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Antibacterial Activities and Phytochemical Screening of *Alhagi pseudalhagi*

[*]Abdul-Hafeez, E.Y.[1]; A.F. Mahmoud[2] and O.H.M. Ibrahim[1]

[1]Ornamental Plants and Landscape Gardening Department, Fac. of Agriculture, Assiut University, Egypt.
[2]Plant Pathology Dept., Fac. of Agriculture, Assiut Univ., amer.mahmoud@agr.au.edu.eg
[*]Corresponding author: Essam.abdul-hafeez@agr.au.edu.eg

Abstract:

The current study was conducted to test antimicrobial activity of aqueous, ethanol, methanol and acetone extracts of camel thorn (*Alhagi pseudalhagi*) against Gram-positive bacteria (*Bacillus subtilis* and *Clavibacter michiganensis* subsp. *sepedonicus*) and Gram-negative bacteria (*Erwinia carotovora* subsp. *atroseptica*), using the agar well-diffusion method. The minimum inhibitory concentration was also determined. Besides, phytochemical constituents of the volatile oil of camel thorn aerial parts were identified using gas chromatography coupled to mass spectrometer (GC-MS) analysis. Data of the antibacterial assay showed significant activity of all extracts against various bacterial strains at the concentration of 256 mg/ml. The methanolic extract showed the highest inhibition zone and the lowest values of minimum inhibitory concentration against all tested bacterial strains. The lowest inhibition zone and comparatively greater minimum inhibitory concentration was induced by the aqueous extract. Ethanol and acetone extracts showed moderate antibacterial activity against all tested bacterial strains. Chromatographic analysis revealed the identification of 66 phytocompounds most of which have been previously reported to possess antimicrobial, antitumor, antiseptic, preservative, insecticidal and antioxidant activities. The most abundant compounds were 1-(3-Furyl)-4b,7,7,9b,11a-pentamethyl-3,8-dioxohexadecahydrooxireno[d]oxireno[7,8]naphtho[2,1-f]isochromen-5-yl acetate; Hexa-t-butylselenatrisiletane; 4-(2-Methyl-cyclohex-1-enyl)-but-3-en-2-one and 1,3-Dimethyladamantane.

Keywords*: antibacterial activity, camel thorn, medicinal plants, phytochemical screening,*

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Introduction:

The genus *Alhagi pseudalhagi* (Bieb.) Desv.syn. *Alhagi maurorum* sensu Baker (non Desv.) is a perennial plant belonging to family Fabaceae (Leguminosae). It is native to tropical and subtropical regions found in Africa, Asia, US, Europe and Middle East. It is common in disturbed urban sites, abundant along riverbanks, canals, irrigation ditches and sometimes in cultivated field. This genus has been reported to have traditional and medicinal uses increasing its remarkable values (Al-Yahaya *et al.*, l985; Srivastava *et al.*, 2014). It is normally used in folk medicine as a remedy for rheumatic pains, bilharziasis, various types of gastrointestinal discomfort and in diseases of the urinary tract and liver (Bolus, 1983). In addition, several previous studies reported promising antimicrobial activity of ethanol, ether and methanol extract of the fresh areal parts of the plant against gram negative and gram positive bacteria (Srivastava *et al.*, 2014).

Alkaloids, flavonoids, and fatty acids are the major active constituents of this genus (Atta and Abo EL-Sooud, 2004). The presence of several constituents was also reported such as fatty acids and sterols, flavonoids, coumarins and alkaloids (Hameda *et al.,* 2012). The volatile fractions of *A. maurorum* were studied by Samejo *et al.* (2012) and found to be consisted of a complex mixture of ketones, acid derivatives, terpenoids, hydrocarbons, heterocyclics and aldehydes. In the leaf oil, drimenol (23.2%), 9-octylheptadecane (9.3%), 4-hexyl-2,5-dihydro-2,5-dioxo-3-furanacetic acid (5.2%), -nonadecanone (4.4%) and pentacosane (4.3%) were found as the major constituents. In the stem oil, neophytadiene (39.3%), *trans*-ionone (5.4%), 6,10,14-trimethyl-2-pentadecanone (5.2%), actinidiolide (4.9%), and nonacosane (4.3%) were the main components. Drimenol, octadecane, eicosane, docosane, tetracosane, and squalene were common volatile constituents of the essential oils.

