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Toxicological and behavioural potency of different plant extracts on *Aedes albopictus* (Diptera: Culicidae) and their qualitative phytochemical analysis

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Abstract
Control of Asian tiger mosquito is very crucial for newly established areas. Botanically based insecticides could be used more as alternative biocontrol agents in the future. The ethyl acetate extracts of some plants naturally growing in Turkey were analysed for potential oviposition deterrent, ovicidal, and skin repellent activities against *Aedes albopictus*. According to the determined oviposition activity index and per cent effective repellence of these plant extracts at constant concentration in the first week, the most effective result belonged to *Salvia verticillata* (-0.9942 and 99.7%, respectively). In these trials for four weeks, the best persistence belonged to *Matricaria chamomilla.* In addition to this test, the ovicidal activities and skin repellent activities for the same plant extracts were determined. Qualitative phytochemical analyses of all plants were performed and showed that alkaloids, flavonoids, terpenoids, coumarins, quinones, tannins, phenols and carbohydrates were present in varying proportions in all plants. All of evidence showed that the plant extracts could be used as potential oviposition deterrents, ovicides and repellents against *Aedes albopictus*.

Keywords: *Aedes albopictus*, oviposition deterrent, ovicidal activity, phytochemical analysis, repellent activity

1. Introduction

Mosquitoes belonging to the Culicidae family are responsible for transmitting viruses such as dengue, yellow fever, Zika and malaria in many parts of the world [1]. In the European continent, these diseases were initially seen as transport cases, but in recent years, they have been identified as local cases, and *Aedes albopictus* has been reported to be the main vector [2-4]. The genus *Aedes* is one of the most important species of Culicidae family. The different species of *Aedes* mosquitoes have a particular ecology, behaviour, geographical distribution and are native to temperate and tropical habitats worldwide. However, some species such as *Aedes aegypti* and *Aedes albopictus* have spread outside their natural areas because of changing environmental influences [5]. One of the reasons for this situation is that waste management systems are insufficient due to rapid human population growth and increasing urbanization.

Aedes albopictus, which is mainly a forest species, is also named the Asian tiger mosquito. In time, this mosquito species have become adapted to rural, suburban and urban human environments. This species is a vector of at least 22 arboviral infections (dengue, chikungunya, yellow fever, and Zika) [5-6]. At the beginning of the 20th century, it spread rapidly to Europe, Africa, America and Australia from Southeast Asia. This mosquito is an aggressive biter with a wide host range including human, domestic, and wild animals.

Various synthetic insecticides have been designed for insect control. However, environmental problems caused by intensive use, toxicity and detrimental effects on non-target organisms and many other reasons such as fast resistance development have led to the search for different control agents [7-9]. Generally, the target of mosquito control studies is to cause larval death in breeding areas by using larvicide, because adulticides may only reduce the mosquito population temporarily. In this regard, many bioactive phytochemicals obtained from natural materials have been seen as weapons in mosquito control studies due to their different effects [10-13].

Some plant crude extracts and essential oils are potential natural resources for insecticides due to their lipophilic structure, which can interrupt the functions of insects, such as their metabolic, biochemical and behavioural properties, and make them ineffective [13]. They have insecticidal activity against various insect species and the potential of being regulators against ovulation and growth. In addition, many different plants have been tested as an insect repellent [14]. Moreover, plant oils contain bioactive components that due to multiple effects, and reduce the chance of resistance development of mosquitoes [15]. These effects are dependent on factors such as the solvent polarity used for extraction, plant species, plant part and mosquito species [16-17]. Most of the plants with insecticidal activities in terms of phytochemical content have been known to belong to Lamiaceae, Asteraceae, Rutaceae, Lauraceae and Ericaceae families [18]. The activity of crude extracts and essential oils obtained from some plants belonging to these families has been reported against larvae and adults of *Aedes* and *Culex* mosquitoes [19-26].

The main aim of our investigation is to determine the effect on oviposition deterrence and ovicidal activity on the eggs of *Aedes albopictus* of ten plants naturally growing in Turkey, as well as to examine the repellent activity of phytochemical extracts against mosquito bites and to analyse the presence or absence of different phytochemicals in the selected plants. The results of this study may be a benefit for the use of these plants or other related projects.

