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Analysis of fruits *Schinus molle* extractions and the efficacy in inhibition of growth the fungi in laboratory

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Abstract: The investigation was carried out during 2013 - 2014 at the laboratories of Plant Protection Dep., Faculty of Agriculture, and Chemistry Dep. Damascus University. the constituents of oil extracts isolated from ripe fruits of *Schinus molle* L. Anacardiaceae family, from grown in Damascus, Syria,. Extracts were prepared from dried and powdered ripe fruits with solvents (n-hexane, petroleum ether). The oil extracts were examined by GC-MS. A total of 10 components were identified. The oil extracts of *S. molle* contained as main components α-phellandrene (24.86% & 22.19%), β-pinene (14.78% & 13.52%), β-Phellandrene (11.02 & 10.00%), and limonene (10.58% & 9.34%) in n-hexane and petroleum ether, respectively. Furthermore, the oil extracts exhibited antifungal al activity using the poison media method against *Botrytis cinerea* fungus. Petroleum ether extract exhibited a high level of antifungal activity where caused full suppression for *B. cinerea* at dose, 1000 ppm,. It was clear that the higher concentration caused higher suppression, and suppression vary according to the type extract. Therefore we recommended to use the Petroleum ether extract of the ripe fruits of *Schinus molle* L. as environment friendly fungicides.
Key words: *Schinus molle* L., *Botrytis cinerea* , GC/MS, Chemical Composition, Antifungal.

Introduction

Schinus molle L. (Anacardiaceae) is a tree originally from South America but has since been introduced to the Mediterranean area. It was widely planted on roadsides, in graveyards and gardens of North Africa as a shade tree. Also known as Brazilian pepper, this plant plays an important role in pharmacology and pharmaceutical chemistry because of its high essential oil content[1]. Local names : Amharic (qundo berbere); Arabic (felfelkazib, filfilrafie); English (pepper tree, California pepper tree, Chilean pepper tree, mastic tree, molle, pepper berry tree, weeping pepper, Peruvian mastic, pink pepper, Peruvian pepper tree). Essential oils from *S. molle* L. berries and leaves were analysed by means of GC–MS. Berry oil contained mainly α- and β-phellandrene (55.4% and 15.4% respectively) along with limonene (14.3%). Leaf oil which also contained α- and β-phellandrene and limonene (30.2%, 9.6% and 9.3% respectively), was characterizied by the presence of sesquiterpenes such as elemol (13.3%), germacrene-D (5.2%), γ-eudesmol (3.2%) and *T*-cadinol (4.7%), which were present only in traces in the oil from the berries.[2]

Analysis by gas-liquid chromatography of the terpene hydrocarbon fraction of the essential oil obtained by steam distillation of the fruit of *S. molle* L. revealed 11 components. Capillary columns and flame ionization detection increased resolution and permitted tentative identification of 6 additional terpene hydrocarbons not hitherto reported in the oil. The compounds found were: α-pinene, β-pinene, -α-phellandrene, β-phellandrene, myrcene, D-limonene, camphene, *p*-cymene.[3] The essential oils (EOs) extracted from the berries of *S molle* L. were analyzed by gas chromatography–mass spectrometry (GC–MS). The major constituents in *S. molle* oil

were α-phellandrene (35.86%), β-phellandrene (29.3%), β-pinene (15.68%), p-cymene (5.43%) and α-pinene (5.22%).[4] The essential oil of *S. molle* L. contained as main components α-phellandrene (20.6%), β-Phellandrene (10.8%), α-pinene (8.7%), β-pinene (5.1%), β-myrcene (6.9%), β-elemene (5.0%), copane (6.5%),germacrene (5.8%),γ-cadinene (6.3%) and α-humulene (5.4%). The essential oil of *S.molle* showed no significant antibacterial activity against gram positive (*Staphylococcus aureus*) but less inhibition towards gram negative bacteria (*Escherichia coli*).[5]

