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In-vitro Toxicity Evaluation of some Phytoextracts against Mealybug *Drosicha mangiferae* (Hemiptera: Pseudococcidae) Infesting Citrus Orchards in Pakistan

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MZM and HAG conceived the idea and planned the experiment. HAG performed the experiment and wrote the manuscript. MAR performed statistical analyses and revised the manuscript. MA provided technical assistance.

Key words
Azadirachta indica, Botanical extracts, Citrus mealybug, *Datura alba*, *Drosicha mangiferae*, *Gardenia jasminoides*, Plant essential oils, *Syzygium aromaticum*, Toxicity.

ABSTRACT

Mealybug *Drosicha mangiferae* (Pseudococcidae: Hemiptera) is one of the destructive insect pests of many agricultural and horticultural crops including citrus. Extensive and injudicious use of conventional synthetic insecticides against *D. mangiferae* have led to many environmental and health problems urging to seek out environment-friendly and safe alternate strategies to control *D. mangiferae*. To this end, the present study evaluated the methanolic extracts and essential oils of eight indigenous plant species for their insecticidal potential against 2nd instar *D. mangiferae* individuals. Standard twig-dip method was used for toxicity bioassays according to Completely Randomized Design. Mortality of mealybug individuals varied with plant materials and increased along with the extract concentration and exposure time. Botanical extracts of *Azadirachta indica* (neem) and *Gardenia jasminoides* (gardenia) were the most effective with minimum LC_{50} (20.00 and 42.19%, respectively) and LT_{50} (47.97 and 71.26 h, respectively) values followed by *Nerium indicum* (oleander). Moreover, the essential oils of *Datura alba* (dhatura) and *Syzygium aromaticum* (clove bud) were the most effective against *D. mangiferae* with minimum LC_{50} (0.80 and 0.90%, respectively) and LT_{50} (61.30 and 68.58 h, respectively) values. These findings substantiate the relative effectiveness of indigenous plant extracts as environment-friendly alternates to hazardous synthetic insecticides and, hence, are recommend to be integrated in pest management programs against *D. mangiferae* and other sucking insect pests.

INTRODUCTION

Citrus is an important fruit crop all over the world including Pakistan. It is being cultivated on approximately 8 million hectares with an annual citrus production of about 122 million metric tonnes (FAO, 2016). Mandarins (*Citrus reticulata* cv. feutrill's early and kinnow) and sweet oranges (*Citrus sinensis* cv. blood red and mosambi) are the most widely cultivated and appraised cultivars of citrus. Pakistan ranks among top citrus producing and exporting countries with an average area and production of about 206,569 hectares and 2.36 million metric tonnes, respectively (GoP, 2017). Sweet oranges, kinnow mandarins, grapefruits and lime are major citrus cultivars of the country. Mandarins (*C. reticulata*) constitute about 80% of citrus production in Pakistan. The province of Punjab and particularly district Sargodha are the production hubs of citrus in Pakistan sharing approximately 95 and 70% of total citrus production of the country, respectively (Ahmad *et al*., 2018).

Although Pakistan is famous worldwide for its best production of kinnow mandarins, per unit area production is far behind other citrus producing countries. Incidence of insect pests and diseases is one of the major reasons for citrus decline in Pakistan (Mahmood *et al*., 2014). Under agro-climatic conditions of district Sargodha (Punjab, Pakistan), canker, slow decline, anthracnose and greening are the major citrus diseases (Anjum and Javaid, 2005), while major insect pests attacking citrus plants include fruit flies (*Bactocera dorsalis* and *B. minax*), leafminers (*Phyllocnistis citrella*), psyllids (*Diaphorina citri*) and mealybugs (*Drosicha mangiferae* and *Planococcus citri*) (Tahir *et al*., 2015). Among these, mealybugs (Hemiptera: Pseudococcidae) are appearing as regular sucking pests of citrus orchards since last decade. Moreover, a recent survey in district Sargodha has found that among mealybug species infesting citrus plants, *Drosicha mangiferae* is the most dominant and prevalent species incurring substantial loss to citrus production both in terms of quantity and quality (Tahir *et al*., 2015).

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Table I.- Different botanical extracts evaluated under laboratory conditions against 2nd instar nymphs of mealybug *Drosicha mangiferae* Green.

Botanical name	Common/ Vernacular name	Family	Major bioactive constituents	Extraction type	Plant parts extracted
Azadirachta indica	Neem	Meliaceae	Azadirechtins and triterpenoids (Benelli *et al*., 2017)	Botanical extract	Leaves and fruits
Cymbopogon citratus	Lemon grass	Poaceae	Citral, citronellol, citronella, myrcene (Dodia *et al*., 2010)	Essential oil	Leaves
Datura alba	Dhatura	Solanaceae	Tropane alkaloids (Moniraand Munan, 2012)	Essential oil	Leaves and seeds
Dodonaea viscosa	Sanatha	*Sapindaceae*	Flavonoids, phenols, tannins, saponins, lupeol, and stigmasterol (Al-Snafi, 2017)	Botanical extract	Tender stems
Gardenia jasminoides	Gardenia	Rubiaceae	Iridoid glycosides (Li *et al*., 2018)	Botanical extract	Leaves and stems
Nerium indicum	Kaner	Apocynaceae	Oleandrin and oleandrigenin (Dodia *et al*., 2010)	Botanical extract	Leaves
Parthenium hysterophorus	Parthenium	Asteraceae	Parthenin (sesquiterpene derivatives) (Dodia *et al*., 2010)	Botanical extract	Leaves and tender stems
Syzygium aromaticum	Clove bud	*Myrtaceae*	Eugenol and E-caryophyllene (Zeng *et al*., 2010)	Essential oil	Buds

