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Insecticidal bioactivity of eco-friendly plant origin chemicals against *Culex pipiens* and *Aedes aegypti* (Diptera: Culicidae)

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Abstract
Mosquitoes are the most important medical insects in the entire world. Natural chemicals have many advantages over the conventional ones in case of mosquito control. The aim of this study is to evaluate the larvicidal and adulticidal bioactivity of some plant origin chemicals against *Culex pipiens* and *Aedes aegypti* (Diptera: Culicidae). The chemicals under investigation of Myristicin, Sage oil and Nutmeg oil were isolated and structurally elucidated using GC/MS, and ^{1}H-NMR, spectra that compared with the authentic samples. Myristicin was the highest larvicidal bioactive compound with LC_{50} values 18.3 ppm against *Cx. pipiens* and *Ae. aegypti*, larvae after 24 h of treatment, respectively. The rest of the tested materials showed also high larvicidal and adulticidal activities that may be related to their volatile bioactive constituents. From the results of this study; its highly recommend the tested natural chemicals to be an alternative eco-friendly source for mosquito control chemicals.

Keywords: mosquito, larvicidal, adulticidal, Nutmeg oil, Sage oil and Myristicin.

1. Introduction
Mosquitoes are the most dangerous insects on earth. It is hard to comprehend the amount of disease and the resulting sickness, death, and economic loss caused by the mosquito. Females are important vectors of many tropical diseases, including Malaria, Sleeping sickness, Filariasis, and numerous viral diseases, such as Dengue, Yellow fever and Japanese encephalitis. In the temperate climate countries they are more important as nuisance pests than as vectors. There are about 3000 species of mosquito, of which about 100 are vectors of human diseases [1]. Control measures are directed mainly against only one or a few of the most important species and can be targeted at the adult or the larval stages [2].
For many decades, the scientists have been engaged in searching the effective and efficient of the mosquito control program based on chemicals. The resistance to conventional insecticides is the major challenge in mosquito control program. The WHO expert committee felt that the resistance in vectors was probably the "biggest obstacle in the struggle against vector-borne diseases" [3]. The conventional insecticides are environmentally non-sustainable and harmful for the non-target biome, moreover, most mosquito species are becoming physiologically resistant [4].
The mosquito larvicidal activity of many different plant extracts and fractions was reported [5-9]. Sukumar *et al* [10] listed and discussed 344 plant species that exhibited mosquitocidal activity. Promsiri *et al.* [11] were evaluated the larvicidal activity of the phytochemicals extracted from 112 medicinal plant species against *Aedes aegypti* mosquito, and the plant species under investigation were collected from the southern part of Thailand. The study extended to assess the effect of the tested plant extracts on non-target guppy fish, *Poecilia reticulata.* Medicinal plant extracts are effective in mosquito control and they may greatly reduce the adverse ecological effect risk and do overcome the obstacle of mosquito pesticide resistance [12]. The search for an eco-safe, low cost and a highly potential insecticide for the control of mosquitoes needs the preliminary screening of plants to evaluate their insecticidal activities.
Plant-based phytochemicals do not have any hazardous effect on the ecosystem. Recent research has proved that effectiveness of plant derived secondary metabolites, such as saponine [13, 14], steroids [15], isoflavonoids [16], essential oils [17], alkaloids and tannins [18] as potential mosquito larvicides. Plant secondary metabolites provide an alternative source in the control of mosquitoes [19, 20].

From the previous review, the search for natural biodegradable botanical mosquitocides that are eco-friendly and may be difficult for mosquitoes to develop resistant strains, is important and urgent as regards environmental sustainability. In this study, we tried to fill this research gap.

The objective of this study was to investigate role of natural chemicals from two medicinal plant origin of sage *Salvia officinalis*, and nutmeg *Myristica fragrans*, as alternative eco-friendly insecticides against the laboratory strains of *Culex pipiens* and *Aedes aegypti* (Diptera: Culicidae).