The plant was mainly pathogenic fungi are the main infectious agents in plants, causing alterations during developmental stages including post-harvest. In fruit and vegetables, there is a wide variety of fungal genera causing quality problems related to aspect, nutritional value, organoleptic characteristics, and limited shelf life.[6] In addition, in some cases fungi are indirectly responsible for allergic or toxic disorders among consumers because of the production of mycotoxins or allergens. The fungus *Botrytis cinerea* Pers.:Fr, the anamorph of *Botryotinia fuckeliana* (De Barry) Whetzel is pathogenic on a wide variety of crop plants.[7] *B. cinerea* causes Gray mold rot or Botrytis blight that affects nearly all species of dicotyledons, including most vegetable and fruit crops, flowers, woody ornamentals, and greenhouse-grown crops. The fungus uses a wide range of infection strategies that allow it to directly penetrate mature-to-senescent leaves and other tender tissues, such as seedlings, floral organs, and mature fruits. The fungus generally infects host tissues in cool damp weather (10 to 25°C) in water droplets.[8], but it also can germinate at high humidity in the absence of water droplets.[9] Generally, phytopathogenic fungi are controlled by synthetic fungicides; however, the use of these is increasingly restricted due to the harmful effects of pesticides on human health and the environment.[10] Fungi are also known to be responsible for the OV-Xavour formation and production of allergenic compounds, which often takes place before the growth of fungi is evident. In addition, fungi such as *Botrytis cinerea*, *Glomerella cingulata*, *Phyllosticta caricae* have been shown to cause spoilage of fruits and vegetables including apple, strawberry, bean, potato, tomato, etc.[11].

Fungicide mixtures are widely used in commercial products. The main advantages of mixture are that they can extend the antifungal spectrum of the single products and delay resistance development to the individual component. Fungicide mixtures may also display a synergistic interaction by which the amount of active ingredients can be reduced[12]. Management of *Botrytis cinerea* is required, as this pathogen and its many special forms affect a wide variety of hosts of economic value. Control of Gray mold rot or Botrytis blight disease has been accomplished primarily by the application of chemical fungicides, long crop rotations, pasteurizing seedbeds with steam or fumigants[6]. Fungicides are the main strategy for chemical control of gray mold[13]. Resistance development to fungicides is due to the emergence of fungicide resistant mutants in wild-type populations upon selection pressure of fungicides in space and time**[14]**. Pesticides have been universally considered for long time as the most efficient solution to control crop diseases. However, synthetic pesticides may enter the food chain and the resistance developed by plant pathogens has rendered some of them ineffective. This has highlighted the need for the use of alternatives compounds that are enviromentally friendly and safe to humans**[15]**. there may be a need to develop new management systems to reduce the dependence on the synthetic agrochemicals. Plant secondary metabolites, such as essential oils and plant extracts are known to possess insecticidal, antifungal, acaricidal, antibacterial and cytotoxic activities[16]. The plant extracts and essential oils can be obtained from different parts like leaves, unripe fruits and ripe fruits of *S. molle* L. Essential oil of leaf contained 24 components; mainly deltacadinene (11.28%) and alpha-cadinol (10.77%) Germacrene D (20.77%) and Betaceryophyllene (13.48%).[17]

In recent years, antimicrobial and antifungal properties of plant extracts have been reported with increasing frequency from different parts of the world [18]. The essential oil of *S. molle* L. growth in Tunisia possessed antimicrobial activity, and could be used in biotechnological fields as a natural preservative ingredients in the food and/ or pharmaceutical industry[4].

However, in Syria few investigations have conducted to find antimicrobial activity of oils and extracts of traditional medicinal plants against plant pathogenic microbes.

The aim of the present study was to evaluate the chemical composition and antifungal activity of the oil extracts from ripe fruits *Schinus molle* L. grown in Syria and to identify whether it could serve as good candidate for the development of environment friendly fungicide.

Material and Method

Plant material

This work was conducted in Department of Plant Protection, Faculty of Agriculture and Dep. Chemistry, Faculty of Science, Damascus University during 2013-2014 to determine the antifungal activity of oil extracts (n-hexane and petroleum ether) of medicinal plant *Schinus molle* L., against *Botrytis cinerea* fungus.

