Original Article

Chemical Compositions of the Peel Essential Oil of *Citrus aurantium* and Its Natural Larvicidal Activity against the Malaria Vector *Anopheles stephensi* (Diptera: Culicidae) in Comparison with *Citrus paradisi*

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Abstract

Background: Recently, essential oils and extracts derived from plants have received much interest as potential bioactive agents against mosquito vectors.
Methods: The essential oils extract from fresh peel of ripe fruit of *Citrus aurantium* and *Citrus paradisi* were tested against mosquito vector *Anopheles stephensi* (Diptera: Culicidae) under laboratory condition. Then chemical composition of the essential oil of *C. aurantium* was analyzed using gas chromatography-mass spectrometry (GC–MS).
Results: The essential oils obtained from *C. aurantium*, and *C. paradisi* showed good larviciding effect against *An. stephensi* with LC_{50} values 31.20 ppm and 35.71 ppm respectively. Clear dose response relationships were established with the highest dose of 80 ppm plant extract evoking almost 100% mortality. Twenty-one (98.62%) constituents in the leaf oil were identified. The main constituent of the leaf oil was Dl-limonene (94.81).
Conclusion: The results obtained from this study suggest that the limonene of peel essential oil of *C. aurantium* is promising as larvicide against *An. stephensi* larvae and could be useful in the search for new natural larvicidal compounds.

Keywords: *Citrus aurantium*, *Citrus paradisi*, Essential oil, Larvicidal activity, *Anopheles stephensi*

Introduction

Mosquitoes are very significant vectors from the medical entomology's point of view. They are responsible for the transmission of many diseases to man and animals such as malaria, dengue, yellow fever, encephalitis or filariasis (Eldridge 2000). Human malaria, as a mosquito-borne disease, caused by parasitic protozoa Plasmodium consider as the most important vector-borne disease, which is transmitted only by females of Anopheles mosquitoes (Lehane 1991). Malaria is still an endemic disease in certain foci located in south and southeast of Iran (Manouchehri et al. 1992, Sedaghat et al. 2003 a,b, Vatandoost et al. 2012). In this part of the country six anopheline mosquitoes including *An. stephensi*, are known as the main malaria vectors (Manouchehri et al. 1976, Sedaghat and Harbach 2005). *Anopheles* (*Cellia*) *stephensi* Liston 1901 is an important malaria vector with wide distribution in the Arabian Peninsula and the Indian subcontinent. It has also distributed in in Khuzestan, Fars, Kerman, Hormozgan, Sistan va Baluchestan and southern

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Kermanshah provinces in Iran (Manouchehri et al. 1976, Vatandoost et al. 2006, Hanafi et al. 2011).

Several methods have been applied for control of the vectors of the disease including using synthetic pesticides as larvicides or imagicides in the malaria control program. Organophosphate compounds are the most common chemical larvicides which are used in control of Anopheles. However, their toxicity to fish and other non-target organisms and the environment are increased (Mittal et al. 1991, Pinkney et al. 1999). Also resistance of anopheles mosquitoes to these compounds has appeared in many areas (Vatandoost and Borhani 2004, Vatandoost et al. 2004). A lot of attention is being paid to the plant extracts or their essential oils as an alternative source of mosquito larval control agents (Isman 2000, Sedaghat et al 2011a, b, Vatandoost et al. 2012).

Citrus aurantium Linnaeus (Rutaceae) which called as bitter orange or marmalade orange is too sour, but the juice of ripe fruit is used as a condiment in Iran. The peel of *C. aurantium* is often used in marmalade and dried peel is used in different food and drinks. The flowers are used in tea and its essential oil is used in perfumes and orange-flower water, which is used to flavor sweets (Kiple and Ornelas 2000). The dried whole fruit or peel of the fruit is used in Asian and Western herbal medicine to treat digestive problems (Wichtl 1994). The hydrolate of the flowers has been used for treatment of mild depression, sedation and as a heart tonic for many years in Iran (Zargary 1986, Ayenechi 1991). The studies on biologic effects of *C. aurantium* indicated potential mosquito repellent, larvicidal and insecticidal activities of this plant (Cetin et al. 2006, Sumroiphon et al. 2006, Yoon et al. 2009).