Plant pathogenic bacteria cause many serious diseases of plants throughout the world and cause economically damaging diseases in agriculture (Vidhyasekaran, 2002). In potato, *Pectobacterium atrosepticum* (*Erwinia carotovora* subsp. *atroseptica*) causes soft rot and blackleg and affects plant health during field production and storage (Ma *et al.*, 2007; Perombelon, 2002). In various aspects, the blackleg disease is similar to the other two major bacterial diseases of potato, namely bacterial ring rot and brown rot caused by *Clavibacter michiganensis* subsp. *sepedonicus* and *Ralstonia solanacearum*, respectively (De Boer *et al.*, 1994; Elphinstone *et al.*, 1996). Bacterial ring rot caused by a bacterium, *Clavibacter michiganensis* subsp. *sepedonicus* (Spieck.& Kotth.) is considered one of the most important bacterial diseases of potato. Nearly all countries that produce potatoes report the presence of this bacterium (Smith and Charles, 1998). Synthetic pesticides have been universally considered for a long time as the most efficient solution to control such plant diseases. These compounds enter the food chain posing a significant human health hazard. This has highlighted the need for the use of alterna-

tive compounds that are environmentally friendly and safe to humans such as essential oils and plant extracts (Srivastava *et al.*, 1996; Tepe *et al.*, 2004).

Therefore, the main objective of the current study was to evaluate the phytochemical constituents of camel thorn and its antibacterial activity against certain Gram-positive and Gram-negative bacterial strains which cause devastating diseases that occurs in major growing areas of the world.

Material and Methods:

Aerial parts of camel thorn were collected from western desert of Assiut region, Egypt during 2014 and 2015 years. Samples were washed with distilled water, divided into small pieces (about 1 cm) and dried in a ventilated place. Air-dried samples were stored at a dark place until the extraction.

Preparing extracts for antibacterial experiment:

Twenty-five grams powder of camel thorn were macerated in 250ml of aqua, ethanol, methanol and acetone at 50% concentration for 48 hours at room temperature under constant shaking. The macerate was filtered with Whatman No. 1 filter paper and the residue was further macerated twice with the same solvent overnight and filtered. The filtrates obtained from each extraction were combined and kept in tightly stoppered bottle in a refrigerator (2-4 °C) as crude extracts. The solvent was evaporated from the crude extract to dryness under reduced pressure using rotary evaporator (Heidolph VV2000) and the residue was freeze-dried (Freeze-dryer Telstar-LyoQuest Plus-55).

Bacterial strains:

Gram-positive (*Bacillus subtilis* and *Clavibacter michiganensis* subsp. *sepedonicus*) and Gram-negative bacteria (*Erwinia carotovora* subsp. *atroseptica*, were isolated from the soil and naturally infected potato collected from farmer fields in Assiut Governorate, Egypt. Bacterial isolates were identified based on their morphological, biochemical as well as physiological characteristics using the standard characterization procedure of Buchanan and Gibbon (2001), Skinner and Lovelock (1979) and Sneath *et al.* (1986).

Antibacterial activity assay:

Crude extract solutions were filtered through a 0.20 µm sterile filter (PES Syringe filter). Antibacterial activities of different extracts were evaluated by the agar well-diffusion method described by El-Zahry *et al.*, (2015), Irobi *et al.* (1994); Murray *et al.*, (1995) and Olurinola, (1996). *B. subtilis* and *E. carotovora* subsp. *atroseptica* were first grown in a nutrient broth medium for 12 h at 26±2ºC before use and standardized to 10^7 CFU /ml. The standardized cell suspensions (150 µl) were spread on a nutrient agar medium. Meanwhile, *C. michiganensis* subsp. *sepedonicus* was grown in nutrient broth yeast extract (NBY), and 150 µl of the standardized cell suspensions (10^7 CFU /ml) were spread on a nutrient broth yeast extract agar (NBYA). Wells were then bored into the agar plate using a sterile 5 mm-diameter cork borer. A sample of the crude extract (100 µl) were introduced into each well and allowed to stand at room temperature for about 2 h and then incubated at 30±2°C for 24 h, where

it was possible to observe inhibition zones. Overall, cultured bacteria with halos equal to or greater than 5 mm were considered susceptible to the tested extract. Streptomycin sulfate salt with concentration of 5.0µg/ml was used as a positive control.