2. Materials and Methods

2.1 Plant material

A selection was made of ten plants belonging to five plant families known for their potential activity against mosquitoes. All plants were collected from different areas in Turkey and transported to the laboratory. The locations where the plant samples were collected and the amounts of oil obtained after extraction are given in Table 1.

Table 1: Selected plants in the study and crude oil yields

Plant species	Plant family	Plant part	Origin and date	Crude oil yield %
Salvia verticillata L.	Lamiaceae	aerial part	Antalya, April 2018	1.72
Matricaria chamomilla	Asteraceae	aerial part	Rize, May 2018	1.97
Leucanthemum vulgare	Asteraceae	aerial part	Rize, July 2018	2.47
Triploaraspermum caucasicum	Asteraceae	aerial part	Rize, July 2018	4.03
Erigeron annuus	Asteraceae	aerial part	Rize, August 2018	2.47
Inula vulgaris	Asteraceae	aerial part	Rize, August 2018	3.33
Citrus sinensis	Rutaceae	fruit peel	Rize, February 2018	2.17
Laurus nobilis	Lauraceae	aerial part	Denizli, April 2018	1.61
Rhododendron ponticum	Ericaceae	flower	Rize, May 2018	1.62
Rhododendron luteum	Ericaceae	flower	Rize, July 2018	1.05

2.2 Mosquito culture

Aedes. albopictus was collected from Rize, Turkey in July 2018 (41.0416 Lat. 40.5771 Lan) / (41.0453 Lat. 40.5784 Lan.). All samples were collected from waste containers and tires at larval stages. Then, live collected larvae were colonised in the laboratory conditions [27-28]. *Ae. albopictus* adults obtained from these colonies (F1) were used in assays.

2.3 Preparation of plant extracts

During the extraction of plant material, it was important to minimize plant parasites and prevent contamination of the extract due to effects such as solvent impurities. For this reason, it was important to clean and wash the plant material with a washing solution before extraction. Before use, fruit peels or the aerial parts of fresh plants were cleaned and thoroughly washed to prevent fungus with a distilled water/alcohol mixture (1:1, v/v). Then, all materials were dried for three days at room temperature (25% humidity), and at end of this time, each of the samples were powdered using an electric blender. The extraction procedure is as in the literature [29]. After a quantity of crude plant extract was reserved for phytochemical analysis, stock solutions (0.1 g/mL) of each sample were prepared in DMSO for bioassay and kept at below 4 °C until analysis. Oil yields obtained from plant materials are given in Table 1.

2.4 Oviposition deterrence activity

For the bioassay test, 25 male and 25 female *Ae. albopitus* were placed into cages (45 × 45 × 45 cm) in insectarium at 27 ± 2 °C and 75 ± 10% relative humidity with a constant photoperiod regime (L:D, 14:10 h). For feeding adults, a plastic cup containing 10% sucrose solution and cotton wick was placed in the cages. Adults were fed with blood in the first two days. Then, five oviposition test cups including white filter paper for oviposition and 100 mL distilled water/plant extract mixture were put to a cage. Each cup to be tested had a final concentration of 200 ppm. The sixth container without extract (only DMSO) was placed as a control. Eggs that the female mosquitoes laid during the test were counted during the 28-days. Tests were performed three replicates and results were evaluated the average number of eggs with three replicates. The oviposition activity index (OAI) was calculated using the suggested formula [30]. The OAI varies from -1 to 1, with 0 indicating no response. This indicates the deterrence of the tested plant extracts.

$$OAI = N_T – N_C / N_T + N_C$$

(N_C = is the number of eggs in the control cup; N_T = is the number of eggs in the experimental cup). The per cent effective repellency (ER%) for all of the plant extract was calculated using the following formula [31].

$$ER\% = (N_C-N_T/N_C)*100$$

2.5 Ovicidal activity

For the ovicidal activity assay, eggs obtained from each test cup containing plant extract were used in the oviposition deterrence experiment. The filter papers were left outdoors for one week until getting completely dry. Then each paper was placed in 250 mL plastic container with approximately 1 cm deep water, to assess egg-hatching rate. These experiments continued for four weeks in parallel with the oviposition deterrence activity tests. Results were evaluated average number of larvae and eggs with three replicates. Percentage hatchability for larvae was calculated with the undermentioned formula [32]. The per cent hatched in the experimental group was compared to that of the control group (control group hatching rate was accepted as 100%).

