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Citrus Waste-Derived Essential Oils: Alternative Larvicides for Dengue Fever Mosquito, *Aedes albopictus* (Skuse) (Culicidae: Diptera)

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Abstract.- Plant extracts are currently studied more and more because of their role in plant protection as well as in urban entomology. Investigations were made to assess the larvicidal potential of citrus waste-derived essential oils at different concentrations (300ppm, 400ppm, 500ppm, 600ppm, 700ppm and 800ppm) against early 4th instar larvae of *Aedes albopictus* (Diptera: Culicidae), a competent mosquito in dengue positive area. Results reveal that the seed and peel extracts of lemon (*Citrus limon* L.) proved to be the most effective larvicides with minimum LC_{50} values after 24 hours (395.59 ppm for seed; 468.69 ppm for peel) and 48 h (247.19 ppm for seed; 392.20 ppm for peel) of exposure, and thus have the potential to be used as an ideal eco-friendly plant extract against larvae of *Ae. albopictus*. Succari (*Citrus sinensis* var. succari) seed and peel extracts proved least effective larvicides by having higher LC_{50} values, after 24 h (905.95 ppm for seed; 1009.44 ppm for peel) and 48 h (759.74 ppm for seed; 1041.53 ppm for peel) of exposure. Extracts from seed were more potent than peel as small doses of seed oils resulted in higher mortality as compared to peel extract.

Key words: Limonoids, day time biting mosquitoes, citrus peel extracts, citrus seed extracts.

INTRODUCTION

Diseases vectored by mosquitoes continue to be a major part of illness and death. Tropical areas are more prone to parasitic diseases and the risk of contracting arthropode-borne illnesses has increased due to climate change and intensifying globalization (Karunamoorthy *et al.,* 2010). Malaria, filariasis, Japanese ncephalitis and dengue fever are the most important diseases vectored by mosquitoes (Service, 1983). Dengue fever is becoming more and more vital public health concern in tropical and sub tropical parts of the world (WHO, 2003) with *Aedes albopictus* as the core mosquito vector in this respect (Yang *et al.,* 2009). Dengue, in south east Asia, is endemic and Bangladesh, India and Pakistan suffered recent outbreak of dengue fever in 2005 (Akram and Ahmad, 2005). Presently controlling this vector with habitat spraying remains the only option to minimize the incidence of dengue fever (Corbel *et al.,* 2004). However, the mishandling of synthetic chemicals in public health programs has caused many problems to human beings as well as the environment (Sarwar *et al.,* 2009). To lighten these problems, major consideration has been on the use of natural plant based products as larvicides which can provide an alternative to synthetic insecticides (Junwei *et al.,* 2006). Plants remain as one of the most important sources as several compounds from plants possess potential insecticidal or repellent properties and are free from harmful effects (Isman, 1995). Many plant products have been reported either as insecticides for killing mosquito larvae or adults or as mosquito repellents for adults (Sukumar *et al.*, 1991). Citrus is one of the most commonly consumed fruits and **is** also known as flavoring agent. The nature has provided it with elements that have mosquitocidal properties (Michaelakis *et al.,* 2009) and the oils from these are effective against dengue and malarial mosquitoes (Lee, 2006). Citrus seeds and peel remain primary wastes; however, a portion of the citrus peel waste is used in animal feed. Moreover, many researchers have defined ways to utilize citrus peel wastes by producing flavonoids or pectin (Leuzzi *et al.,* 2000).

Pakistan is amongst the important citrus producing countries of the world and several citrus cultivars have been grown in Pakistan since long but

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they have not had their mosquitocidal potential assessed. The identification and eventual use of citrus oils is beneficial to developing countries like Pakistan and its Southeast Asian neighbors. Keeping in view the importance of citrus, present laboratory studies were undertaken to evaluate the mosquitocidal properties of citrus waste-derived essential oils from various cultivars of citrus family (Rutaceae).