Determination of Minimum Inhibitory Concentrations (MIC):

Minimum inhibitory concentration is defined as the lowest concentration of antimicrobial compounds able to inhibit any visible bacterial growth on the culture plates. This is determined from the readings on the culture plates after overnight incubation. MIC was determined by agar dilution method (EUCAST, 2000), through the incorporation of varying concentrations of antibacterial extract into an agar medium. Serial dilutions of aqueous, ethanol, methanol and acetone extracts at concentrations of 0.5-256 mg/ml were used according to the international guidelines given by the NCCLS (2000). The MIC was recorded as the lowest concentration (highest dilution) of antibacterial extract with no visible growth. Streptomycin sulfate salt with concentration of 5.0µg/ml served as a positive control.

Extraction of essential oil:

Dried samples (100 g) were subjected to hydro-distillation for 3 hours using the Clevenger apparatus for essential oils (Clevenger, 1928) in which water is heated to produce steam, which carries the most volatile chemicals and aromatic material. Essential oils are usually float on the surface Hydrosol (a component of distilled water). Extracted essential oil is stored in a clean Eppendorf glass, in the dark at 4 °C.

Chromatographic analysis:

Chromatographic analysis was performed using Shimadzu GC-MS in electron impact mode. The ionization voltage was 70e V as well as temperature of the ion source and injector were 250°C and 200°C, respectively. Capillary column used was a DB-WAX (60 × 0.2 mm ID and 0.25 micron film thickness; J & W, USA). The temperature of the furnace was held at 45°C (isothermal for 1 min) and was increased to 100^{o}C in rate of 10^{o}C/min and held 1 min, then increased to 220°C in rate of 5ºC/min and held 1 min, then increased to 290°C in rate of 10ºC/min and held 10 min. Helium was used as a carrier gas at a flow rate of 1 ml/min, with injector volume of 1 µl 1:20 split ratio. A mixture of 1 µl of alkanes was analyzed to determine retention time (RT) standards for GC-FID. To preserve the index of each peak, the main program was established, which replaced the RT of each peak of *n*-alkanes confirmed at GC chromatogram. Qualitative analysis of volatile compounds was carried out by identification of mass spectra with a spectral reference.

Statistical analysis:

The results were analyzed using ANOVA test and the means differences were regarded as significant using LSD test at 5% level of probability. The obtained antibacterial results were stated in as mean ±SD for three replicates according to Gomez and Gomez (1984).

Results:

Antibacterial activity assay:

Bacterial strains were isolated from the soil and naturally infected potato collected from farmer fields in

Assiut Governorate, Egypt. The selected bacterial strains were identified as *B. subtilis*, *C. michiganensis* subsp. *sepedonicus* and *E. carotovora* subsp. *atroseptica* based on their morphological, biochemical as well as physiological characteristics using the standard characterization procedure of Skinner and Lovelock (1979); Sneath *et al.* (1986) and Buchanan and Gibbon (2001) (Table 1).

The antibacterial activities of various extracts of *A. pseudalhagi* were carried out by the well diffusion method and the results are shown in Table 2. All the investigated plant extracts exhibited different degrees of antibacterial activities at the concentration of 256 mg/ml. The highest activity was obtained against *B. subtilis* followed by *E. carotovora* subsp *atroseptica* and *C. michiganensis* subsp. *sepedonicus*. On the other hand, among all the investigated extracts, the highest antibacterial activity was obtained by methanolic extract against *Bacillus subtilis* (15.00±0.82) comparing with positive control. Ethanolic extracts recorded higher inhibition zones against *E. carotovora* subsp *atroseptica* and *C. michiganensis* subsp. *sepedonicus* (14.00±0.82 and 12.25±0.50, respectively) in comparison with the methanolic followed by acetone extract which showed the smallest inhibition zone comparing with the positive control. Aqueous extracts exhibited the lowest inhibition zones against all bacterial strains in comparison with other extracts and the positive control. The minimum inhibitory concentration (MIC) of plant extracts against the bacterial strains varied significantly. The MIC value of the same plant extract has changed according to the tested organism (Table 2). The methanolic extract showed the best MIC (16 mg/ml) against *B. subtilis* followed by *E. carotovora* subsp *atroseptica* and *C. michiganensis* subsp. *sepedonicus* (32 mg/ml). The highest MIC value (256 mg/ml) was recorded when aqueous extract was used against any of the tested bacterial strains.