% hatchability = (Number of larvae hatched / Total number of eggs) * 100

2.6 Skin repellent activity

Ten crude extracts *Salvia verticillata*, *Matricaria chamomilla*, *Leucanthemum vulgare*, *Triploaraspermum caucasicum*, *Erigeron annuus*, *Inula vulgaris*, *Citrus sinensis*, *Laurus nobilis*, *Rhododendron ponticum*, *Rhododendron luteum* were tested for repellency against *Ae. albopictus* bites. This study was based on the human feed technique [19, 33], which included counting the number of bites of the volunteer who put his/her hand into the mosquito cage. For this purpose, cages (45 × 45 × 45 cm) containing 50 mosquitoes were set up, and these mosquitoes were not fed with blood for 48 hours. Approximately 3 mL of extract (from 10% DMSO stock solution) was applied to the forearm of three different volunteers. Then, extract applied arm was inserted into the cage for 2 minutes every 15 minutes for around one hour. The DMSO only treated arm was used as a control. Tests were repeated two times for each plant extract by each human volunteer. The repellent effect (%) was calculated with the undermentioned formula [32].

Repellency % = (Ta – Tb / Ta)*100

Ta and Tb are the number of control and test groups mosquitoes, respectively.

2.7 Qualitative analysis of phytochemical substances in plant extracts

Qualitative tests were performed to identify natural chemical components such as alkaloids, flavonoids, terpenoids, coumarins, quinones, tannins, saponins, phenols, sugar reductions and carboxylic acids. Standard procedures in the literature were used for these tests [34-36]. These reactions revealed the presence or absence of bioactive components in the plant extracts tested.

2.7.1 Dragendorff's and Mayer's test for alkaloids

Plant extract (5 mL) and 1% hydrochloric acid (5 mL) was stirred in a water bath at 65 °C and filtered. Dragendorff's reagent (a few drops) was added into the filtrate (2 mL). The reddish-brown precipitate formed was a positive result for alkaloids. Some of Mayer's reagent were mixed in 2 mL of filtrate and turbidity or cream precipitates were observed.

2.7.2 Shidona test for flavonoids

Two pieces of magnesium and a few drops of conc. hydrochloric acid were mixed with 2 mL of extract. The change from an orange to red of colour indicated the presence of flavonoids.

2.7.3 Salkowski test for terpenoids

A few mL of chloroform was added to the plant extract and this mixture was homogenised. Then, attentively, few drops of conc. sulphuric acid were added. A red-brown colour formed at the interphase indicated the presence of terpenoids.

2.7.4 Alkaline reagent test for coumarin

Plant extract (1 mL) was mixed with 10% sodium hydroxide. The formation of yellow colour was a positive result for coumarins.

2.7.5 Alcoholic KOH test for quinone

When the extract was treated with 10% alcoholic KOH, a reddish-blue colour formation indicated the presence of quinones.

2.7.6 $FeCl_3$ test for tannin

1 mL of 10% ferric chloride solution was added to 1 mL of each extracts. The dark-blue or greenish-black colour formation was a positive result for the tannins.

2.7.7 $FeCl_3$test for phenol

2 mL of extract was treated with 10% iron (III) chloride solution. The appearance of a blue-green colour indicated the presence of phenol-like compounds.

2.7.8 Froth test for saponin

Plant extract (2 mL) was diluted with hot water. This mixture was shaken vigorously to obtain a stable persistent froth.

2.7.9 Fehling test for carbohydrate

Plant extract (1 mL) was treated with 1 mL of Fehling solution A and B (1:1) and heated in a water bath for 5 -10 minutes. The appearance of a reddish-orange precipitate shows the presence of carbohydrates.

2. 8 Statistical Analysis

Weekly and plants based OAI, ER%, hatchability% differences were analyzed. Plants and time based skin repellent activity differences were analyzed. Whole tests performed with MANOVA in IBM SPSS 22 ®.