Preparation of Plant Extracts

The ripe fruits of *S. molle* plant were collected during the month of November 2013 from University Damascus Garden, Syria. The ripe fruits of *S. molle*, were air-dried in a shaded area at ambient temperature. The ripe fruits were ground by a blender and 50 grams of their powders were soaked and stirred in 500 ml of solvents (n-hexane or petroleum ether) for 48 hours at laboratory condition followed by filtration in first through four layers of muslin, then through Whatman No.1. The solvent (n- Hexane or Petroleum ether) was evaporated at lower temperature under reduced pressure in rotary flash evaporator to get the oil extracts were stored in dark vials at 4°C for future uses.

Extracts Analysis:

Analysis of extracts were carried out by GC-MS chromatography (GC-agilent 7986, indictor: inert-MS) in Atomic Energy Commission(AECS)- Damascus, Syria. This instrument was fitted with HP-5MS capillary column (30cm×0.25mm i.d., film thickness 0.25μm). The temperature injector and indictor 250 °C .The oven temperature program was 60-270°C (2.5°C per min.).. The identity of components was ascertained based on the spectra and compared with library and literature data. Also, the identification of each compound was confirmed by comparison of its retention index with those of authentic compounds.

Isolate fungi:

The fungus specie tested was *Botrytis cinerea* were isolated from strawberry fruits by the hyphae point and monosporic techniques, and identified based on their macroscopic and microscopic features [19].

Effect of Plant Extract on *B. cinerea* Mycelia Growth:

The effect of *S. molle* L. extracts on diameter growth of *B. cinerea* on Potato Dextrose Agar (PDA) was evaluated by the Poison Food Technique[20]. The plant extracts were added to PDA (at 45°C) to give a final concentration 100, 200, 400,600,800,1000 and 1500 ppm for each extract and then the resulting media were poured in Petri dishes (9 cm in diameter). Hexane or Petroleum ether was added to medium in control plates. Then, inoculums discs (5 mm in diameter) from five days growing cultures of *B. cinerea* placed in the center of Petri plates containing PDA and extracts. Each treatment was tested on 5 plates as replications. The plates were incubated in 27°C. After seven days (when the fungus overgrow on control plates), radial growth of *B. cinerea* was recorded for each plate. The percentage of fungal growth inhibition was calculated as formula[21]:

Growth inhibition% = [(growth in control – growth in sample)/growth in control] × 100.

The ED_{50} value for each fungus, which was defined as the concentration of fungicide causing 50 % inhibition of mycelial growth, was determined according to[22].

Data Analysis

The experiment was conducted using a completely randomized design. All statistical analyses were carried out using Spss 20. soft ware.

Results and Discussion

Chemical properties of *S. molle* L. ripe fruits oil

The composition of oil extracts of ripe fruits *S. molle* is shown in the table 1, Figures 1 and 2. a total of 82.38% ±2was identified. The major components identified were α-phellandrene (24.86% & 22.19%), β-pinene (14.78 % & 13.52%), β-Phellandrene (11.02% & 10%), Limonene (10.58%& 9.34%) and Cyclohexane methanol,4-ethyl-,alpha.,alpha.,4-trimethyl-3-(1-methylethenyl)-,[1R-(1-alpha,3.alpha.,4.beta,)], (2.58% & 2.10

%) in n-Hexane and petroleum ether extracts, respectively. On the other hand, the higher components fatty acids were in petroleum ether extracts compared with n-Hexane extracts, were the percentage of Oleic acid 18:1 (8.31% & 4.23%) and Linoleic acid 18:2 (8.32 % & 5.69%), respectively. We Suggesting that petroleum ether was the best solvent to extract fatty acids Oleic acid and Linoleic acid from ripe fruits of *S. molle*. B-pinene is a monoterpene and α-pinene an organic compound of the terpene. These are two structural isomers of pinene found in nature. Limonene is hydrocarbon classified as a cyclic terpene. The above identified constituents in the present study were reported by earlier workers [5,23,24]. A recent report from Saudi Arabia showed that the leaf oil of *S.molle* contained, *p*-cymene, α-terpinene and β-pinene as the main volatile constituents[25]. In contrast, An investigation from Brazil showed the presence of sabinene and limonene as the main constituents[26]. The composition may differ by season and the region that the plant material was collected.