GC-MS analysis of volatile oil:

The results of *A. pseudalhagi* GC-MS analysis led to the identification of number of compounds. These compounds were identified through mass spectrometry attached with GC and the mean structure are presented in Fig. 1. The nature of active principles with their retention time (RT), area %, molecular formula, probability and molecular weight (MW) in the volatile oil fraction are shown in Table 3. The results revealed the presence of 66 various phytocompounds in aerial parts volatile oil. The most abundant metabolites to all fractions are 1-(3-Furyl)-4b,7,7,9b,11a-pentamethyl-3,8-dioxohexadecahydrooxireno[d]oxireno[7,8]naphtho[2,1-f]isochromen-5-yl acetate; Hexa-t-butylselenatrisiletane; 4-(2-Methyl-cyclohex-1-enyl)-but-3-en-2-one; 1,3-Dimethyladamantane; 2-hydroxy-2-methyl-propionic acid and 5-Chloro-6-nitroandrostane-3,17-diyl diacetate.

Table (1): Morphological, biochemical and physiological characteristics of the isolated bacteria.

Characteristics			Starins					
			1		2		3	
1	Shape of cell		Rod		Rod		Rod	
2	Motility		+		-		+	
3	Gram staining		+		+		-	
4	Endospore production		+		ND		ND	
5	Hydrolysis of casein		+		-		+	
6	Gelatin liquefaction		+		-		+	
7	Urea test		-		-		-	
8	Nitrate reduction		+		-		+	
9	Starch hydrolysis		+		-		-	
10	Levan production		-		-		-	
11	Catalase test		+		+		+	
12	Indole formation		-		-		-	
13	Aesculin hydrolysis		+		+		+	
14	Methyl red test		+		ND		+	
15	Oxidase		-		-		-	
16	H_2S Production		+		-		-	
17	Citrate utilization		ND		-		+	
18	Reducing compound from sucrose		ND		ND		+	
19	Acid from:		Glucose	+	Glycerol	-	Glucose	+
-			Arabinose	+	Lactose	-	Maltose	+
-			Xylose	+	Rhamnose	-	Sucrose	+
-			Mannitol	+	Salicin	-	Mannitol	+
20	Growth in NaCl:	2.0 %	+		+		+	
-		5.0 %	+		W		+	
-		7.0 %	+		-		-	
-		10.0 %	-		-		-	
21	Growth at:	5 °C	-		-		-	
-		10 °C	+		W		W	
-		30 °C	+		+		+	
-		40 °C	+		-		-	
-		50°C	-		-		-	
Identity			*Bacillus subtilis*		*Clavibacter michiganensis* subsp. *sepedonicus*		*Erwinia carotovora* subsp. *atroseptica*	

(ND) = not determined, (W) = weak reaction, (+) = positive reaction, (-) = negative reaction

Table (2): Antibacterial activity of camel thorn extracts by different solvents against different bacterial strains

Bacterial strain	Solvent	Zone of inhibition (mm) ±SD	MIC (mg/ml)
Bacillus subtilis	Aqua	8.00±0.82 F**	256
	Ethanol	14.50±0.58 AB	64
	Methanol	15.00±0.82 A	16
	Acetone	14.50±0.58 AB	64
	Positive Control*	14.00±0.82 B	0.5
Erwinia carotovora subsp. *atroseptica*	Aqua	6.75±0.50 G	256
	Ethanol	14.00±0.82 B	128
	Methanol	12.25±0.50 C	32
	Acetone	12.00±0.82 CD	128
	Positive Control*	12.50±0.58 C	0.5
Clavibacter michiganensis subsp. *sepedonicus*	Aqua	6.25±0.50 G	256
	Ethanol	12.25±0.50 C	128
	Methanol	11.25±0.96 D	32
	Acetone	10.25±0.50 E	128
	Positive Control*	12.00±0.82 CD	0.5

* Positive Control (Streptomycin sulfate salt) 0.5 mg/ml
** Means within the same column followed by the same letter are not significantly different (P≤0.05) based on LSD.