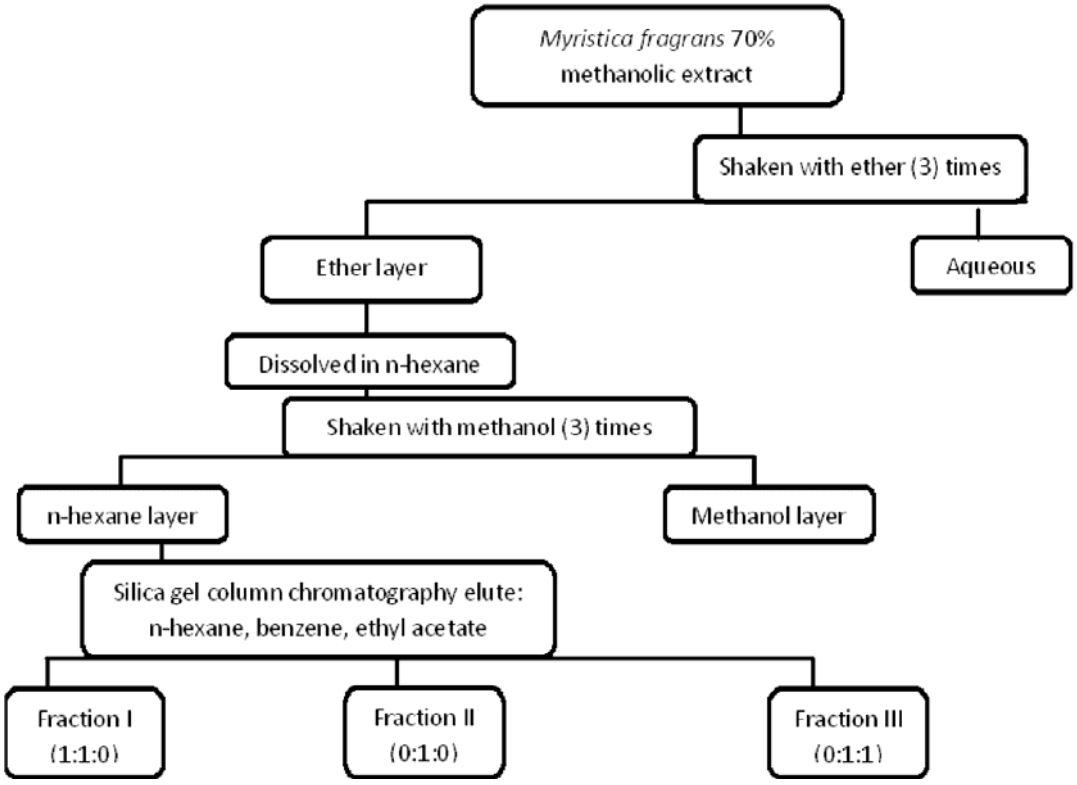
2. Materials and Methods

2.1 Extraction of Nutmeg Oil and Isolation of Myristicin

The nutmeg kernel seeds (*Myristica fragrans*) were imported from Tanzania by Harraz Herbs Company for medicinal and aromatic plants, Cairo, Egypt. Seeds were powdered in the blander and the oil extracted by steam distillation method. 50 g powdered plant material was placed in a distillation flask of 500 ml, contained water about 2/3 of its volume. The operation proceeded by heating the flask at water boiling point 100 ˚C, heat was applied to the flask and the volatile oil was carried with the steam to a cold condenser, the lighter oil rises to the top of the separator. The essential oil collected was dried over anhydrous sodium sulphate, weighed and stored in a sealed vial dark colored at 4˚ C. The yield percentage of essential oil was determined using the formula described by Rao *et al.* [21].

$$\text{Yield (\%)} = \frac{\text{Amount of essential oil recovered (g)}}{\text{Amount of plant material distilled (50 g)}} \times 100$$

Isolation of myristicin according to (Essam and Maythan) [22], with some modification as shown in **(Scheme (1)**. 50 gram of the crude powder of seeds was refluxed with 350 ml of 70% methanol (1:7) in a soxhlet apparatus for 8 hours.



Scheme 1: Flow diagram of fractions of the nutmeg methanol extract on the silica gel column.

A partial purification of myristicin from the n-hexane phase preceded using open glass column (2.5 x 21 cm) filled with silica gel G200 special for column chromatography. The residue was dissolved in 1-2 ml hexane and the mobile phase was n-hexane: benzene: ethyl acetate, 1:1:1.

The elusions were collected for each of mobile phase used and numbered as fractions; all fractions were tested on T.L.C. plates for the presence of myristicin. Only the positive results elution were collected and dried under vacuum by a rotary evaporator. The myristicin spots were detected on a TLC aluminum sheet silica gel *60F254* in comparison with the standard spot using the same mobile phase in the column chromatography. The RF values (mobility relative to solvent front) were measured to represent the distance; a compound moved in chromatography relative to solvent front [23]. The collected elution after dryness was referred to as "partial purification" for myristicin. Acetone solvent used for crystallization to obtain the pure myristicin.