Table 1: Anlysis of *S. molle* L. oil extracts by GC/MA chromatography.

Component	Retention time (min.)		Percentage %	
	n-Hexane	Petroleum ether	n-Hexane	Petroleum ether
Alpha-pinene	7.94	7.91	2.54	1.94
Beta-pinene	12.06	11.95	14.78	13.52
Alpha-phellandrene	13.49	13.32	24.86	22.19
Limonene	14.87	14.74	10.58	9.34
Beta-phellandrene	15.44	15.29	11.02	10.00
Benzene,1-methyl-2-(1-methylethyl)	15.87	15.78	2.09	1.80
Octanoic acid methyl ester	24.04	23.98	4.01	3.42
Cyclohexanemethanol,4-ethyl-,alpha., alpha.,4-trimethyl-3-(1-methylethenyl)-,[1R-(1-alpha,3.alpha.,4.beta,)]	60.96	60.92	2.58	2.10
Octadec-9-enoic acid (Oleic acid 18:1)	101.58	101.83	4.23	8.31
9,12-octadecadienoic acid (linoleic acid 18:2)	101.99	102.21	5.69	8.32

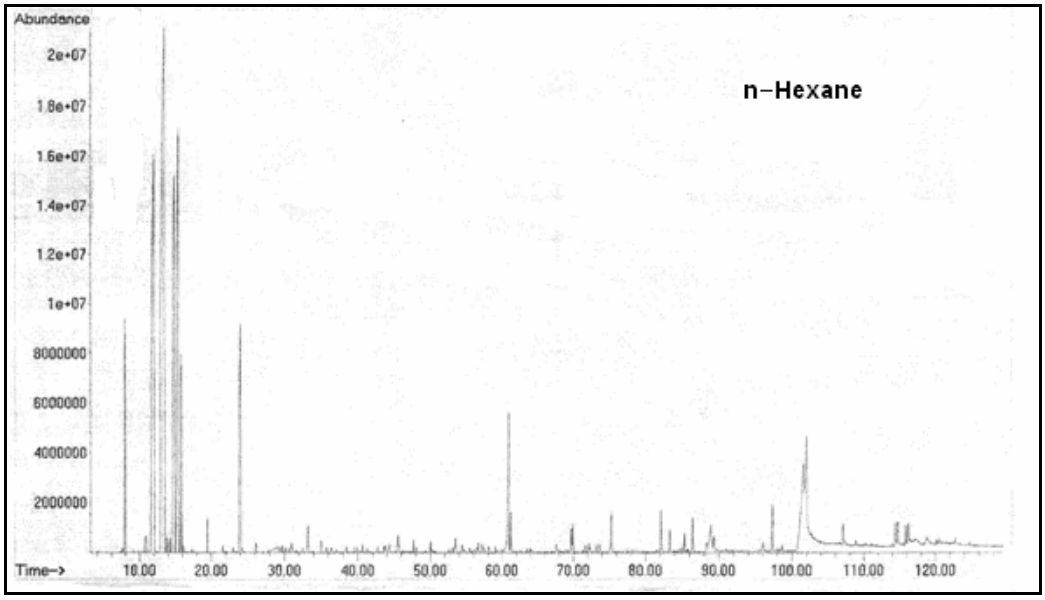

Fig. 1. Chromatogram of oil extract from *Schinus molle* (n-hexane).

