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Toxicity of methanolic fruit peel and seed extracts from *Citrus sinensis* (L.) Osbeck against papaya mealybug, *Paracoccus marginatus* (Williams and Granara de Willink)

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Abstract
Plant extracts contained secondary metabolites, which may protect the plants from insect herbivores, pathogens and vertebrate herbivores in one or many ways. The present study was conducted with the aim of evaluating the toxic property of the *Citrus sinensis* (L.) Osbeck fruit peel and seed extracts on third instar nymphs of *Paracoccus marginatus* (Williams and Granara de Willink), at the Department of Agricultural Entomology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai during August 2018 to March 2019. Probit analysis was carried out for the methanol extract from *C. sinensis* peels and seeds on *P. marginatus.* The LC_{50} values for the methanolic seed and peel extract from *C. sinensis* on *P. marginatus* in different methods of toxicity tests were 0.90 % and 1.06 %, respectively by leaf contamination method, 1.07 % and 1.28 %, respectively by dry film method and 0.53 % and 0.57 %, respectively by topical application method. The LT_{50} values for the *C. sinensis* methanolic seed and peel extract on *P. marginatus* were 38.75 hours and 44.72 hours, respectively by leaf contamination method, 40.62 hours and 45.45 hours, respectively by dry film method and 26.40 hours and 27.91 hours, respectively, by topical application method. Among the three different toxicity methods tested by using methanol extract of *C. sinensis* peels and seeds against *P. marginatus*, topical application method proved to be most effective against *P. marginatus* having lowest LC_{50} values. This study results concludes that the methanolic seed and peel extract are toxic to the nymphs of *P. marginatus.*

Keywords: Citrus sinensis, peel and seed, toxicity, *Paracoccus marginatus*, LC_{50}, LT_{50}

Introduction
Mealybugs, which belong to the family Pseudococcidae, under the order Hemiptera, are the "hard to kill pests" with more than 2000 different species. The prevalence of mealybugs are severe in introduced countries rather than their countries of origin. One such example is papaya mealybug, *Paracoccus marginatus* (Williams and Granara de Willink) (Downie and Gullan, 2004) [6]. Muniappan *et al.* (2008) first reported the invasion of papaya mealybug into Asia in countries like Java, Indonesia and India. In India, the incidence was first recorded in July 2007, at Tamil Nadu Agricultural University, Coimbatore. Now, the occurrence of papaya mealybug is seen throughout the country. The major host plants of papaya mealybug are papaya, congress grass, red gram, silk cotton, bhendi, mulberry, cassava, potato, banana, mango, teak, brinjal, grapevine, guava, cotton, chilli, jatropha and tomato (Krishnan *et al.*, 2016) [10]. The feeding behaviour of papaya mealybug is similar to that of other sap feeders as by inserting its stylets into the epidermis of the leaf, fruits and stem. At the time of feeding, a toxin substance gets injected into the plant that leads to chlorosis, stunting, leaf distortion and heavy build-up of honeydew, ultimately leading to death of the plant. The papaya mealybug is a pernicious insect pest attacking both horticultural and agricultural crops of economic importance. Hence, it is necessary to find out the appropriate management strategies to control this insect pest.

Plants can able to produce a variety of secondary metabolites like alkaloids, terpenoids, flavonoids, phenols, aminoacids and sugars. These secondary compounds may protect the plants from insect herbivores, pathogens and vertebrate herbivores in one or many ways. *Citrus* is a genus of flowering trees and shrubs in the family, Rutaceae. *Citrus* plant extract seems to be a source of many insect controlling agents. *Citrus* oil, which is extracted from the

Seed, peel and segment membrane, is a mixture of over hundred compounds that can be categorized into three main fractions *viz.,* non-volatile compounds, oxygenated compounds and terpene hydrocarbons (Cholke *et al.,* 2017) [5]. This may be an inherent characteristic of *C. sinensis* extracts for its insecticidal properties. The present study reveals the insecticidal activity of the methanolic peel and seed extracts of *Citrus sinensis* (L.) Osbeck on papaya mealybug.

Materials and Methods

The laboratory experiments were carried out at the Natural Pesticides Laboratory, Department of Agricultural Entomology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai during August 2018 to March 2019.