Note: the (eco) toxicological data for the product “hesperidine” are incomplete. No more data are available on its chronic toxicity. For the solvents used in this experiment relevant (eco) toxicological data are available. Only methanol exhibits a significant acute toxicity. The other organic solvents such as dimethyl sulfoxide, petroleum Ether (40/60), 2-propanol, 1-butanol and acetic acid are of relatively low acute toxicity and do not exhibit chronic toxicity.

Except for petroleum ether (40/60) all organic substances in this experiment are biologically easily degradable and mineralizable. The evaluation data for the ecotoxicity of the solvents indicate a low to medium toxicity for aquatic organisms.

2.2 Extraction of Sage Oil

Leaves of the *Salvia officinalis* were collected from Sousse

city which located 140 kilometers (87 miles) south of the capital Tunis, Tunisia. The botanical material identified by the expert Prof Dr. Ebrahim Mashaly, in the herbarium of Botany Department, Faculty of Science, Mansoura University. The plant material were collected during the flowering phase (November/2013).

The techniques used for extraction was hydrodistillation. The leaves had been air-dried until weight stabilization. Crushing done to produce a fine power. The hydrodistillation have been realized with 500 g of dried sage leaves and 2.5 liters of distilled water [24]. This technique is the most frequently applied method to produce essential oils from sage. The organic phase has been treated with anhydrous sodium sulphate (Na_2SO_4) to eliminate all water.

GC and GC/MS Analysis of the essential oils was performed using a Hewlett-Packard 5890 GC apparatus with FID detector [25]. The carrier gas was helium at a flowing rate of 1.2 ml.min^{-1}. Injector temperature was 240 °C, the injector and detector temperature was 250 °C and the oven temperature 240 °C with a heating rate of 9 °C min^{-1}. The column used was DB-5 fused silica capillary column (25 m x 0.25 mm i.d., 0.25 µm film thickness). The samples were injected in split mode with an injected volume of 0.2 µl. The injection concentration of essential oil was 1% in hexane. The relative amounts of the individual components are based on the peak area obtained with FID response factor correction. The identification of the essential oil components was performed by GC-MS analysis using a Hewlett-Packard 5890 gas chromatograph coupled to a Hewlett-Packard 5972 Mass Spectrometer under the same conditions as in GC analysis but using a 30 m x 0.25 mm id, 0.25 µm film thickness DB-5 column, ionization energy 70 eV, scan time 1 s, mass range 40-300 m/z. Mass spectra correlations were done using Hewlett-Packard Wiley 275.L, or with authentic compounds and The components were identified based on the comparison of their retention indices relative to n-alkanes series and mass spectra with those of authentic samples.

2.3 Mosquitoes Culture

Susceptible strains of *Cx. Pipiens* and *Ae. aegypti* (Diptera: Culicidae) were obtained from the laboratory of Entomology, faculty of Science, Mansoura University, which kept for 2 subsequent successful years.

They were maintained and all the experiments were carried out at 27 ± 2 °C and 75–85% RH. Yeast suspension (5%) was used as food source for larvae, and adults male feed on 10% sugar solution while females were received blood meals periodically from rabbits for egg production.

2.4 Mosquitoes Larvicidal Bioassay Test

The natural products and the essential oils from different tested plant species were evaluated at the level of 5, 10, 15, 20 and 25 ppm in dechlorinated tap water. Propylene glycol was used as emulsifying agent at a concentration of 0.01%. Dechlorinated tap water mixed with 0.01% propylene glycol and used as control. Standard **WHO** test protocol [26] was used with slight modification in the procedure. Twenty five larvae from the 3rd larval stage were put into a container dish containing 145 ml dechlorinated tap water plus 5 ml of the test solution of each concentration. 4 replicates for each concentration. Observation on larval mortality was recorder after 24 h and 48 h. The larvae considered dead, when they did not react to the needle touching. Probit analysis is the base of the used program LDP-line for analyzing the mortality data and detecting the lethal concentrations (LC_{50} and LC_{90}).