MATERIALS AND METHODS

Extraction of oil

Citrus fruits were collected from local market as well as research area of the university and identified in the Pomology laboratory, Institute of Horticultural Sciences, University of Agriculture, Faisalabad during 2008-2009. The fruits were washed and seeds and peel were separated manually. The seed were dried in the oven at 60°C for 48 hours, powdered in the blander and later placed in the Soxhlet apparatus for oil extraction using di-ethyl ether as a solvent (Vogel, 1978). Cold press method was used for oil extraction from peel. The extracts were dissolved in acetone to prepare a stock solution from which a series of concentration ranging from 300 to 800 ppm of dissolved extract were prepared in water (Murugan *et al.,* 2007).

Mosquito rearing

Ae. albopictus larvae and the adults were collected round the year, from all artificial containers and natural habitats and reared in the laboratory by standard rearing procedures (Bhat and Kempraj, 2009). The larvae were reared in steel trays approximately 3inch deep and fed on Tetramin (artificial diet) until the adults emerged. Adults were maintained in a cage (70×35×35 cm) and females were fed with blood of white rats every alternate day whereas the males were fed with 10% sugar solution soaked on cotton pad, which were suspended in the middle of the cage. A glass beaker with strips of moistened filter paper was provided in the cage for oviposition. The population was maintained in the laboratory at a set condition of 27±2^{o}C, 75-80% RH and L14:D10.

Bioassay

Larvicidal bioassays were done on F1 generation by using six different concentrations (300, 400, 500, 600, 700, 800 ppm). One ml of acetone was mixed with 199 ml of distilled water for check treatment. Each treatment was replicated four times, each replicate containing 200ml of the oil solution was placed in 250ml glass beakers. Twenty five early fourth instar larvae of *Ae. albopictus* were exposed to each concentration (Mohtar *et al.,* 1999) and the number of dead mosquito larvae were recorded after 6, 12, 24 and 48 hours of exposure.

Data analysis

Mortality data was corrected by Abbott's formula (Abbott, 1925) and then analyzed by Probit analysis (Finney, 1971), using Minitab statistical software for dose and time mortality regression lines. Significant differences among LC_{50} and LT_{50} values were inferred by non overlapping of 95% fiducial limits (Ahmad *et al.,* 2003). The data on percent mortalities were analyzed with Statistix version 8.1 (Analytical Software, 2005) and means were compared with least significant difference (LSD) test at 5% level of significance.

RESULTS AND DISCUSSION

Citrus peel and seed extracts exhibited strong larvicidal activities against *Ae. albopictus*. With respect to percent mortality, seed and peel oils of lemon and kinnow yielded highest percent mortalities after 24 and 48 h of exposure while succari seed and peel oils caused minimum percent mortalities (Fig. 1).

Lemon seed extracts proved to be the most effective larvicide for having the lowest LC_{50} values after 24 and 48 hours of exposure (395.59ppm and 247.19ppm, respectively) while Succari seed extract proved relatively least effective larvicide by having the highest LC_{50} values after 24 and 48 hours of exposure (905.95ppm and 759.74ppm, respectively) (Table I).

Among peel extracts, lemon peel extract also proved to be the most effective larvicide by having lowest LC_{50} values after 24 and 48 hours of exposure (468.69ppm and 392.20ppm respectively) while Musambi and Succari proved least effective by having highest LC_{50} values particularly after 24 hour of exposure (1067.92ppm and 1009.44ppm, respectively), latter both were statistically at par. Succari peel extract proved least effective among the tested varieties after 48 hours of exposure (1041.53ppm) (Table II).