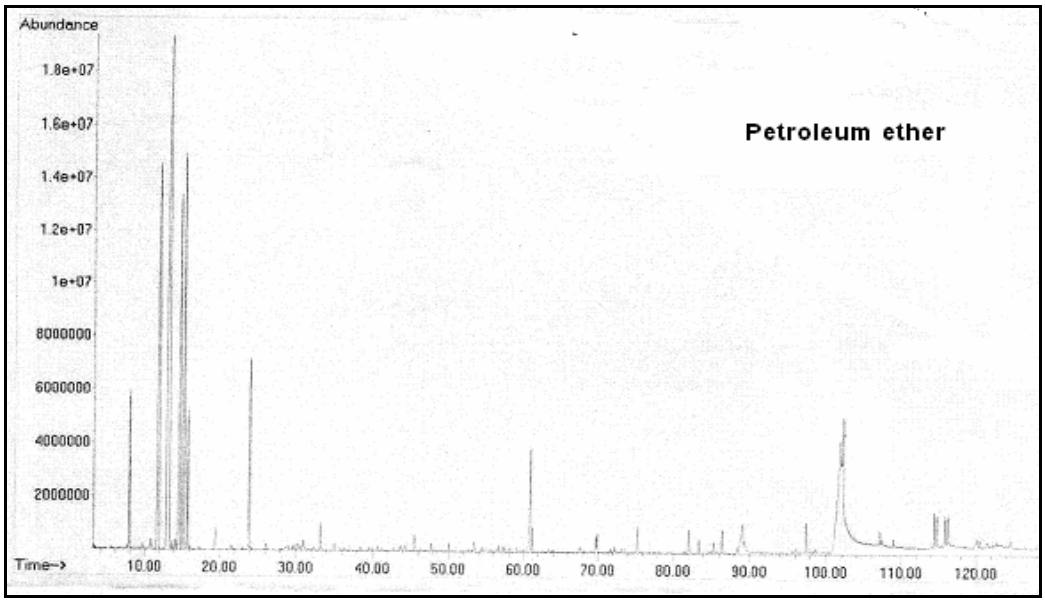

Fig. 2. Chromatogram of oil extract from *Schinus molle* (petroleum ether).

Effect of oil Extracts on *B. cinerea* Mycelia Growth:

Different doses (100, 200,400, 600, 800, 1000 and 1500 ppm) of the extracts from ripe fruits *S. molle* L. were tested against *B. cinerea* to determine their antifungal activity *in vitro* tests. The results of revealed that The oil n- hexane extract of *S. molle* L. showed moderate antifungal activity (table 2 and fig 3) against *B. cinerea* fungus. Significant inhibition of mycelia growth of the fungus *B. cinerea* at highest concentration 1000 and 1500 ppm. Where, the lowest concentration (100 ppm) of oil hexane extracts minimum inhibition of mycelia growth. However, percentage inhibition of growth varies from 16.78% to 93.57% in different concentrations of *S. molle* L. n- hexane extract. Pharmacological properties of the essential oil and hexane extract of *S. molle* have already been investigated by various workers and include anti-inflammatory [27], analgesic [28]. The oil extract (petroleum ether) of *S. molle* L. at (1000 ppm) dose was most effective in reducing fungus growth were gave complete inhibition of growth of the tested fungus. while, the lowest concentration (100 ppm) of oil petroleum ether extract, gave the percentage of fungal growth inhibition (34.56%). As mentioned by[29] the leaf essential oil hydrodistilled from *S. molle* grown in Costa Rica was characterized in terms of its chemical composition, antioxidant activity, ability to induce cytotoxicity and the mechanism of cell death involved in the process. Furthermore, data analysis showed the differences between extracts, between doses, as well as between their interaction are significant ($p<0.05$). The comparison of means showed maximum inhibition of *B. cinerea* growth was found at highest doses, 800, 1000 and 1500 ppm (Table 2). It was followed by the concentration 600, 400,200 and 100 ppm of the plant extracts as compared to control which showed least inhibition on mycelia growth. Data, also, indicate that the concentration of the oil extracts increased, the reduction in mycelia growth of the tested fungus was significantly increased. [30]The highest growth inhibition of all fungi was observed with *Achillea santolina* at 1000 ppm., where, the growth inhibition of *F. oxysporum* and *R. solani* was 42.2% and 42.0% respectively.