2.5 Mosquito Adults Vapor Toxicity Test

The isolated natural chemicals and essential oil of different tested plant species were dissolved in acetone to make different concentrations. A card board sheet of size (18 x 11 mm) and thickness (1 mm) equal to commercially available mosquito mats were treated with tested material of different concentrations. Control card board sheets were treated with acetone under similar conditions. Acetone was allowed to evaporate at room temperature from the mats. Adulticidal activity of the tested materials was evaluated at four concentrations (0.07, 0.13, 0.20, and 0.27 mg/mat) to produce a range of mortality from 10 to 100% along with control. The mosquito mat machine was used for evaporating the tested materials from the treated cards when kept on for 15 min. the technique used for adult toxicity vapor test was performed by Vartak and Sharma [27], with some modifications. Fifty female mosquitoes from each of of *Cx. pipiens* and *Ae. aegypti* (2-5 days old sugar fed, blood starved) were collected and gently transferred in two different mosquito glass cages (70 X 85 cm^2). The mosquitoes were allowed to acclimatize in the holding tube for 1 h and then exposed to test cards for 1 h. Adult mortality with different concentration of different natural chemicals were record at 1 h after the treatment. The experiment was repeated twice for each concentration. Data of adult mortality were analyzed statistically for LD_{50} and LD_{90} values.

2.6 Statistical Analysis

Ldp-line a computerized software program was used to determine the dose-response relationship by probit analysis [28]. Mortality data were corrected using Abbott's formula [29].

3. Results and Discussion

3.1 Spectral Analysis of the Tested Natural chemicals

3.1.1 GC and GC/MS analysis of essential oils

The essential oils collected from different plant materials of nutmeg kernel, *M. fragrans* and Sage leaves, *S. officinalis* were less dense than water and exhibited a colorless or pale yellow color for nutmeg oil and yellow greenish color for sage leaves oil.

Nutmeg essential oil was extracted by steam distillation method with a yield of 2.5 – 3.6 gm / 50 gm. The compounds identified in the oil sample are presented in **Table 1**. GC and GC/MS analysis of the nutmeg oil resulted in the identification of 12 major constituents which account for 88.08 % of the oil. The spectrum characterized by high percentage of Myristicin, Sabinene, 4-Terpineol, α-Pinene and Limonene. This is in accordance with the previous reports found in literature [30, 31].

GC and GC/MS analysis of the sage oil resulted in the identification of 12 major constituents which account for 79.49 % of the oil (**Table 2**). Its chemical composition is characterized by major constituents of α-thujone, camphor, 1-8- Cineol and camphene. In previous studies, the essential oils of some sage populations from Montenegro and Serbia have been studied. In the oil of sage leaves collected from different locations, α-thujone, β- thujone, borneol and manool were the major components. Similar to the results, Raal *et al* were identified the Composition of the essential oil of *S. officinalis,* that collected from Serbia. Camphor was the first major constituent besides thujone and 1, 8-cineole [32]. Furthermore, Tomasz *et al* confirmed the presence of terpenoids as major components of the sage essential oil obtained by

hydrodistillation [33].

Table 1: Chemical composition of the nutmeg kernel seed oil corresponding to the GC/MS chromatogram.

No.	Rt (min)	Compound*	content%
1	6.24	α-Pinene	10.31
2	7.51	Sabinene	15.37
3	8.49	α-myrcena	2.40
4	8.84	Limonene	6.75
5	9.52	ɣ-Terpinolene	3.98
6	10.11	Terpinolene	1.69
7	10.39	Linalool	0.97
8	12.30	4-Terpineol	14.92
9	14.16	Safrole	4.21
10	16.57	Isoeugeunol	3.76
11	17.78	Myristicin	20.58
12	18.02	Elimicin	3.14
13	20.62	Myristic acid	0.91
14	21.35	Palmitic acid	0.08

Rt refers to the spectrum retention time.

*Constituents identified by mass spectra comparison of their retention indices relative to n-alkanes series and mass spectra with those of authentic samples.

Table 2: The chemical composition of the sage leaves oil corresponding to the GC/MS analysis.

No.	Rt (min)	Compound*	content%
1	8.63	*Cis*-Salvene	0.30
2	9.34	α-Thujene	2.45
3	9.52	α-Pinene	0.36
4	9.68	Camphene	6.15
5	9.87	Sabinene	0.10
6	9.98	β-Pinene	0.45
7	10.36	Limonene	0.38
8	10.39	1-8-Cineole	14.92
9	10.54	ϒ-Terpinene	0.18
10	11.15	α-Thujone	30.75
11	11.39	β-Thujone	5.34
12	11.61	Camphor	16.72
13	12.00	α-Terpineol	0.41
14	12.76	Bornyl acetate	0.98

Rt refers to the spectrum retention time.

