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Extraction and chemical composition essential oil of *Kelussia odoratissima* and comparison its larvicidal activity with Z-ligustilide (Major Constituent) against *Anopheles stephensi*

Mahmoud Osanloo, Amir Amani, Hassan Sereshti, Mansoureh Shayeghi and Mohammad Mehdi Sedaghat

Abstract
In this study larvicidal activity of extracted essential oil of *Kelussia odoratissima* (KO) and its major component i.e. Z-ligustilide (LIG) were evaluated against *Anopheles stephensi* and compared with together during May 2016. Yield of extraction of essential oil from KO was 0.1% from dried plants. Components of essential oil were identified by GC-MS analysis. Z-ligustilide (64.24%), 2-octen-1-ol acetate (7.17%), thymol (4.72%), E-ligustilide (4.42%) and eugenol (2.51%) were 5 major components of KO. Larvicidal tests were performed in line with WHO guideline. Calculated LC_{50} and 90% for LIG (i.e. 8.73 and 15.70 ppm) higher than total essential oil (i.e. KO: 4.77 & 8.44 ppm). Essential oil has more effective than its major components and beside that, has low cost and lower risk in occurrence of resistance in different population of mosquito larvae.

Keywords: Larvicidal activity, major component, *Kelussia odoratissima*, Z-ligustilide & *Anopheles stephensi*

1. Introduction
More than 17% of all infectious diseases around the word are vector borne disease such as malaria, japanese encephalitis, and lymphatic filariasis [1, 2]. Unfortunately, they caused more than 1 million death annually; among these diseases, malaria alone caused the death of 429000 of people just in 2015 [1, 2]. For control of malaria, control of larvae used in 55 countries [2]. Continuous use of industrial larvicides such as deltamethrin, malathion and temephos lead to environmental pollution and occurring resistance in various population of mosquitos e.g. *Aedes aegypti, Culex pipiens* and *Anopheles stephensi* [3-5]. Essential oils have been suggested as alternative sources due to biodegradability, negligible effects on non-target organisms and safety to mammalians and environment [6-8]. In recent years, larvicidal activity of many essential oils have been reported, for instance LC_{50} of *Bunium persicum* determined at 27.72 and 20.61 ppm against *An. stephensi* and *Cx. pipiens*, respectively [9]. In another study revealed that, larvicidal activity of *Zhumeria majdae* lower than mentioned essential oil; (i.e. LC_{50} increased to 61.34 and 88.51 ppm) [10]. There are many efforts were made to control of *An. stephensi*, as the main malaria vector in many parts of the word. For instance reported LC_{50} of many essential oils such as *Citrus aurantium and Citrus paradise, Cionura erecta and Cupressus arizonica* against *An. stephensi;* i.e. 31.20, 35.71, 77.30 and 79.30 ppm, respectively [11-13].
Essential oil has many components, commonly few components have highest ratio than others. Recently, for better development of botanical insecticides, in many researches larvicidal activity of essential oil with their major components have been compared. For instance larvicidal activity of α-pinene and turpentine compared with essential oils of *Eucalyptus grandis* against *Aedes aegypti* [14]. Similarly, larvicidal activity of Thymol i.e. major components of essential oils of *Coleus aromaticus* and *Trachyspermum ammi*, compared against various population of mosquito [15, 16].
LIG or 3-butylidene-4,5-dihydrophthalide is the major component of multitude umbelliferae medicinal plants [17-19]. Two important plants in traditional Chinese and Iranian medicine are *Radix Angelica* sinensis and *Kelussia odoratissima* that practicable to relieved various disease. LIG is major ingredients of the both mentioned essential oils [20-23]. In this research, larvicidal activity of LIG evaluated against *An. stephensi* and compared with total extracted essential oil of KO.

2. Materials and Methods

2.1 Extraction of essential oil

Seven Kilo gram of fresh branch tips and leave of KO were collected in May 2016 from Chahar Mahal and Bakhtiari region, Iran. Plants were dried in shade condition with air circulation (decreased weight to 1Kg) and then cut in small pieces. In each step 200 g of dried sample subjected to hydro-distillation, using Clevenger type apparatus for 5h. The extracted oil dried over anhydrous sodium sulfate. Percentage content of oil was calculated based on plant dry weight and essential oil kept in colure container and away from sun lights at 4-8 °C [21].