Data in table (2) show that oil hexane extract was lowest effective against *B. cinerea* . The corresponding ED_{50} reached 650 ppm. On the other hand, the growth of *B. cinerea* was highly affected by oil petroleum ether extract, showing the ED_{50} of 290 ppm. Extracts of *S. molle* have been shown to have potent broad-spectrum antibacterial, antifungal, and diuretic properties. Certain toxic properties of this plant against insects and fungi have also been reported[31,32,33]. Our result demonstrated that the *S. molle* extracts and their concentrations had considerable effect on the growth rate of *B. cinerea*. The petroleum ether extract of *S. molle* indicated considerable antifungal activity against *B. cinerea* followed by hexane extract This activity could be attributed to the presence of fatty acids (Oleic acid and linoleic acid) in the petroleum ether solvent. This report is totally in agreement with an earlier investigation[34] The main components of dry and fresh seed oil of *Melia azedaracht* L. were oliec acid ($C_{18:1}$) 19.90 % and 20.03 % , and Linoleic acid ($C_{18:2}$) 68.76% and 65.40%, respectively. Oil extract of dry and fresh seed Chinaberry caused full suppression for *B. cinerea* at dose, 300 and 400 ppm, respectively. Similar studies have been carried out by different researcher on antifungal activity of plant extract. [35]reported that plant extracts and essential oils of *Cinnamomum zeylanicum* and *S.aromaticum*were effective at

500 ppm against the mycelia growth *Fusarium oxysporum* f. sp. *Cubense.*Also, the antifungal activity of *Cinnamomum zeylanicum* essential oil. The results proved the oil had fungicidal properties against *F. oxysporum* and *Penicilium digitatum*. The essential oils were mainly composed *Cinnamic aldehyde* (37.6%), ciannamic acetate (23.7%), ciannamyl benzoate (14.6%) and other compounds[36]. This antifungal activity is attributed to the presence of active principles such as Alpha-phellandrenes, pinene and oliec acid in the oil of *S.molle* .these results are in agreement with many researchers i.e.[4, 25]. Again, the higher the oil extracts concentration was the lower growth and vice versa. These results are in agreement with those of [30, 37]. These findings suggest that ripe fruits of *S. molle* are potential sources of antifungal compounds.

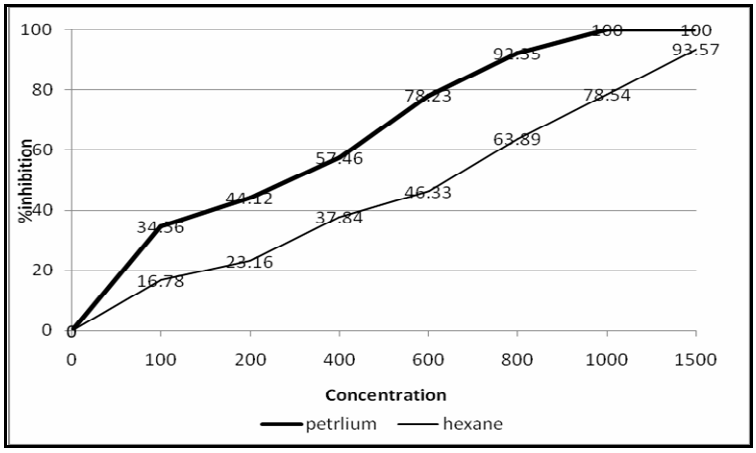

Fig. 3: Effect of different conc. of petroleum ether and n-hexane extract on inhibition percent of mycelia growth of *B. cinerea.*

Table 2: Inhibition percent of mycelia growth of *B. cinerea* by different concentrations of *S. molle* L. extracts on PDA. and ED_{50}

Concen. (ppm)	Oil extracts of *S. molle* L.	
	n-hexane	Petroleum ether
100	16.78	34.56
200	23.16	44.12
400	37.84	57.46
600	46.33	78.23
800	63.89	92.35
1000	78.54	100
1500	93.78	100
ED_{50}	650	290

Data given are mean of five replicate

L.S.D ($p \leq 0.05$) : Between concentrates : 3.17, L.S.D ($p \leq 0.05$) : Between solvents : 4.25 L.S.D ($p \leq 0.05$):Between interactions : 3.89

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