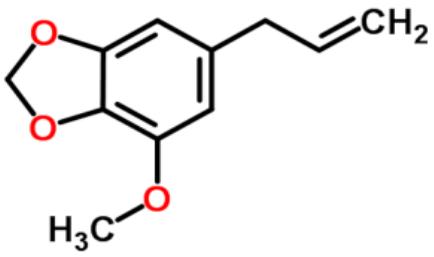
*Constituents identified by mass spectra comparison of their retention indices relative to n-alkanes series and mass spectra with those of authentic samples.

3.1.2 Chromatographic and Spectral Analysis of the Isolated Myristicin

50 g nutmeg kernel seeds powder yielded 0.85 g of myristicin. The bioactive aromatic cumarin were characterized by comparing the physical and spectroscopic properties with the authentic samples.

Hesperidin spectral data:

^{1}H-NMR study was performed to determine the structure of isolated



Myristicin spectral data

Myristicin (4-methoxy-6-[2-1propenyl] 1, 3-bezodioxole), belonging to the apiol group of compounds, was isolated and identified from the hexan fraction of *M. fragrans* alcoholic extract. Separation by TLC fraction II (Rf= 0.70) was done, and the pertinent ^{1}H NMR data support the structure of myristicin with characteristic signals (δ ppm) at 3.30 (br. d., -CHr), 3.90 (s, -OCH3), 5.10 (br. d., CH2=), 5.93 (br. m., CH=), 5.94 (s, -OCH20-), and 6.40 (br. m., Aromatic 1-1); Various other small signals are due to the minor components in the mixture. The data were consistent with data obtained from authentic sample of commercial myristicin and those reported in the literature [34].

3.2 Mosquito Larvicidal Activity

The findings of larvicidal investigation indicate the activity of essential oils of *M. fragrans* followed by *S. officinalis* and isolated compound of myristicin against 3rd instar larvae of *Cx. pipiens* and *Ae. aegypti*. *Cx. pipiens* are the most non-significantly susceptible towards toxicity of the tested materials with LC_{90} value of 119.2, 125.2, and 147.9 ppm, respectively (Table 3) and LC_{50} values in the range of 22.9, 24.4, and 28.5 ppm as compared to *Ae. aegypti,* with LC_{90} values of 121.2, 120.5, and 151.4 ppm and LC_{50} values of 22.9, 25.1 and 28.8 ppm, after 24 h of treatment (Fig. 1, 2). The recorded non-significant susceptibility of *Cx. pipiens* was documented by Manimaran, *et al* [35].

The increase of exposure time led to the increase of mosquito larval mortality with the following values of LC_{50} 15.2, 16.8, and 17.9 ppm for *Cx. pipiens* larvae. While, Ae. *aegypti* larvae showed non-significant tolerance with LC_{50} values of 16.8, 18.4, and 19.7 ppm, against the tested materials of myristicin, sage oil and nutmeg oil, respectively (Table 3 & Fig 3, 4). It was observed, in general, that *Cx. pipiens* was more susceptible towards all the tested chemicals than *Ae. aegypti.* The insecticidal activity of myristicin has been reported as contact toxicity and antimetabolic activity against 4th instar larvae of Lepidopterous insect *Spilarctia obliqua* after 24 h, the LD_{50} was 104 µg/larvae [36]. Sage oil composed of high value of terpenoids (Thujone, camphor and cineole) that were reported with strong larvicidal effect against *Anopheles stephensi*, as an essential leaf oil extract from *Eucalyptus tereticornis* [37].