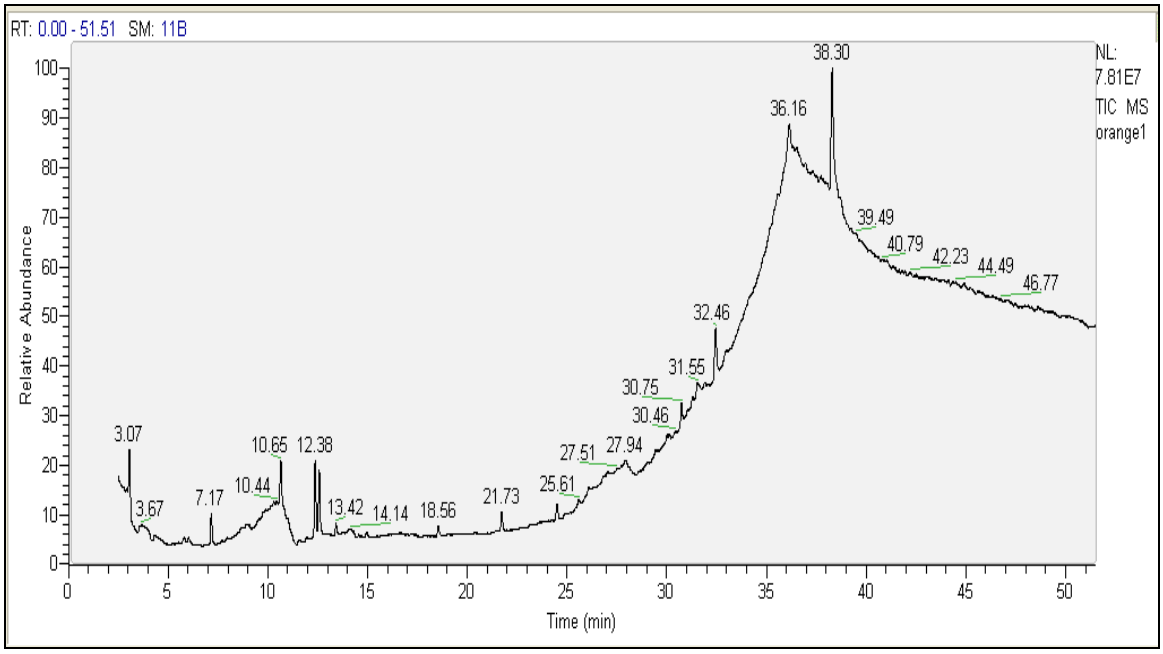

Fig. (1): GC / MS chromatogram of volatile organic components derived from *A. pseudalhagi*.

Discussion:

According to many previous studies, *A. pseudalhagi* is rich in potentially useful structures as sources of new antimicrobial and chemotherapeutic agents. Therefore, the current work was devoted to studying the antibacterial effects of various extracts from locally collected *A. pseudalhagi* plants in addition to identifying its volatile oil components using GC-MS analysis. The antibacterial assay revealed that all extracts showed varying degrees of antimicrobial activity on the microorganisms tested. Among all solvents, methanol and ethanol were the best extractants. An acceptable explanation for the superiority of both methanol and ethanol is found by Cowan (1999) that methanol and ethanol contained a large proportion of soluble polar compounds than acetone. It has been documented that different solvents have diverse solubility capacities for different phytochemical constituents. Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction.

The antibacterial activities significantly differed depending on taxonomic characteristics of the plant species as well as biological characteristics of the tested bacteria which may explain the variations in the antibacterial activity of plant extracts tested. In classifying the antibacterial activity as Gram-positive or Gram-negative, it would be generally expected that a much greater number would be active against Gram-positive than Gram-negative bacteria (McCutcheon *et al.*, 1992). Supporting this view, our results indicated that all extracts showed higher activity against Gram-positive bacteria (*B. subtilis*). Nevertheless, the growth of the Gram-negative bacteria (*E. carotovora* subsp *atroseptica*) was inhibited by any of the extracts especially ethanol. This antibacterial effect may be attributed to antioxidant compounds such as polyphenols, flavonoids and others which play a vital role in removing free radicals and in cytotoxic effects (Sulaiman *et al.*, 2011). The cytotoxic effects of phenolic compounds may depend on their lipophilicity, which is very important for the penetration into cells. On the other hand, lipids and proteins present in biological membranes facilitate the solubility of polyphenols, and differences in cell membrane structures and metabolic activation of chemicals can also affect the activity of polyphenols (Szliszka *et al.*, 2009).