2.2 Analysis of essential oil by GC-MS

The GC-MS analyses were done using a 6890 GC system coupled with 5973 network mass selective detector (Agilent Technologies, Santa Clara, USA). Separation of the components of the essential oil was carried out on an HP-5MS silica fused columns (30 m length; 0.25 mm i.d; and 0.25 µM film thickness 5% phenyl-methylpolysiloxane). The GC-MS column temperature was programmed as follows: initial temperature was set at 40 °C and fixed for 1 min, then, increased with rate of 5 °C/min to final temperature of 250 °C and hold for 60 min. Temperature of injection port and detector was fixed at 250 and 230 °C, respectively. Other instrument parameters were set as, split flow: 25 mL/min, septum purge: 6 mL/min and column flow rate: 1 mL/min. Helium gas with purity of 99.99% was used as carrier gas. Mass spectra were taken at 70 eV ionization energy and full scan mode. The scanned mass range was set at 50–350 m/z.

Components of essential oil were identified by comparison of their retention indices (RIs) determined with reference to a homologous series of C9–C24 n-alkanes. Firstly, this was confirmed by chromatographic injection of available analytical standard compounds (C9–C24 n-alkanes) and comparison of their retention times with those obtained for the essential oil. If standard compounds were not available, the identification was carried out by comparison with traditional retention indices. The identification was also confirmed by comparison of their mass spectra with those stored in the Wiley7n.l MS computer library. The linear temperature programmed retention indices (RIs) of all the constituents were calculated from the gas chromatogram by interpolation between bracketing n-alkanes (equation (1).

$$RI = 100[(tR(i) - tR(z)/tR(z+1) - tR(z)) + z]$$ Equation 1

Where z is the number of carbon atoms in the smaller n-alkane, and tR(i), tR(z) and t are the retention times of the desired compound, the smaller n-alkane and the larger n-alkane, respectively. In addition, the search match factor (SMF), rank number (RN) in the mass library, and five highest peaks in the mass spectra were prepared and used for identification of the components.

2.3 Applied method for evaluation of larvicidal activity

Required third and fourth instar larvae of *An. stephensi* were obtained from anophelini insectarium, Tehran University of Medical Sciences. The larvae are reared constantly at recommended condition i.e. 28 ± 2 °C, 12:12 dark and light periods and relative humidity of 65 ± 5%. Larvicidal bio assay were done in line with WHO recommended test in laboratory, with some modifications [24]. Standard solutions of each sample were prepared by dissolving in ethanol. By adding 0.5% v/v from samples to containers contains no chlorine water (199 mL with depth of 8 cm) desired concentrations of samples were prepared i.e. 0.63 – 15 and 1.25 – 25 ppm for KO and LIG, respectively. Homogenized containers with separate rubber probe before adding batches of 25 larva of *An. stephensi* by net. Dead larvae were counted after 24h of exposing in all concentration. The tests were performed in 16 repetitions at 4 different replicates. In each replicates 2 control groups were considers that ethanol added to those with similar mentioned manner.

2.4 Statistical analysis

Lethal concentrations (LC) at 50 and 90% of each samples (i.e. KO and LIG) were calculated using SPSS software (v 22) and probit analysis [21]. In this study, LC_{50} & 90 of LIG compared with KO. Evaluation overlaps between confidence intervals (CI) of 2 groups is common way for comparing various LC. If overlaps no occurred, imply differences are significance [25-27].

3. Result and Discussion

3.1 Determining components of KO by GC-MS analysis

Totally, thirty components (96.65%) of essential oil were identified by GC-MS analysis. Z-ligustilide (64.24%), 2-octen-1-ol acetate (7.17%), thymol (4.72%), E-ligustilide (4.42%) and eugenol (2.51%) were 5 major components (Table 1). Yield of extracted essential oil by hydro-distillation from dried KO was 0.1% (w/w).

Like to this study, LIG were identified as the major component of KO in our previous study (77.73%) [28]. And also in the literature content of LIG in KO reported in range of 49- 86% [29, 30]. LIG ($C_{12}H_{14}O_2$) with molecular weight of 190.242 g/mol is member of phthalide family, its structure drawn by Chem Draw software v 8.0 (Fig. 1).

Table 1: Identified components of essential oil of *K. odoratissima* using GC-MS analysis.

NO	Components	RI*	%	NO	Components	RI	%
1	Propyl benzene	910	0.25	16	α-Humulene	1421	0.21
2	α-Fenchene	921	0.26	17	(Z)-b-Farnesene	1427	0.31
3	Para-cymene	990	0.31	18	β-Acoradiene	1438	0.37
4	Limonene	999	0.36	19	Cadina-1,4-Diene	1446	0.27
5	(E)-b-ocimene	1018	0.61	20	(D)- Germacrene	1453	0.29
6	ɣ-terpinene	1026	0.31	21	2-Tridecanone	1469	0.27
7	2-Nonanone	1049	0.32	22	Cuparene	1472	0.43
8	α-Terpinolene	1058	0.21	23	β-Himachalene	1474	1.7
9	2-octen-1-ol acetate	1256	7.17	24	(Cis)-ɣ-bisabolene	1476	0.51
10	Thymol	1286	4.72	25	(D)-Cadinene	1489	0.86
11	Eugenol	1315	2.51	26	Cadina-1(2),4-diene	1502	0.32
12	α-Cubebene	1317	1.93	27	β- Germacrene	1530	0.17
13	α-Copaene	1344	0.51	28	ButylidenePhthalide	1606	1.97
14	(E)-Caryophyllen	1386	0.65	29	(Z)-Ligustilide	1692	64.24
15	(cis)-Thujopsene	1400	0.19	30	(E)-Ligustilide	1764	4.42