Table 3: Larvicidal bioactivity of the essential oils and natural chemicals of the medicinal plants against *Cx. pipiens* and *Ae. aegypti.*

Tested material against mosquito species		Activities							
		24 h treatment				*48 h treatment*			
		LC_{50} (ppm)	**LC_{90} (ppm)**	**χ^2**	**Slope**	**LC_{50} (ppm)**	**LC_{90} (ppm)**	**χ^2**	**Slope**
Myristicin	***Cx. pipiens***	22.9	119.2	0.3	1.8±0.24	15.2	68.0	0.6	1.8±0.23
Sage oil		24.4	125.2	0.9	1.8±0.25	16.8	92.3	0.7	1.7±0.23
Nutmeg oil		28.5	147.9	0.4	1.8±0.25	17.9	98.5	0.2	1.7±0.25
Myristicin	***Ae. aegypti***	22.9	121.2	0.9	1.9±0.23	16.8	87.3	0.6	1.8±0.23
Sage oil		25.1	120.5	0.4	1.9±0.24	18.4	106.8	0.8	1.7±0.24
Nutmeg oil		28.8	151.4	0.5	1.8±0.24	19.7	117.2	0.9	1.8±0.23

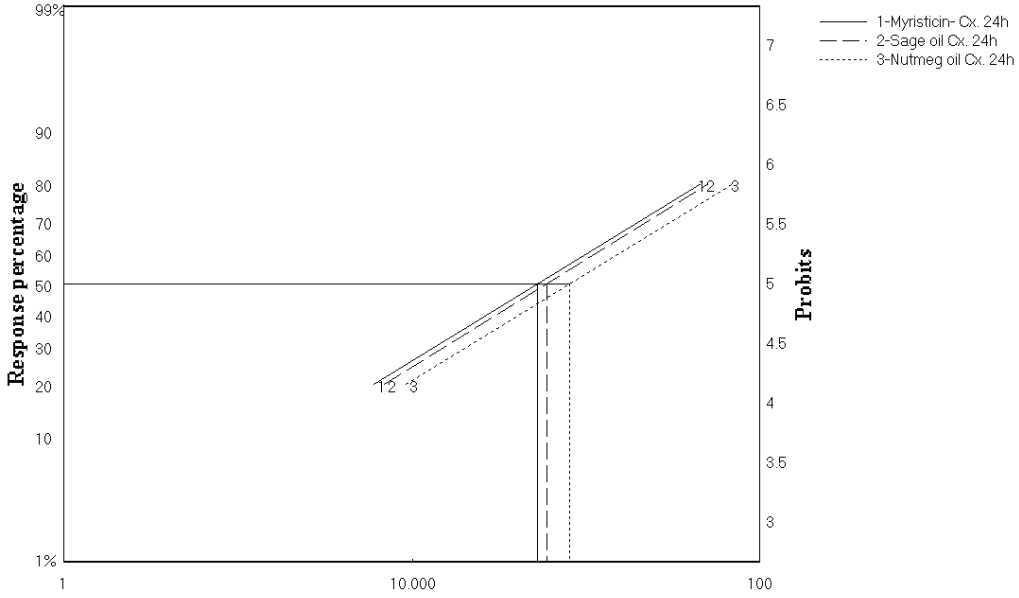


Fig 1: Relative toxicity of the tested materials against *Cx. pipiens* mosquito larvae after 24 h treatment

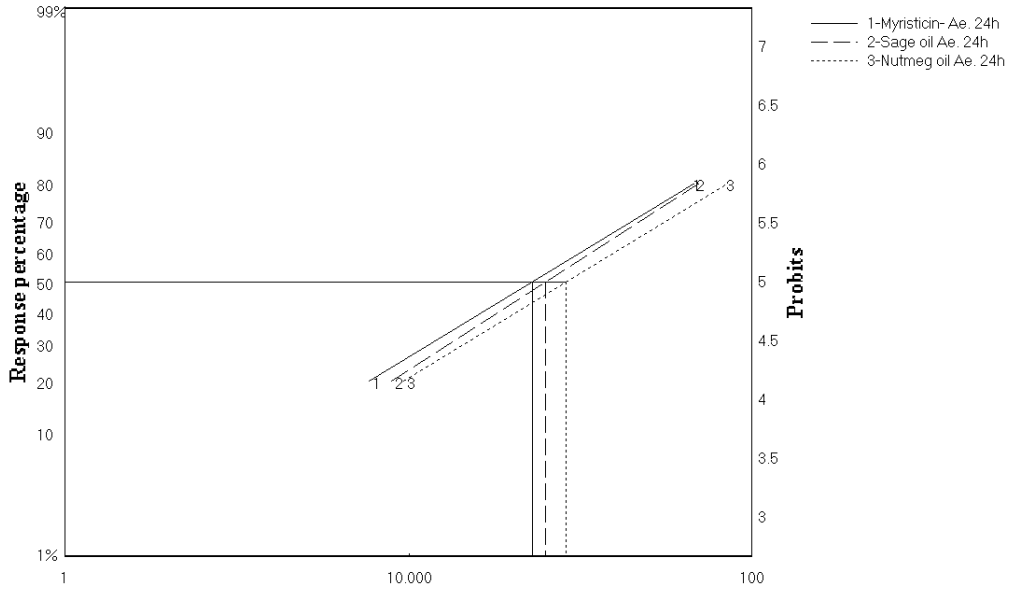


Fig 2: Relative toxicity of the tested materials against *Ae. aegypti* mosquito larvae after 24 h treatment.