Mass culturing of test insect

The papaya mealybug was cultured on potato sprouts. Seed potatoes were washed with water and disinfected by using 1 % carbendazim solution. The disinfected potatoes were treated with 1 % gibberellic acid for half an hour, to encourage sprouting (Mani *et al.,* 2013) [11]. The treated potatoes were sown in a tray containing sterilized wet sand (sterilized in sunlight for 2 - 3 days prior to sowing) and it was covered with black cloth. Then the tray was kept in a dark place and the sand was sprinkled with water every day, to maintain moisture. After one week, the potatoes with good sprouts were selected and provided with egg mass (4 to 5 overacts for each sprouted potato) and the culture was maintained. Out of which, 80 per cent of the third instar nymphs were used for the bioassay and 20 per cent crawlers of papaya mealybug were used for multiplication (Sankar *et al.,* 2013) [20].

Sample preparation

Peels and seeds of *C. sinensis* were collected from fruit juice centres. Collected materials were shade dried until the moisture content was completely removed. The dried samples were ground and sieved in 30 mesh sieve in order to get uniform particle size.

Extraction of bioactive compounds

The bioactive compounds from the fruit peels and seeds of *C. sinensis* were extracted in Soxhlet apparatus using methanol as an extraction solvent (Ruberto *et al.,* 2002) [19], at the Department of Soils and Environment, Agricultural College and Research Institute, Madurai. Sample (30 g) was taken in the Soxhlet thimble and extracted with 300 ml of solvent at 60^{o} C for 6 hours. After extraction, the extracts were filtered and concentrated under vacuum at 337 mbar pressure in rotary vacuum evaporator to obtain the viscous concentrate (Patil, 2009). The solvent extract (oil residue) was stored in 30 ml glass vials and kept in refrigerator until further use.

Experimental bioassay

Bioassays were conducted by leaf contamination, dry film and topical application method, using crude methanolic peel and seed extracts of *C. sinensis* at five different concentrations (0.5 %, 1 %, 1.5 %, 2 % and 2.5 %). The lower and upper limits of the concentrations were determined by the preliminary toxicity test. The extracts were diluted in water (Amusan *et al.,* 2005) [2] along with triton X-100 as a surfactant (0.01 %). The experiment was replicated four times, with ten third instar nymphs of *P. marginatus* per replication. The experiment was conducted in Completely Randomized Block Design.

Leaf contamination method

Oral toxicity test was carried out by leaf contamination method (Amusan *et al.,* 2005; Murugan *et al.,* 2012) [2, 15]. The papaya leaf discs (14 cm diameter) were dipped in the methanolic peel and seed extracts of prepared concentrations, individually, for 10 seconds and air dried for 10 minutes. Then it was placed in a plastic Petri dish (14.5 cm diameter) lined with moist filter paper, to maintain the turgidity of the leaf disc. Ten third instar nymphs of *P. marginatus* were released in each Petri dish containing the treated leaf. Control was maintained by dipping the papaya leaf disc in water containing triton X-100.

Dry film method

Contact toxicity test was conducted by dry film method (Preetha *et al.,* 2009) [17]. The plastic Petri dish of 14.5 cm diameter was evenly coated with the methanolic peel and seed extracts of prepared concentrations, individually. Ten third instar nymphs of *P. marginatus* were released in each treated Petri dish and allowed to crawl for one hour. After one hour, they were transferred to the uncontaminated leaf, which was contained in a Petri dish, lined with moist filter paper. Control was maintained by allowing the mealybug to crawl on the Petri dish coated with water containing triton X-100.

Topical application method

Contact toxicity test was also carried out by topical application method (Karamaouna *et al.,* 2013; Mostafa *et al.,* 2018) [9, 13]. The papaya leaf disc of 14 cm diameter was placed in a plastic Petri dish of 14.5 cm diameter. Earlier, the Petri dish was lined with moist filter paper, in order to maintain the turgidity of the leaf disc. Ten third instar nymphs of *P. marginatus* were transferred to the papaya leaf disc and it was sprayed with the methanolic peel and seed extracts of prepared concentrations, individually, using a hand atomizer and allowed the mealybug to feed on the leaf (Rani *et al.,* 2011) [18]. Control was maintained by spraying the *P. marginatus* nymphs with water containing triton X-100.