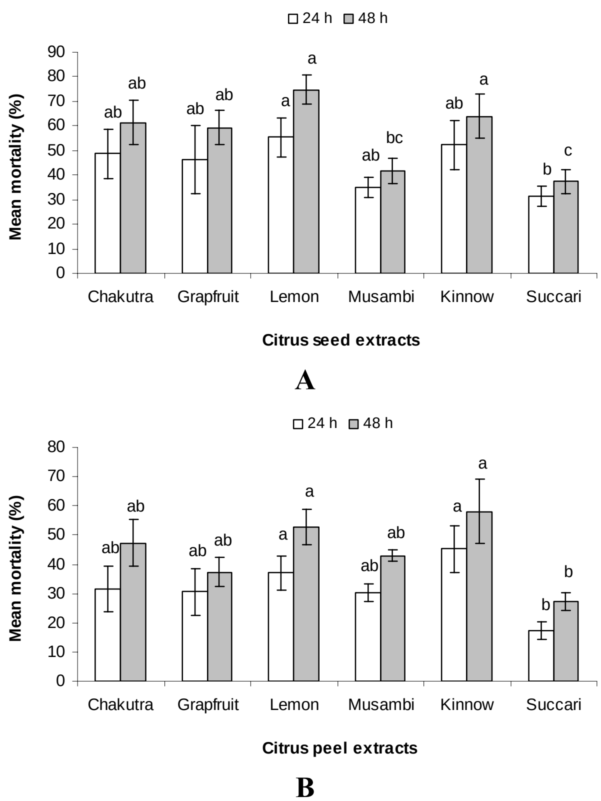

Fig. 1. Effect of citrus seed extracts (A) and citrus peel extract (B) on the mortality of mosquito larvae. Bars represent mean percent mortality (± S.E.) at different time intervals. Bars of specific time duration, sharing the same letters are not significantly different at 5% level of significance (Least significant difference test [LSD], Statistix 8.1)

In terms of lethal time to kill 50% population of the subjected *Ae. albopictus* larvae, Lemon and grapefruit seed extracts took minimum time to kill 50% population (20.01 h, 27.11 h respctively), both were statistically at par, while Succari and Musambi seed extracts took longest duration (74.07 h, 63.55 h respectively) and were statistically at par (Table III).

Lemon peel extract also took minimum time to kill 50% larval population (32.88 h) followed by Kinnow, Chakutra and Musambi (42.24 h, 49.30 h and 55.95 h respectively), latter three were statistically at par while Succari peel extract took highest time to kill 50% larval population (88.48 h) (Table IV).

Plant extracts are a rich source of bioactive compounds that have been exploited over the past few decades in order to limit the use of synthetic chemicals. Our preliminary studies on the use of citrus limonoids against adults as repellents provided good base for studying these compounds against ,ol. *Ae. albopictus* larvae (Hafeez and Akram, 2010). The ether extracts of peel and seed parts of the fruits of different citrus varieties have been investigated for use as eco-friendly insecticides instead of eco-enemy synthetic insecticides. Results on the larvicidal effects of these extracts reported in the present study, confirm their potential for the control of larval population and management of *Ae. albopictus* population. Lemon seed and peel extracts demonstrated best lethality by killing *Ae. albopictus* larvae in shorter period of time, however seed extracts of all the tested varieties proved comparatively very effective than peel oils by having less LC_{50} values, shorter time to cause 50% mortality and highest percent mortalities. Lemon extracts revealed stronger toxicity compared to other citrus varieties owing to a mixture of many para-menthane type molecules, which are well known for their larvicidal activities (Michaelakis *et al.,* 2009). The varying results of different extracts with respect to LC_{50} and LT_{50} values are probably due to the difference in level of toxicity of insecticidal ingredients of each citrus variety (Tawatsin *et al.,* 2006).

As plant extract present high mortality in various life stages of *Ae. albopictus*, therefore, this intervention for public authorities (Silva *et al.,* 2003) to use plant products (Rutaceae) will provide ideal approach in public health management especially mosquito control. Plant extracts are safer for non target organisms including man, therefore, plant based formulations would be more feasible from environmental perspective than synthetic mosquitocides (Bhat and Kempraj, 2009). Amer and Mehlhorn (2006) studied the essential oils from 41

Table I.- **LC_{50} values of citrus seed extracts against 4th instar larvae of *Aedes albopictus.***