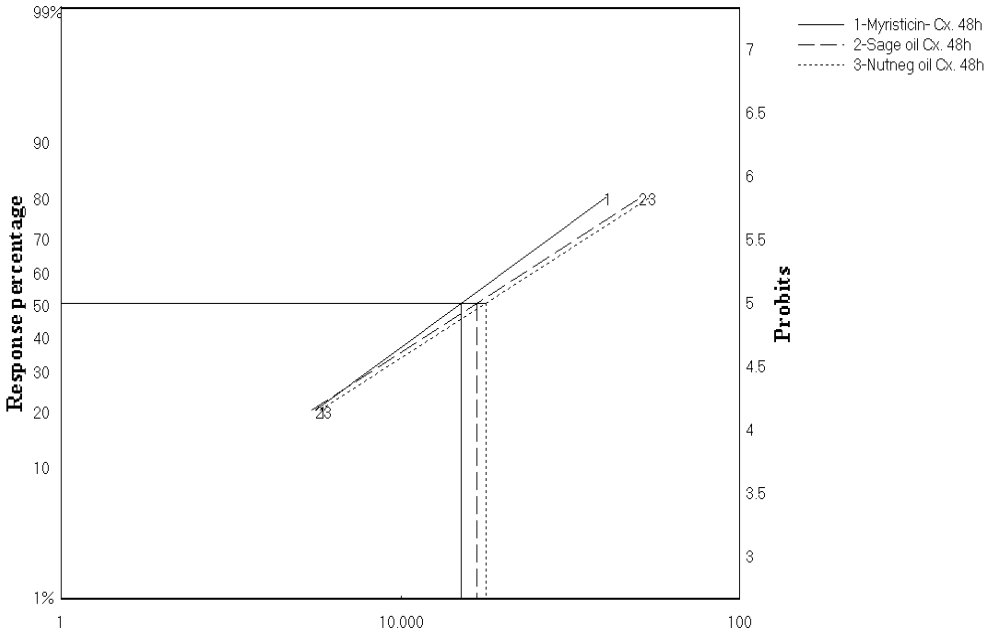


Fig 3: Relative toxicity of the tested materials against *Cx. pipiens* mosquito larvae after 48 h treatment.