Citrus paradisi Macfadyen (Rutaceae) or grapefruit like other citrus fruits contains many phytochemicals which contribute to a healthy diet (Fellers et al. 1990). It is used in Persian traditional medicine to treat infected injuries, some digestive problems, cold and helps to lower cholesterol. Recent studies have shown the potential of *C. paradise* to favorably affect metabolic syndrome, lipid and sugar metabolism (Fujioka et al. 2006, Goldwasser et al. 2010, Ogura et al. 2011). Its seed extract has shown the antimicrobial properties against bacteria and fungi (von et al. 1999).

The objective of this work was to study the effect of the peel essential oils of ripe fruits of *C. aurantium* and *C. paradisi* against fourth instar larvae of *An. stephensi* under laboratory conditions and determine the chemical composition of *C. aurantium* essential oil.

Materials and Methods

Collection of plant materials

Fresh plants samples of ripe fruit of *C. aurantium* and *C. paradisi* were collected in October 2012 from Babol, Iran (52º 41’E, º36 32’N, elevation: -5 m above sea level) and Jiroft (57º 45’E, 28º 37’N, elevation: 650 m above sea level) respectively. The plants were identified and authenticated and the voucher specimen was deposited at Vector Biology Laboratory, Department of Medical Entomology, Tehran University of Medical Sciences, Iran.

Extraction of essential oils

The peel essential oils of fresh (50 g) of *C. aurantium* and *C. paradisi* were hydrodistilled using a Clevenger-like apparatus (Model: British Pharmacopoeia, Manufactured by Pyrexfan Company, Iran and mantle model EM manufactured by Bibby Scientific Company, United Kingdom) for 3 hours at 70 °C. The yields were averaged over four experiments and calculated according to fresh weight of the plant materials. The obtained oil was dried over anhydrous Na_2SO_4 and transferred into an airtight amber-colored vial at 4 °C for further experimentation by

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gas chromatography–mass spectrometry (GC and GC-MS).

GC and GC/MS analysis of essential oil

GC analysis was carried out using an HP6890 gas chromatograph equipped with flame ionization detector and an Hp-1 capillary column (30 m× 0.25 mm I.D., film thickness 0.25 µm) and split ratio, 1:25. The GC settings were as follows: initial oven temperature was held at 40 °C for 1 min, rising to 250 °C at 5 °C/min. The injector temperature was maintained at 250 °C. The detector temperature was at 230 °C. The carrier gas used was Helium at a flow rate of 1 ml/min.

GC-MS was performed on Agilent Technology 5973 mass selective detector connected with an HP 6890 gas chromatograph. The oil of *C. aurantium* was analyzed using an HP-1MS (Fused silica) with the same column and temperature programmed as above. The MS operated at 70 eV ionization energy. Quantitative data were obtained from the electronic integration of the Flame Ionization Detector (FID) peak areas.

Determination of oil composition

Identification of the oil components were assigned based on retention indices which were calculated by using retention times of n-alkanes that were injected after the oil at the same chromatographic conditions. The compounds were identified by comparison of their relative retention indices and with those in the literature. In addition, computer searching followed by matching of the mass spectra data with those stored in the computer library. The percentage of each component is presented in Table 1.

Mosquito culture

Fourth instar larvae *Anopheles stephensi* was used in this study. All larvae of *An. stephensi* were obtained from laboratory culture of Department of Medical Entomology, Tehran University Medical Sciences (TUMS). The *Anopheles* colony was maintained at 27 °C with 12: 12 light and dark photoperiod in 60±10% relative humidity.