* Retention indices

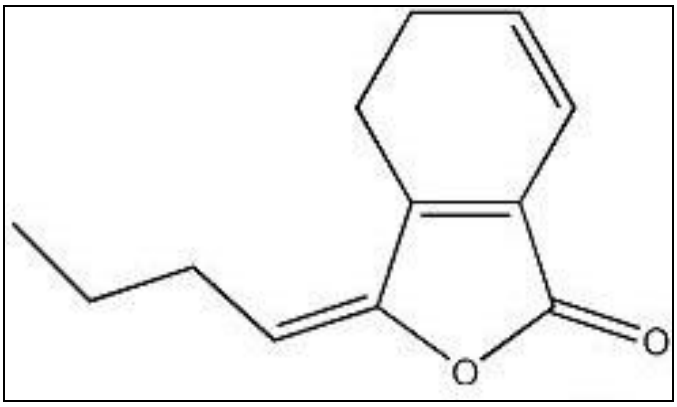

Fig 1: Structure of Z-ligustilide.

3.2 Evaluation larvicidal activity of KO in comparison with LIG

Larvicidal activity of LIG against *An. stephensi* was evaluated for the first time; larvicidal activity of LIG began from 2.5 ppm and increased by increasing the concentration of LIG (Figure 2). At concentration of 20 ppm or higher, perfect larvicidal activity was seen. Larvicidal activity of KO against *An. stephensi* depicted in Figure 3. Its activity was began from 1.25 ppm and like to LIG enhance with increasing concentration of sample. Achieved full larvicidal activity at concentration of 10 ppm and higher.

Regression equation parameter and lethal concentration of samples illustrated in Table 2. LC_{50} of LIG and KO calculated at 4.77 and 8.73 ppm, and their LC_{90} were 8.44 and 15.7 ppm respectively. Since no overlaps between both value (i.e. LC_{50} and 90), larvicidal activity of KO significantly better than LIG.

From the literature, insecticidal activities of LIG against various groups of insects were evaluated; its LC_{50} % against various biotype of adult silverleaf whitefly (*Bemisia tabaci*) were determined, i.e. biotype B (268 ppm) & biotype Q (254 ppm) [31]. In addition, its larvicidal activity against common fruit fly (*Drosophila melanogaster*) were proven i.e. LC_{50}% 2.54 ± 0.19 µmol/mL of diet [32]. LIG has variety of biological and pharmacological activities including; reduce vascular resistance and protective effect against ischemic brain injury [20, 33]. Besides that, extensively used in medicine researches as protector agent; against neurotoxicity that caused by β-amyloid [34] or cisplatin (chemotherapeutic drug) [35]. Additionally, its anti-inflammatory effect has been proven in numerous studies both in *in vivo* and *in vitro* [36-38].

LC_{50} & 90% of KO was reported in our previous study (i.e. 4.88 and 9.6 ppm) [28], these value comparable with results of this study. By regarding these results could be told larvicidal activity of KO is repeatable. Another reported application of KO are including; using as anti-oxidant [39] and anti-inflammatory [40] and prevention of pulmonary hypertension [41].

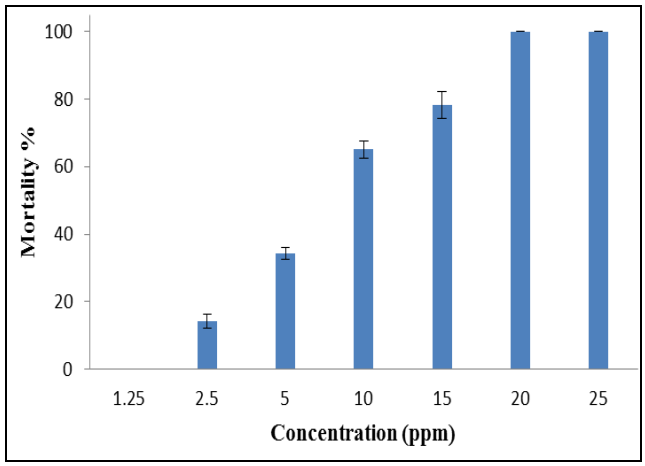

Fig 2: Larvicidal activity of z-ligustilide against *An. stephensi*.

