activities of the fruits volatile oil and the micropropagated plants hexane extract.

	Fungi		**Gram positive bacteria**		**Gram negative bacteria**	
	***Aspergillus fumigatus* (RCMB 02569)**	***Candida albicans* (RCMB 05039)**	***Streptococcus pneumoniae* (RCMB 010010)**	***Bacillus subtilis* (RCMB 010068)**	***Pseudomonas aeruginosa* (RCMB 010048)**	***Escherichia coli* (RCMB 010052)**
Fruits volatile oil	14.1±0.37	NA	16.9±0.44	19.3±0.25	NA	12.2±0.58
Micro-propagated plants hexane extract	20.1±0.58	17.3±0.25	20.6±0.44	21.3±0.67	NA	19.1±0.34

Standard antimicrobials						
Amphotricin B	23.7±0.1	25.4±0.1	-	-	-	-
Ampicillin	-	-	23.8±0.2	32.4±0.3	-	-
Gentamicin	-	-	-	-	17.3±0.1	19.9±0.3

NA = No activity, data are expressed in form of mean zone of inhibition ± standard deviation.

CONCLUSION

Foeniculum vulgare var. *vulgare* contains various important volatile oils. In this study, different extracts of *in vivo* wild and cultivated cultivars as well as *in vitro* tissues of the wild plant were investigated. GC-MS analysis of the volatile oils of cultivated, wild fennel as well as the aerial parts of the wild fennel showed different medicinally important volatile oils but the most interesting point was the reduction of estragole percentage in the fruits of the wild fennel in comparison of the fruits of the cultivated fennel. Also, GC-MS analysis of callus and micro-propagated plants showed valuable volatile oils particularly apiole which showed significantly higher percentages in the micro-propagated plants. Furthermore, biological screening revealed different valuable activities for the oil of wild fennel such as analgesic, anti-inflammatory, antipyretic, anxiolytic, antispasmodic and antimicrobial activities. The most interesting was that micro-propagated plants extracts showed activity much stronger than the extracts of the wild plant against the tested microorganisms and also showed activity against *Candida albicans* contrary from the wild plant extracts that showed no activity against it.

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