Citrus extract	Observation (h later)	$LC_{50}{}^{a}$ ppm (95% FL)	Slope±SEM	χ^{2b} (df=5)	*P*
Chakutra (*Citrusgrandis*)	24	446.24 (410.56-476.39)^{B}	1.84±0.74	2.83	0.24
Musambi (*Citrus sinensis* var. musambi)	24	784.81 (753.71-816.31)^{C}	0.71±0.66	0.21	0.89
Grapefruit(*Citrus paradisi*)	24	473.32 (437.85-510.71)^{B}	1.18±0.31	1.50	0.47
Kinnow(*Citrus reticulata*)	24	417.13 (402.08-445.38)^{B}	1.37±0.29	1.52	0.46
Lemon (*Citrus limon*)	24	395.59 (377.75-400.47)^{A}	1.48±0.29	1.54	0.46
Succari (*Citrus sinensis* var. succari)	24	905.95 (828.90-1092.1)^{D}	0.67±0.30	0.42	0.81
Chakutra (*Citrus grandis*)	48	360.83 (313.13-396.38)^{B}	1.65±0.30	0.98	0.61
Musambi (*Citrus sinensis* var. musambi)	48	557.12 (470.15-918.40)^{C}	0.88±0.27	0.29	0.86
Grapefruit(*Citrus paradisi*)	48	362.11 (300.47-404.86)^{B}	1.34±0.51	0.11	0.98
Kinnow(*Citrus reticulata*)	48	344.84 (294.45-380.52)^{B}	1.66±0.34	0.80	0.67
Lemon (*Citrus limon*)	48	247.19 (222.63-273.17)^{A}	1.23±0.35	1.83	0.40
Succari (*Citrus sinensis* var. succari)	48	759.74 (648.90-983.3)^{D}	0.89±0.28	1.13	0.57

LC_{50} values recorded after the same time interval within an extract denoting the same superscript are not different at the 5% level of significance (Ahmad *et al.,* 2003). FL, upper and lower fiducial limits of the respective LC_{50} value.
a= Lethal concentration (ppm) to kill 50% population, b= Chi square

Table II.- **LC_{50} values of citrus peel extracts against 4th instar larvae of *Aedes albopictus.***

Citrus extract	Observation (h later)	$LC_{50}{}^{a}$ ppm (95% FL)	Slope ± SEM	χ^{2b} (df=4)	*P*
Chakutra (*Citrus grandis*)	24	605.62 (538.24-757.72)^{B}	1.57±0.32	1.15	0.56
Musambi (*Citrus sinensis* var. musambi)	24	1067.92 (838.21-1209.50)^{C}	0.56±0.23	0.06	0.97
Grapefruit (*Citrus paradisi*)	24	836.76 (615.47-803.2)^{BC}	0.79±0.29	0.85	0.64
Kinnow (*Citrus reticulata*)	24	610.93 (516.29-728.33)^{B}	1.02±0.29	1.62	0.45
Lemon (*Citrus limon*)	24	468.69 (432.44-514.63)^{A}	1.85±0.31	0.15	0.93
Succari (*Citrus sinensis* var. succari)	24	1009.44 (736.51-907.57)^{C}	0.85±0.26	0.91	0.63
Chakutra (*Citrus grandis*)	48	458.62 (413.66-516.43)^{AB}	1.45±0.47	1.35	0.50
Musambi (*Citrus sinensis* var. musambi)	48	672.56 (551.92-601.33)^{C}	0.41±0.28	0.54	0.76
Grapefruit (*Citrus paradisi*)	48	609.28 (513.85-550.88)^{B}	0.99±0.18	1.48	0.48
Kinnow (*Citrus reticulata*)	48	402.45 (380.55-431.55)^{A}	0.85±0.28	1.08	0.58
Lemon (*Citrus limon*)	48	392.20 (360.39-420.55)^{A}	2.17±0.30	0.67	0.72
Succari (*Citrus sinensis* var. succari)	48	1041.53 (682.01-1238.30)^{D}	0.70±0.30	0.37	0.82

LC_{50} data recorded after the same time interval within an extract denoting the same superscript are not different at the 5% level of significance (Ahmad *et al.,* 2003). FL, upper and lower fiducial limits of the respective LC_{50} value.
a= Lethal concentration (ppm) to kill 50% population, b= Chi square

Table III.- **Time mortality response of *Aedes albopictus* larvae against citrus seed extracts.**

Citrus extract	$LT_{50}{}^{a}$ h (95% FL)	Slope ± SEM	χ^{2} (df=2)	*P*
Chakutra (*Citrus grandis*)	30.20 (27.06-34.23)^{C}	0.79±0.08	6.51	0.10
Musambi (*Citrus sinensis* var musambi)	63.55 (49.29-97.55)^{D}	0.53±0.08	4.80	0.01
Grapefruit (*Citrus paradisi*)	27.11 (21.63-35.50)^{A}	0.37±0.07	0.63	0.41
Kinnow (*Citrus reticulata*)	29.14 (26.66-26.09)^{AB}	0.98±0.09	19.17	0.00
Lemon (*Citrus aurantifolia*)	20.01 (17.65-22.40)^{A}	0.76±0.20	0.003	0.95
Succari (*Citrus sinensis* var succari)	74.07 (56.63-116.21)^{D}	0.57±0.10	7.09	0.00