Observations were made on the number of dead nymphs in each treatment, at 12, 24, 36, 48, 60 and 72 hours after treatment (Amusan *et al.,* 2005; Huang *et al.,* 2013) [2, 8]. The dead nymphs were identified by its dull blackish inactive appearance. The Lethal Concentration (LC_{50} and LC_{95}) and Lethal Time (LT_{50} and LT_{95}) were calculated in excel based probit analysis software. The fiducial limits, the boundaries in which, $LC_{50,}$ $LC_{95,}$ LT_{50} and LT_{95} values located were calculated and the chi square values were also estimated.

Statistical analysis

The dose mortality and time mortality relationships in case of the crude methanolic peel and seed extracts of *C. sinensis* were subjected to Probit analysis (Finney, 1952) [7] and the $LC_{50,}$ $LC_{95,}$ LT_{50} and LT_{95} were calculated by using excel based Probit analysis software prepared by Dr. M. R. Srinivasan, Tamil Nadu Agricultural University, Coimbatore.

Results and Discussion

Extracts from the *Citrus* plant parts was reported to have a mixture of over hundred compounds including terpene hydrocarbons, aldehydes, terpene alcohols, organic acids and non-volatile compounds (Cholke *et al.,* 2017) [5], which may be an inherent characteristic of its insecticidal property. In this

study, the toxicity parameters *viz.*, LC_{50}, LC_{95}, LT_{50} and LT_{95} to the papaya mealybug, *P. marginatus* were evaluated for the methanolic *C. sinensis* peel and seed extracts.

The dose mortality response of *P. marginatus* to methanol extract of *C. sinensis* peel and seed by leaf contamination method, dry film method and topical application method are presented in the Table 1. The results indicated that topical application method of toxicity test caused 50 per cent mortality of *P. marginatus* with minimum concentration of 0.57 % methanolic peel extract and 0.53 % methanolic seed extract. In leaf contamination method, median lethal concentration of *C. sinensis* peel and seed extract were 1.06 % and 0.90 %, respectively. The lethal concentration required to cause 50 per cent mortality by dry film method were 1.28 % (peels) and 1.07 % (seeds). The lethal concentration 95 was 1.81 to 2.28 % for topical application method, 2.99 to 3.43 % by leaf contamination method and 3.48 to 4.54 % through dry film method. Among the three different toxicity methods tested by using methanol extract of *C. sinensis* peels and seeds against *P. marginatus*, topical application method proved to be most effective against *P. marginatus* having lowest LC_{50} values *viz.*, 0.57 % (peels) and 0.53 % (seeds).

The results of the timemortality response of *P. marginatus* tested by three different toxicity tests are presented in the Table 2. Among the three toxicity tests, the topical application method of methanolic peel and seed extract had killed 50 per cent of tested insects within 27.91 hours and 26.40 hours, respectively. It was followed by leaf contamination method and dry film method, which had LT_{50} values of 44.72 hours (peels), 38.75 hours (seeds) and 45.45 hours (peels), 40.62 hours (seeds), respectively. The results denoted that the topical application of methanolic *C. sinensis* peel and seed extract was the best method to cause mortality of *P. marginatus* early. All the other methods took double the time to cause 50 per cent mortality. Nearly 95 per cent mortality could be caused by topical application method within 63.02 (seed) to 65.84 (peel) hours, whereas, it took 92.83 (seed) to 94.52 (peel) hours in leaf contamination method and 92.99 (seed) to 112.19 (peel) hours in dry film method.

The results of the current investigation are supported by the findings of Amusan *et al.* (2005) [2]; Bagavan *et al.* (2009) [3]; Murugan *et al.* (2012) [15] and Martins *et al.* (2017) [12]. The LC_{50} value for the ethanolic peel extract of *C. sinensis* against *Aedes aegypti* (L.) larva was found to be 0.4 ppm (Amusan *et al.*, 2005) [2]. Bagavan *et al.* (2009) [3] reported that hexane extract from the peels of *C. sinensis* against *Aphis gossypii* (Glover) had an LC_{50} value of 0.02%. The results of Murugan *et al.* (2012) [15] revealed that *C. sinensis* peel extract was more toxic to mosquito species, having LC_{50} values of 0.03 %, 0.02 % and 0.04 % to *Anopheles stephensi* (Liston), *A. aegypti* and *Culex quinquefasciatus* (Say) larvae, respectively. The LC_{50} value from the *C. sinensis* and *C. limon* fruit peel essential oil against *Dysmicoccus brevipes* (Cockerell) were 2.21 % and 0.72 %, respectively (Martins *et al.*, 2017) [12].