3. Results

As a result of qualitative phytochemical analyses, firstly, tannin and phenolic compounds and secondly alkaloid, terpenoid and coumarin compounds were observed to be intense in ethyl acetate extracts of all plants in different ratios. Saponin or carbohydrate derived components were not found. The phytochemical constituents of the investigated plants are summarized in Table 2.

Table 2: Qualitative phytochemical test results of ethyl acetate extract of plants

Plant species	Phytochemicals								
	Alkaloids	Flavonoids	Terpenoids	Coumarins	Quinons	Tannins	Phenols	Saponins	Carbohydrates
Salvia verticillata L.	++	±	+++	±	++	+++	+++	-	±
Matricaria chamomilla L.	+	±	++	+	+	++	++	-	±
Leucanthemum vulgare	++	+	+++	++	±	++	++	-	-
Triploaraspermum caucasicum	++	+	++	++	-	+++	+++	-	-
Erigeron annuus (L.) Pers	++	++	+	++	++	+++	+++	-	-
Inula vulgaris (Lam.) Trevisan	++	+	+++	++	++	+++	+++	-	-
Citrus sinensis	++	+	++	++	-	++	++	-	++
Laurus nobilis	++	-	++	+	+	+++	+++	-	+
Rhododendron ponticum	++	-	++	++	±	++	++	-	±
Rhododendron luteum	++	-	+++	+	±	-	-	-	±

-: absent; ±: trace; + or ++: moderately; +++: abundance

3.1 Oviposition deterrent effect

In this study, it was displayed that the crude extracts of ten different plants possessed the different degrees of oviposition deterrent activity against *Ae. albopictus* adults in the laboratory media. Oviposition deterrent activity was evaluated as the oviposition activity index (OAI) and percentage effective repellency (ER%). The OAI ranged from -0.0009 (*C. sinensis*, second week) to -1 (*M. camomilla*, third week) according to the results (Table 3). In the first week, the highest OAI belongs to *I. vulgaris* and the lowest OAI belongs to *S. verticillata*. Therefore, the highest efficiency of the oviposition deterrent activity was determined to belong to *S. verticillata*. OAI values increased in the second and fourth weeks. Although a gradual increase was detected for OAI in the second and fourth weeks, the OAI result for *R. luteum* in the fourth week was lower than in the third week (efficiency is higher than in the third week). Statistical analysed showed significant differences between OAI weekly (f=30.632 p=$1.38X10^{12}$). Post hoc test indicated that first week result significant difference 2^{nd} and 4^{th} week results (p<0.05). Second week results also showed significant differences from 3^{rd} week results, 3^{rd} week results significant difference 4^{th} week results (p<0.05). There is a significat differences between OAI for plants (f=2.87 p=0.006). The ER% ranged between 5.7471 (*C. sinensis*, second week) and 100 (*M. camomilla*, third week). The ER% results were correlated with the OAI. ER% results statistical analysis showed same differences level with OAI.

Table 3: Oviposition deterrent of *Ae. albopictus* to ethyl acetate extracts of plants used

Plant species	First week		Secondweek		Third week		Fourth week	
	OAI	ER%	OAI	ER%	OAI	ER%	OAI	ER%
Salvia verticillata	-0.9942	99.7063	-0.3992	55.4849	-0.9978	99.8896	-0.3932	46.5950
Matricaria chamomilla	-0.9559	97.7113	-0.6959	81.4876	-1.0000	100	-0.2967	51.1111
Leucanthemum vulgare	-0.9322	96.3896	-0.6137	75.0242	-0.9975	99.8718	-0.6288	72.5926
Triploaraspermum caucasicum	-0.9002	94.2904	-0.2782	41.1013	-0.3095	45.1073	-0.0371	45.2992
Erigeron annuus	-0.8746	92.5831	-0.3145	43.7395	-0.9677	98.3411	-0.3085	58.1766
Inula vulgaris	-0.3305	38.7625	-0.5737	71.6680	-0.9899	99.4911	-0.8889	93.3333
Citrus sinensis	-0.9913	99.5595	-0.0009	5.7471	-0.8195	89.1280	-0.2760	42.2939
Laurus nobilis	-0.8083	89.1257	-0.1489	25.7252	-0.7938	88.1018	-0.7591	85.2535
Rhododendron ponticum	-0.6889	81.1091	-0.3281	48.6982	-0.9694	98.4394	-0.3150	39.2729
Rhododendron luteum	-0.7924	87.1317	-0.2884	42.9498	-0.5231	68.0545	-0.5426	65.1818