Larvicidal bioassay

Tests of mosquito larval activity were conducted with reference to the standard method recommended by the World Health Organization (WHO 2005). Since the essential oils do not dissolve in water ethanol 99.0% was used as co-solvent. Different concentrations of the essential oils in distillated water and the co-solvent were prepared. The oil-ethanol-water solution was gently stirred for 30 seconds with a glass rod to ensure a homogeneous test solution and each glass beaker was left at room temperature. After 15 minutes 20 larvae were taken with a fine mesh strainer and transferred gently to a 400 ml glass beaker. Control group included batches of mosquitoes from the colony exposed to water and the solvent alone. The larvae were exposed to the concentrations of 10, 20, 40, 80 and 160 ppm of essential oil in distilled water for 24 hours at room temperature. In the control beakers only 1 ml of solvent was added to each beaker. Mortality was recorded after 24 h of exposure while during the test no food was given to the larvae. Each treatment was done with five replicates.

Statistical analysis

Toxicity and activity were reported as LC 50 and LC90, representing the concentrations in ppm that killed 50% and 90%, respectively of larvae in 24 h. The LC50 and LC90 values and their 95% confidence intervals of each essential oil were calculated by log concentration-probit equation (Finney 1971) using the SPSS 16.0 probit procedure. Controls with mortality between 5–20% were corrected using Abbott's formula (Abbott 1925). When mortality in controls exceeded 20%, test results were rejected or repeated. Comparison of the LC_{50} and LC_{90} values were analyzed using ANOVA Test with SPSS ver-

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sion 17.0. Differences between means were considered significant at P≤ 0.05.

Result

Yields and chemical constituents of essential oil

The yields of peel essential oils of *C. aurantium* and *C. paradisi* were 0.7±0.12% and 0.85±0.14% (w/w) based on fresh weight respectively. The chemical composition of *C. aurantium* peel essential oil is presented in Table 2. A total of 21 compounds were identified representing about 98.62% of the oil. The results revealed that terpenoids in the oil were predominant. The main constituents in the *C. aurantium* peel essential oil were Dl-limonene (94.81%), β-myrcene (1%) and α-pinene (0.65%) respectively.

Larvicidal activity of essential oils

The LC_{50} and LC_{90} values of *C. aurantium* and *C. paradisi* oils against *An. stephensi* larvae were 31.20 ppm and 73.83 ppm, 35.71 ppm and 70.23 respectively (Table 1). Both peels essential oils at the 80 ppm concentrations killed more than 90% of the fourth instars larvae (Fig. 1). The mortality rates in the control groups were lower than 5% in all concentrations, no correction were applied. The probit regression lines of *An. stephensi* exposed to different interval concentrations of a *C. aurantium* and *C. paradisi* extractions are shown in Fig 2. The statistical test (ANOVA) showed that there was no significant statistical difference in mortality rate among two essential oils (P≤ 0.628).

Table 1. Probit regression line parameters of *Anopheles stephensi* to peel essential oil extraction of *Citrus aurantium* and *C. aurantium* at different interval concentrations

Species	A	B±SE	LC_{50} , 95% C.I.	LC_{90}, 95% C.I.	X^2 (df)	Heterogeneity P-value
C. aurantium	-5.12	3.43±0.28	28.17 **31.20** 34.62	63.30 **73.83** 90.08	3.15 (2)	> 0.05
C. paradisi	-5.51	3.55±0.29	32.28 **35.71** 39.62	82.03 **70.23** 100.47	2.54 (2)	> 0.05

A= y-intercept, B= the slope of the line, SE= Standard error, LC50, 95% CI= lethal concentration causing 50% mortality and its 95% confidence interval, LC90, 95% CI= lethal concentration causing 90% mortality and its 95% confidence interval, X2= heterogeneity about the regression line.

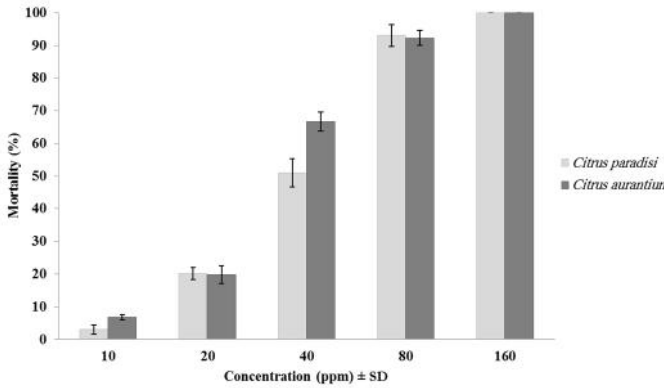

Fig. 1. Percentage of larval mortality of *Anopheles. stephensi* after treatment with *Citrus aurantium* and *C. paradise*