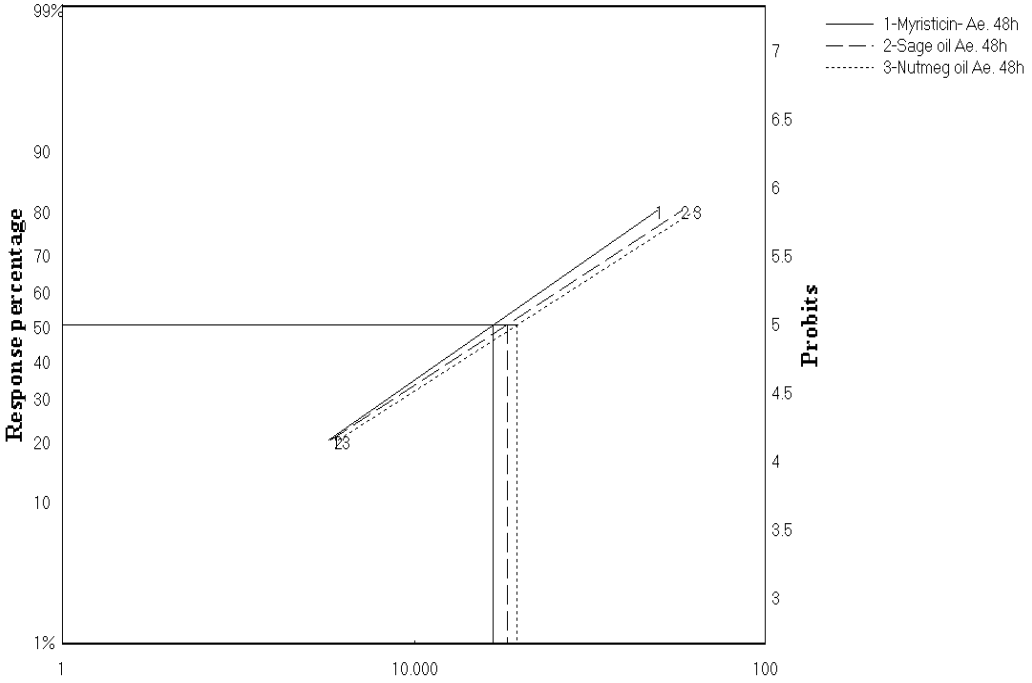


Fig 4: Relative toxicity of the tested materials against *Ae. aegypti* mosquito larvae after 48 h treatment.

3.3 Adulticidal Vapor Toxicity

Results of adulticidal vapor toxicity indicates that myristicin compound was the highest bioactive against *Cx. pipiens* and *Ae. aegypti* with LD_{50} values 0.12 and 0.15 mg/mat, respectively. However, sage oil and nutmeg oil have got less adulticidal bioactivity against *Cx. pipiens* and *Ae. aegyti,* with LD_{50} values 0.28, and 0.34 mg/mat, while, the LD_{50} values were 0.37, and 0.57 mg/mat in the case of *Ae. aegypti.*

It was observed in general, that *Cx. pipiens* more susceptible towards the response of 3 tested materials with respect to the evaluated adulticidal toxicity (Table 4, Fig. 5).

Survey of literature shows directs that the bioactivities of the tested plant materials evaluated in the present investigation against *Cx. pipiens* and *Ae. aegypti*, have not been documented. However, thujone, camphor and cineole were the major components of sage leaves oil *S. officinalis,* they were documented as mosquitoes toxic and repellent natural compounds but were isolated from different plant sources [38, 39].

Table 4: Adulticidal vapor toxicity of the essential oils and natural chemicals of the medicinal plants against *Cx. pipiens* and *Ae. aegypti*.

Tested material against mosquito species		*Activities*					
		LD_{50} (mg/mat)	**Lower limit**	**Upper limit**	**LD_{90} (mg/mat)**	***RR***	**Slope**
Myristicin	***Cx. pipiens***	0.12	0.10	0.15	0.63	1.00	1.8±0.29
Sage oil		0.28	0.23	0.44	1.79	1.00	1.6±0.32
Nutmeg oil		0.47	0.32	1.23	3.73	1.00	1.4±0.33
Myristicin	***Ae. aegypti***	0.15	0.13	0.18	0.77	1.33	1.7±0.29
Sage oil		0.34	0.26	0.57	1.92	1.20	1.7±0.33
Nutmeg oil		0.57	0.37	1.87	4.54	1.20	1.4±0.34

RR: Resistance Ration

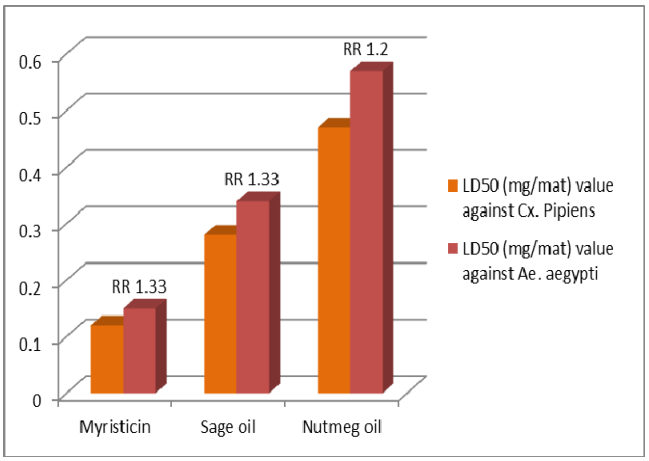


Fig 5: Does response relationship of the tested natural chemicals against female adult mosquito of *Cx. pipiens* and *Ae. aegypti*.

4. Conclusion

The screening results suggested that myristicin and the essential oils of sage leaves, *Salvia officinalis*, and nutmeg seed kernel, *Myristica fragrans*, are promising as alternative eco-friendly larvicides and adulticides against the laboratory strains of *Culex pipiens* and *Aedes aegypti* (Diptera: Culicidae).

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