3.2 Ovicidal activity effect

The ovicidal activity was calculated with a percentage hatching rate relative to the control group (Table 4). Results according to the experiment were ranged between 22.8227 (*S. verticillata*, first week) and 94.5431 (*L. vulgare*, second week). The lowest hatching rate was observed for *S. verticillata* in the first week, *R. ponticum* in the second week and third weeks and *E. annuus* in the fourth week. The decrease trend egg hatching was found to be *I. vulgaris, C. sinensis, R. ponticum, R. luteum* in the second week and *I. vulgaris* and *C. sinensis* in the fourth week in contrast to the other results. There are no significant differences between weeks related to the ovicidal acitivity (p<0.05). Plants ovicidal effects showed significant differences between 10 plants as a different degree (f=2.68 p=0.01). Post hoc test showed *Matricaria camomilla* results significantly different other plants. Furthermore, *Salvia verticillata* results showed significant diffrences from *Triploaraspermum caucasicum* and *Laurus nobilis* (p<0.05).

Table 4: Ovicidal activity of different plant extracts against eggs of *Aedes albopictus*

Plant species	Percentage of egg hatching			
	1st week	2nd week	3rd week	4th week
Salvia verticillata L.	22.8227	72.2002	48.9798	62.6593
Matricaria chamomilla L.	25.4123	46.1180	NH	71.6133
Leucanthemum vulgare	35.1406	94.5431	63.2231	89.0515
Triploaraspermum caucasicum	53.6363	80.5574	78.3533	74.2569
Erigeron annuus (L.) Pers	50.8364	61.6332	85.9395	27.7853
Inula vulgaris (Lam.) Trevisan	86.0482	80.1296	49.6833	40.27683
Citrus sinensis	66.8160	62.3712	54.8287	53.4115
Laurus nobilis	69.0003	70.9659	50.8347	89.2748
Rhododendron ponticum	92.6771	44.6609	40.0148	49.4079
Rhododendron luteum	81.3918	57.0151	50.9212	84.5833
NH: Not hatching				

3.3 Repellent activity

Percentage repellence effects ranged between 45.2381 (*L. vulgare*, 45 min) and 95.9936 (*R. ponticum*, 15 min). Results obtained in the first 15 minutes were higher than those at 30 minutes were except for *E. annuus*, *I. vulgaris* and *C. sinensis*. The 45 min results showed a decreasing trend relative to the 30 min results except for *I. vulgaris*, *L. nobilis* and *R. luteum*. The highest decrease was found to belong to *L. vulgare* throughout the period, and the lowest decrease was found to belong to *M. chamomilla.* There is significant difference between time (f=7.954 p=0.001) and plant species (f=2.21 p=0.037) for repellency tests.

Table 5: Percentage of repellency of different plant extracts against *Ae. albopictus* using bites test

Plant species	Concentration (mg/cm^2)	% of repellency		
		15 min	30 min	45 min
Salvia verticillata L.	0.1	-94.6705	-91.3690	-84.5238
Matricaria chamomilla L.	0.1	-95.4365	-88.6905	-85.0000
Leucanthemum vulgare	0.1	-90.9722	-84.6970	-45.2381
Triploaraspermum caucasicum	0.1	-94.6581	-93.3036	-85.8333
Erigeron annuus (L.) Pers	0.1	-74.7692	-93.0556	-88.7879
Inula vulgaris (Lam.) Trevisan	0.1	-84.1538	-89.2381	-90.8088
Citrus sinensis	0.1	-88.4091	-93.3333	-72.5000
Laurus nobilis	0.1	-93.3036	-72.5000	-81.6667
Rhododendron ponticum	0.1	-95.9936	-85.0000	-80.3571
Rhododendron luteum	0.1	-90.1786	-78.3333	-84.3434