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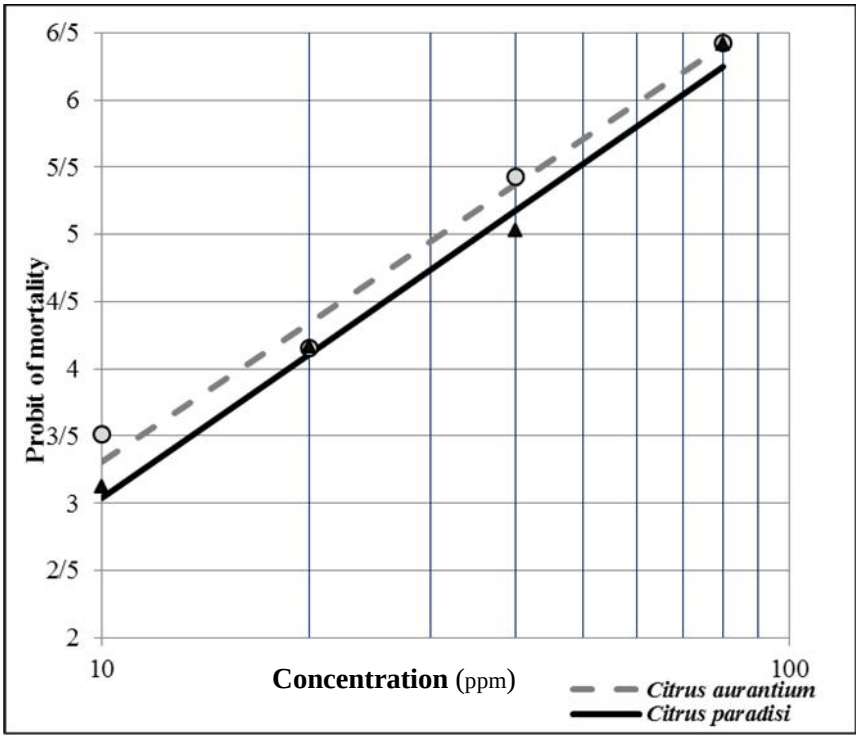

Fig. 2. Probit regression line of *Anopheles stephensi* exposed to different interval concentrations of a *Citrus aurantium* in comparison with *C. paradisi* essential oils

Table 2. Chemical constituents of peel essential oil from *Citrus aurantium*

n	Compound	KI [a]	Concentration (%)
1	α-Pinene	909	0.30
2	β-Pinene	947	0.65
3	β-Myrcene	958	1.00
4	Dl-Limonene	997	94.81
5	Trans-Ocimene	1016	0.19
6	Gamma-Terpinene	1029	0.01
7	Linalool Oxide	1039	0.04
8	1-Octanol	1043	0.13
9	Trans-Linalool Oxide	1044	0.02
10	Isoterpinolene	1045	0.01
11	Nonanal	1054	0.02
12	α-Terpinolene	1057	0.44
13	Linalyl acetate	1234	0.32
14	Sabinene Hydrate Acetate	1235	0.03
15	Neryl Acetate	1338	0.03
16	geranyl acetate	1358	0.13
17	trans-Caryophyllene	1386	0.03
18	Germacrene-D	1453	0.08
19	Nerolidol	1536	0.06
20	Palmitic Acid	1929	0.06
21	Trans-Oleic Acid	1930	0.26
Total			**98.62**

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Discussion

During past five decades using of chemical insecticides against vector mosquitoes have been developed the resistance in vectors to the insecticides and also hazards to the environment (Mittal 1991, Gunasekaran et al. 2004, Vatandoost et al. 2012). Although chemical larval control is considered as a major component in malaria prevention strategies, this part of control has side-effects on human and animal health and also the environment (Sedaghat et al. 2010, Vatandoost et al. 2012). In order to reduce the dependency on chemical insecticides, alternative methods including botanical insecticides for the control of vectors are considered. Certain plant's essential oils or extracts have been found effective for mosquito larval control. It is important to identify chemical constituents of the indigenous plans and their efficacies of essential oils as natural larvicides.