The results of GC-MS analysis led to identification of 72 various phytocompounds. Our results are in accordance with those of Samejo *et al.* (2012) which revealed the presence of 2-Nonadecanone as one the abundant compounds. They also revealed that the volatile fractions of *A. maurorum* consisted of a complex mixture of different substances, with ketones (leaf – 4.4%, stem – 5.2%), acid derivatives (leaf – 1.5%, stem – 1.8%), terpenoids (leaf – 26.8%, stem – 18.7%), and hydrocarbons (leaf – 19.3%, stem – 50.6%). Also, heterocyclics (5.2%) were present in leaves, and aldehydes (0.2%) in stems. Likewise, literature survey revealed that flavonoids, fatty acids, cou-

marins, sterols, vitamins, and alkaloids are the active constituents of *Alhagi* species (Awaad *et al.*, 2006).

The results of the current work provide important and novel findings which represent a basis for more future studies on the components of *A. pseudalhagi* volatile oil. Comparing to previous literature, many new compounds were identified in the current work, which need to be extensively studied. Encouraging results were found regarding the antibacterial action of various extracts which resemble safe and inexpensive antibacterial agents against certain economically important agricultural pathogenic bacteria.

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Website: http://www.aun.edu.eg/faculty_agriculture/arabic *E-mail: ajas@aun.edu.eg*

النشاط المضاد للبكتريا والمحتوى الفيتوكيميائي لنبات شوكة الجمل *Alhagi pseudalhagi*

عصام يوسف عبد الحفيظ[١]، عامر فايز احمد محمود[٢]، عمر حسني محمد إبراهيم[١]

[١] قسم نباتات الزينة وتنسيق الحدائق، كلية الزراعة، جامعة أسيوط، مصر

[٢] قسم أمراض النبات، كلية الزراعة، جامعة أسيوط، مصر

الملخص:

أجريت الدراسة الحالية بهدف دراسة النشاط المضاد للبكتريا لمستخلصات من نبات شوكة الجمل (*Alhagi pseudalhagi*) باستعمال أربعة مذيبات (الماء ،الايثانول ،الميثانول والأسيتون) ضد سلالات بكتيرية موجبة لجرام (*Bacillus subtilis* and *Clavibacter michiganensis* subsp. *sepedonicus*) وسلالة أخرى سالبة لجرام (*Erwinia carotovora* subsp. *atroseptica*) وأجري الاختبار بطريقة الانتشار في حفر الأجار agar well-diffusion method. كما تم تقدير أدنى تركيز مثبط MIC للمستخلصات ضد البكتريا. بالاضافة إلي ذلك، تم تقدير المحتوى الفيتوكيميائي للزيت الطيار المستخلص من الأجزاء الخضرية لنبات شوكة الجمل وذلك باستخدام جهاز كروماتوجرافيا الغازات المتصل بوحدة مطياف الكتلة GC-MS. وأظهرت نتائج اختبار النشاط المضاد للبكتريا فعالية معنوية لجميع المستخلصات ضد جميع السلالات البكتيرية وذلك عند تركيز ٢٥٦ ملجم/مل. وكانت أكبر منطقة تثبيط وأقل قيمة لأدنى تركيز مثبط عند المعاملة بمستخلص الميثانول. في حين أظهر المستخلص المائي أصغر منطقة تثبيط وأعلى قيمة لأدنى تركيز مثبط. وكانت فعالية مستخلصي الايثانول والأسيتون متوسطة تجاه جميع السلالات البكتيرية المختبرة. كما أمكن من خلال التحليل الكروماتوجرافي تعريف ٦٦ مركباً معظمها ثبتت مسبقاً فعاليتها المضادة لنشاط البكتريا والأورام السرطانية وكمواد مطهرة ومواد حافظة ومضادة للحشرات ومضادة للأكسدة. وكانت أعلاها تركيزا في الزيت 1-(3-Furyl)-4b,7,7,9b,11a-pentamethyl-3,8-[2,1-f]isochromen-5-yl acetate; dioxohexadecahydrooxireno[d]oxireno[7,8]naphtha Hexa-t-butylselenatrisiletane; 4-(2-Methyl-cyclohex-1-enyl)-but-3-en-2-one and 1,3-Dimethyladamantane.