4. Discussion

Results on oviposition deterrence as well as ovicidal and repellent activity of ethyl acetate extracts of *S. verticillata, M. chamomilla, L. vulgare, T. caucasicum, E. annuus, I. vulgaris, C. sinensis, L. nobilis, R. ponticum* and *R. luteum* were reported in the present study and confirmed for use as natural agents instead of synthetic insecticides against *Ae. albopictus* (Tables 3-5). One of the most important factors affecting the extraction of bioactive components from plants is the extraction solvent. Since the components to be obtained are organic compounds, polar/apolar organic solvents are generally selected for this purpose. The selection of extraction solvent in this study was made according to results of our previous study [37] because of the activity of this extract solvent against the *Ae. albopictus* larvae. Previously reported studies in the literature provide information about various phytochemicals extracted from plants that showed properties as oviposition deterrents and ovicidal agents against medically important mosquitoes such as *Ae aegypti* and *Ae albopictus*, among others [8, 38-40]. The oviposition area is selected according to olfactory, visual and tactica responses of the female individuals [41]. Gravid females seek suitable places during their oviposition activity. However, they may or may not lay several eggs before flying in the presence of oviposition deterrent [42]. Similarly, our observations of the deterrence tests revealed that the access to crude extract was applied to test paper, but flew away without/few eggs laying activity. Xue *et al*. (2006) indicated that the oviposition deterrent activity against *Ae. albopictus* of 21 different commercial insects repellent included 12 botanical extracts [43]. Tawatsin *et al*. (2006) also reported that a high oviposition deterrence for some essential oils [16]. Although we used crude extracts contrary to abovementioned study, our result gave high repellent activity and some extracts persisted over two weeks (*M. chamomilla*, *L. vulgare*, and *I. vulgaris*). It can be said that the observed deterrence and persistency properties are caused by the amount of alkaloid, terpenoid, coumarin compounds and the interactions among them.

The test results with ovicidal activity revealed egg hatchability failure *Ae. albopictus* prefer the lay eggs on moist surfaces or above the water. The eggs can live until contact with water. The effects of ovicidal compounds depend on the penetration of eggshells [44]. During the egg-laying period and waiting during the drying period, crude extracts penetrated the egg from the egg-laid paper. Our study revealed that *S. verticillata*, *M. chamomilla* and *L. vulgare* had a high percentage of ovicidal effects. The other crude extracts had less than the abovementioned plants during the fresh time. Elango *et al*. (2010) reported the different degree of ovicidal effects of three different plant leaf extracts on *An. subpictus* [38]. Phasomkusolsil *et al*. (2012) reported that the ovicidal effects of herbal essential oils against *Ae. aegypti*, *An. dirus* and *Cx. quinquefasciatus* [40]. They also noted that the dose affected the degree of potency. Our results gave high ovicidal effects at a fixed volume, although did not try different doses. Govindajaran *et al*. (2011) reported remarkable ovicidal activity for *Ocimum basilicum* on *Ae. ageypti*, *An. stephensi and Cx quinquefasciatus* [32]. They also indicated that repellent activity against these three mosquito species.

These results appear to be a combination of the odours of terpenoids, the bitterness of low molecular phenolic compounds and tannins or the deterrent effects of alkaloids in the plant content. The present results show the highest degree of protection of whole tested plant extracts. A fixed volume was used for this study but many studies report that the relation to dose and time different depending on mosquito species [38, 45, 46]. Amer and Mehlhorn (2006) reported a maximum 8-hour efficiency and 100% repellence against *Ae. ageypti*, *Cx. quinquefasciatus* and *An. stephensi* for the five different types of plant oil [47]. Prajapati *et al*. (2005) reported the ovicidal / oviposition deterrence and skin repellent activity of 10 different plant essential oils against *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* [8]. Our one-dose results give high and persistent protection effects against *Ae. albopictus*.

5. Conclusion

This study gave toxicological and behavioural potency of some plants on *Ae. albopictus*. These data give a chance to improve new biological-based control products for *Ae. albopictus* with naturally derived Turkish plants. This situation may give a chance to decrease the high amount of chemical or synthetic insecticide usage and the problem of contamination from their usage.

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