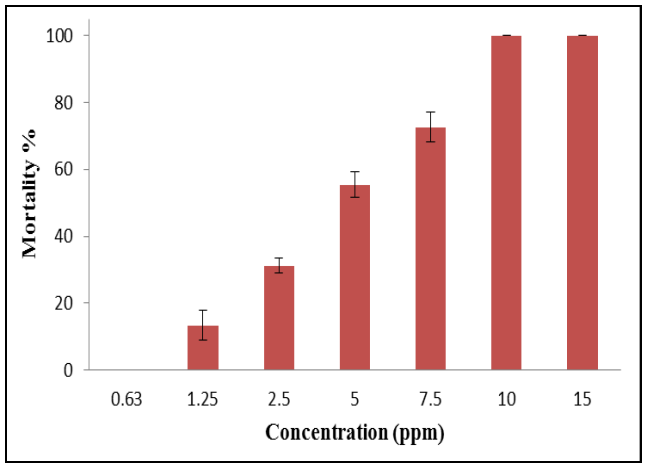

Fig 3: Larvicidal activity of essential oil of *Kelussia odoratissima* (KO) against *An. stephensi*.

Table 2: Regression equation for larvicidal activity of essential oil of *Kelussia odoratissima* (KO) and its major component z-ligustilide (LIG) against *An. stephensi*, calculated parameter by probit analysis.

Samples	intercept	Slope ± Standard error	larvicidal efficacy, 95% CI (ppm)		χ2 (df)
			LC_{50}	LC_{90}	
KO	-.3.006	4.365 ± 1.14	4.77 (3.68– 6.04)	8.44 (6.96- 11.36)	29.68 (2)*
LIG	-1.607	0.184 ± 0.007	8.73 (6.10– 11.81)	15.7 (12.44- 23.31)	75.761 (4)*

χ2 = Chi-square, df = degree of freedom, Since significance lower than 0.15, heterogeneity factor is used in the calculation of confidence limits.

In the literature; many comparisons between larvicidal activities of essential oils with their major constituents have been done. Results were very different; in many research larvicidal activities of essential oils better than major constituent. For instance; rotundifolone was major component (70.95%) of *Mentha x villosa Hudson* essential oil, larvicidal activity of total essential oil against *Aedes aegypti* (LC_{50} 45 ppm) significantly better than rotundifolone (LC_{50} 62.5 ppm) [42]. In another study larvicidal activities of six species of Greece Juniperus family against *Culex pipiens* were evaluated. The most efficient samples was essential oil that derived from the wood of *Juniperus drupacea* (LC_{50} 26.47 mg/mL), while larvicidal activity of their major constituents were 33.83 – 94.88 mg/mL [43]. In another study larvicidal activity of α-pinene (LC_{50} 15.4 ppm) evaluated against *A. aegypti*. This ingredient is major component in both essential oils of *Eucalyptus grandis* (52%) and *turpentine* (45%); their reported LC_{50} were 32.4 and 14.7 ppm, respectively [14]. In other word, larvicidal activity of α-pinene was better than essential oil of *Eucalyptus grandis* and worse than *turpentine*.

In some researches, larvicidal activity of major ingredients showed better efficacy than total essential oils e.g. Thymol is

major components of essential oils of *Coleus aromaticus* (82.68%) and *Trachyspermum ammi* (66.96%) [15, 16]. Calculated LC_{50} for Thymol against *Culex tritaeniorhynchus, Aedes albopictus, Anopheles subpictus* were 28, 24, 22 ppm respectively, while correspond value in for essential oil of *Coleus aromaticus* increased to 72, 67 and 60 ppm respectively. And also, larvicidal activity of Thymol against *An. stephensi* (LC_{50} 48 µg/mL) was better than total essential oil of *Trachyspermum ammi* (LC_{50} 80 ppm).

Synergistic effects in constituents of essential oils were acceptable in usage them as anti-fungal and anti-bacterial agent [44, 45]. By considering results of previous mentioned researches and this research, it seems this effect also occurs in larviciding purpose.

Besides, occurring resistance to larvicides was mostly seen in formulations with one active agent in comparison with those having multi components [46-49]. Essential oils have many constituents with different mode of action e.g. main site action of flavonoids, oleic acid, and palmitic acid is acetylcholinesterase [50] while alkaloids have rapid knockdown effect [8].

4. Conclusion

The present study concluded that using essential oils as larvicides beside the lower LC_{50} or 90% have many advantages in contrast to their major components. Preparing in much lower cost than extraction or synthesis of major components and reducing the risk of resistance.

5. Acknowledgment

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6. Conflict of Interest

There is no conflict of interest to the authors.

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