The yield of oil obtained of *C. aurantium* was 0.7%. It was nearly as same as reported from Pakistan (Siddique et al. 2011) but less than as reported in Egypt (Hifnaway et al. 2004). Gas chromatography–mass spectrometry of peel essential oil of *C. aurantium* revealed the presence of 21 components. In this study, major constituent of peel essential oil of *C. aurantium* was Dl-limonene, 94.81 % of the oil. In previous studies, various constituents of the oil of *C. aurantium* were reported. Although limonene is the most abundant constituent in the oils obtained from all similar studies, its percentage were varied, based on the origins of the plant (Caccioni et al. 1998, Boussaada and Chemli 2007, Moraes et al. 2009, Hosni et al. 2010). The result is in agreement with the results obtained from other study in Iran (Hosni et al. 2010). The other constituents of the peel oil were α-pinene (0.30%) and β-pinene (0.65%), while the results obtain from a study in Pakistan α-pinene and β-pinene were reported 0.476% and 0.176% respectively (Siddique et al. 2011). The result of the larval bioassay tests showed that essential oils of *C. aurantium* and *C. paradise* have a same level of bioactivity against *An. stephensi* larvae. Previous studies have demonstrated the same level of larvicidal activity against mosquito larvae. The LC_{50} of the essential oil of *C. paradise* were 47.3 ppm and 85.1 ppm for *Ae. aegypti* and *Ae. albopictus*, respectively (Morales-Saldañaet al. 2007). In another study reported by Boussaada and Chemli, the content of limonene in Tunisian *C. aurantium* from 87 % to 92.2% on fresh weight basis (Boussaada and Chemli 2007). The results nearly similar from the study of Caccioni et al. (1998) which reported limonene (94.3%) myrcene (1.88%) as the main constituents of *C.s aurantium*. In another study on the peel oil composition of Brazilian *C. aurantium*, limonene (97.5–98%), myrcene (1.2–1.45%) and octanol (0.34–0.54%) were found as the main constituents (Moraes et al. 2009). The results of constituents of the peel oil of *C. aurantium* obtained from this study are similar with all prior studies.

Our previous studies on the same strain of *An. stephensi* larvae revealed that the efficacy of *Eucalyptus camaldulensis*, *Cupressus arizonica*, *Coriandrum sativum* and *Heracleum persicum* oils were less than the efficacy of *C. aurantium* and *C. paradise* oils, while the efficacy of *Foeniculum vulgare* and *Kelussia odoratissima* are more than the two Citrus (Sedaghat et al. 2010, 2011ab, Vatandoost et al. 2012).

According to the classification of Vatandoost et al. (2012) the level of larvicidal activity of *C. aurantium* and *C. paradise* demonstrated them as active plants. If we accept Cheng et al. (2003) suggestion, these plants should be considered as very active. Based on both above classifications these two plants

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need more attention as they lied in active or very active categories.

Results on the larval mortality of *C. aurantium* and *C. paradise* oils against *An. stephensi* confirm their potential to control of the mosquito populations. It seems that the presence of a high amount of *Dl-limonene* in *C. aurantium* oil, could demonstrate its efficacy against *An. stephensi* larvae. In brief, this study clearly illustrated the potential use of *C. aurantium* and *C. paradise* as natural mosquito larvicides.

Conclusion

Essential oils from aromatic plants are the complex mixture of constituents with several usages in health and medical sciences. The essential oils of *C. aurantium* and *C. paradise* oils show larvicideal activity against *An. stephensi* the main vector of malaria in Indo-Persian areas including southern areas of Iran. The results obtained from this study suggest that the limonene of peel essential oil of *C. aurantium* is promising as larvicide and could be useful in the search for new natural larvicidal compounds.

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