Karamaouna *et al.* (2013) reported that essential oils from the peels of lemon, *C. Limon* and orange, *C. sinensis*, extracted by using Clevenger apparatus were more or equally toxic to the vine mealybug, *Planococcus ficus* (Signoret), by the topical application method of toxicity tests, having LC_{50} values rangedfrom 2.7 to 8.1 mg per ml. The result of Smith (2015) [21] was in contrary with our results, who conducted contact toxicity test on long tailed mealybug, *Pseudococcus longispinus* (Targioni Tozzetti) and reported that the LC_{50} value of *Citrus limon* (L.) Burm. f. essential oil was 48.0 %. The effect of *Citrus* essential oil to the leaf feeding insect was tested by Ali *et al.* (2017) and reported that the *C. Limon* essential oil had an LC_{50} value of 24.20 % on *Spodoptera littoralis* (Boisduval) larva by leaf contamination method of toxicity test.

The methanolic extract of peels and seeds of *C. sinensis* by topical application method of bioassay required minimum time interval (27.91 hours and 26.40 hours, respectively) to cause 50 % of mortality of *P. marginatus.* This result was correlated with the insecticidal effect of seed extract of *C. sinensis* and *Citrus reticulata* (L.) on *Aedes albopictus* (Skuse), which had LT_{50} values of 18.49 hours and 31 hours, respectively (Bilal *et al.*, 2012) [4].

It is concluded that the topical application of *C. sinensis* seed extract 10000 ppm on third instar papaya mealybug, *P. marginatus*, exhibited an LC_{50} value of 0.53 % and LT_{50} value of 26.40 hours was considered to be an effective treatment against *P. marginatus.*

Table 1: Dose mortality response of *P. marginatus* to methanol extract of *C. sinensis* peel and seed

Treatment	χ^2	LC_{50} (%)	Fiducial limits (50 %)		LC_{95} (%)	Fiducial limits (95 %)	
			LL	UL		LL	UL
Leaf contamination method							
Peels	13.36	1.06	0.91	1.24	3.43	2.49	4.71
Seeds	8.98	0.90	0.75	1.06	2.99	2.20	4.07
Dry film method							
Peels	4.18	1.28	1.10	1.48	4.54	3.12	6.60
Seeds	6.82	1.07	0.92	1.25	3.48	2.57	4.72
Topical application method							
Peels	3.61	0.57	0.44	0.75	2.28	1.64	3.18
Seeds	1.20	0.53	0.41	0.68	1.81	1.40	2.34

χ^2 = Chi square
LL = Lower Limit
UL = Upper Limit
LC_{50} = Lethal Concentration which kills 50 per cent of the exposed nymphs
LC_{95} = Lethal Concentration which kills 95 per cent of the exposed nymphs

Table 2: Time mortality response of *P. marginatus* to methanol extract of *C. sinensis* peel and seed

Treatment	χ^2	LT_{50} (h)	Fiducial limits (50 %)		LT_{95} (h)	Fiducial limits (95 %)	
			LL	UL		LL	UL
Leaf contamination method							
Peels	11.97	44.72	41.02	48.77	94.52	78.37	113.99
Seeds	6.64	38.75	34.97	42.93	92.83	74.57	115.55

Dry film method							
Peels	5.21	45.45	41.05	50.32	112.19	87.80	143.35
Seeds	4.88	40.62	36.93	44.68	92.99	76.18	113.51
Topical application method							
Peels	4.03	27.91	24.97	31.20	63.02	53.26	74.58
Seeds	6.05	26.40	23.37	29.83	65.84	54.44	79.62

χ^2 = Chi square
LL = Lower Limit
UL = Upper Limit
LT_{50} = Lethal Time which kills 50 per cent of the exposed nymphs
LT_{95} = Lethal Time which kills 95 per cent of the exposed nymphs

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