LT_{50} data recorded within an extract denoting the same superscript are not different at the 5% level of significance (Ahmad *et al.,* 2003). FL, upper and lower fiducial limits of the respective LT_{50} value.
a= Lethal time of an extract to kill 50% population

Table IV.- Time mortality response of *Aedes albopictus* larvae against citrus peel extracts

Citrus extract	LT_{50}^{a} h (95% FL)	Slope ± SEM	χ^2 (df=2)	*P*
Chakutra (*Citrus grandis*)	49.30 (42.56-60.27)^{B}	0.80±0.08	2.81	0.09
Musambi (*Citrus sinensis* var musambi)	55.95 (47.18-71.36)^{B}	0.76±0.09	4.86	0.05
Grapefruit (*Citrus paradisi*)	72.28 (56.14-109.18)^{C}	0.61±0.10	7.34	0.01
Kinnow (*Citrus reticulata*)	42.24 (36.08-52.63)^{B}	0.64±0.09	0.29	0.60
Lemon (*Citrus limon*)	32.88 (28.74-34.85)^{A}	0.65±0.07	2.24	0.14
Succari (*Citrus sinensis* var succari)	88.48 (70.01-127.20)^{C}	0.87±0.12	8.61	0.00

LT_{50} data recorded within an extract denoting the same superscript are not different at the 5% level of significance (Ahmad *et al.,* 2003). FL, upper and lower fiducial limits of the respective LT_{50} value.
a= Lethal time of an extract to kill 50% population

plants against three genera: *Aedes, Anopheles* and *Culex*, *Citrus limon* showed promising efficacy. Six Brazilian plants oil tested showed better results from *Citrus citratus* with LC_{50} values of 69 ppm (Cavalcanti *et al.,* 2004). Tiwary *et al.* (2004) applied *Zanthoxylum armatum* DC (Rutaceae) against three mosquito disease vectors and found it very effective. Sumroiphon *et al.* (2006) evaluated the bioactivity of citrus seed against mosquito larvae and obtained results based on 24 hours exposure against 4th instar larvae. The lethal concentration for 50 percent mortality of ethanol extracts for *Aedes aegypti* and *Culex quinquefacsiatus* was 2,267.71 and 2.639.27 ppm respectively. In contrast, citrus extracts in the present findings against larvae of *Ae. albopictus* showed higher LC_{50} values. Similarly, the orange peel (*Citrus sinensis*) showed the great potency against household insect pest especially mosquitoes (Ezeonu *et al.,* 2001). The seed extracts in the present study documented more lethality than peel extracts as these contain potent active ingredients that resulted in high mortality and morbidity with short duration. High larval mortality (60-80%) was noticed in seed extracts of some varieties (Fig. 1A) which may be due to the chemical constituents present in the seed extracts that arrest the metabolic activities of the larvae (Senthilkumar *et al.,* 2009). The phytochemicals may inhibit the activity of neuro-secretory cells or may disrupt epidermal cells due to which tanning or cuticular oxidation process is affected (Jeyabalan and Murugan, 1999). Silva *et al.* (2003) reported plant products (Rutaceae) to combat the problems associated with public health especially mosquitoes that in turn will provide valuable replacement of dangerous chemicals.

Plant based insecticides are a promising tool for targeting mosquitoes in their larval stages (Amer and Mehlhorn, 2006) and mosquito control is inevitable in different parts of the world and is still in a state of evolution. In conclusion, citrus peel and seed extracts showed promising results against larvae of *Ae. albopictus,* therefore, citrus extracts could be used in places where mosquito breed and for the control of *Ae. albopictus* larvae.

As citrus farming is one of the important commercial and industrial activity of the world and the commercial exploitation of the citrus waste against mosquitoes have the advantage that they can be used locally, cost effective, eco-friendly and may help to boost the local economy.

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