D. mangiferae is one of the most economic exotic insect pests of a wide range of agricultural and horticultural crops in South East Asia including Pakistan. Although it is predominantly a pest of mango orchards but for last few decades, it has attained the status of major pest of citrus orchards in the district Sargodha (Afzal *et al*., 2018). Every year, this mealybug species infests and incurs considerable qualitative and quantitative loss to indigenous citrus crop (Franco *et al*., 2004; Arshad *et al*., 2015). These mealybugs are considered as 'hard-to-kill' pests because these are difficult to eradicate with synthetic insecticides and have least absorption of pesticides due to presence of waxy mealy powder on dorsal surface of body (Chaudhari, 2012). Usually, there is no effective and operative chemical control option available against *D. mangiferae* mealybugs infesting different horticultural crops including citrus (Tanwar *et al*., 2007). Farmers utilize various synthetic insecticides to eradicate *D. mangiferae* infestations and often use over-dose rates (often double or triple) because of unsatisfactory and partial control of *D. mangiferae* (Mani and Shivaraju, 2016). Most of these pesticides are highly persistent and cause problems of pesticide residues and environmental contamination (Edwards, 2013).

Therefore, it is imperative to seek out new control tactics for mealybug control on citrus plants which would be more biorational and environment-friendly than conventional synthetic chemicals. In this regard, plant-derived insecticidal compounds appear as one of the biorational pest control potions. For instance, botanical extracts of many plants have been demonstrated to effectively control various phytophagous insect pests including mealybugs (Regnault-Roger, 1997; Prishanthini and Vinobaba, 2014; Lanjar *et al*., 2015; Badshah *et al*., 2017; Khan *et al*., 2019). Keeping in view the above cited situation, the present study was aimed to screen out some indigenous plant extracts and essential oils for their toxicity against *D. mangiferae* nymphs under laboratory conditions.

MATERIALS AND METHODS

Culture of mealybugs

Third instar female individuals of *D.* mangiferae were collected from citrus (*C. reticulata* cv. kinnow mandarin) orchard (32°08'21"N; 72°40'11"E) located in the vicinity of the College of Agriculture, University of Sargodha. Collection was carried out during the 1st fortnight of January upon emergence of early batches of mealybugs and it was ensured that no pesticidal application was made in the orchard against mealybugs yet. These individuals were brought to the laboratory under cool conditions and were reared at 27±2°C and 65±5% relative humidity in plastic cages (90 x 60 cm) up to F_2 generation on the young seedlings of *C. reticulata*. For bioassays, only healthy and active 2nd instar individuals were utilized.

Extraction of botanicals

Toxicity potential of essential oils and botanical extracts of eight indigenous plant species (Table I) was assessed under laboratory conditions against 2nd instar nymphs of *D. mangiferae*. For this purpose, different plant parts as described in Table I were collected from the vicinity of the College of Agriculture, University of Sargodha and washed with clean tap-water and were shade-dried for about a week at room temperature (26°C) and then were powdered using an electric blender. Soxhlet apparatus (Sigma-Aldrich, Germany) was used for the botanical extracts using 1:10 (w/v) methanol as extraction solvent, while essential oils were extracted by hydro-distillation using clevenger-type apparatus. Excess of extraction solvent was removed from the crude plant extracts using a rotary evaporator (Büchi R-3000; Büchi Laboratoriums-Technik, Flawil, Switzerland) set at 41°C. Plant essential oils and extracts were stored in dark colored hermetic glass vials in the refrigerator at 4°C until their utilization in toxicity bioassays.

Bioassays

Standard twig-dip bioassays were conducted to determine the toxicity of botanical extracts and essential oils against 2nd instar nymphs of *D. mangiferae*. In brief, unsprayed 5 cm long twig-tips of *C. reticulata* plants were collected washed with clean tap-water and were dried at room temperature (26°C). Their stems were wrapped in moist cotton plug to ensure their freshness for at least three days. Bioassays were laid out according to their label-recommended dose rates according to CRD design with 5 replications per treatment. Twigs were dipped for 5-10 sec in three different concentrations of essential oils (2.0, 1.0 and 0.5% v/v) and botanical extracts (40, 20 and 10% v/v) and after air-drying for 30 min were transferred to Petri plates (diameter 9 cm). Control treatment included distilled water used for the preparation of botanical concentrations. Using camelhair brushes, ten healthy and active 2nd instar mealybug nymphs were released on treated citrus twigs and Petri plates were incubated at 27±2°C and 65±3% relative humidity in an environment chamber set with 16:8 h light–dark photoperiod. Data regarding the mortality of mealybug individuals were recorded at 12, 24, 48 and 72 h post-treatment.

Statistical analysis

Statistical analysis of data was carried out using SPSS® version 20.0 (IBM Corp., Armonk, NY, USA). Data regarding percent mortality of mealybugs in response to plant essential oils and methanolic extracts insecticides were subjected to factorial analysis of variance (ANOVA) followed by Fischer's least significant difference (LSD) test at 5% probability level in order to compare the treatments. Prior to ANOVA, mortality data was corrected using Abbott's formula. Median lethal concentration (LC_{50}) and median lethal time (LT_{50}) values were calculated by probit regression analysis using POLO-PC®.

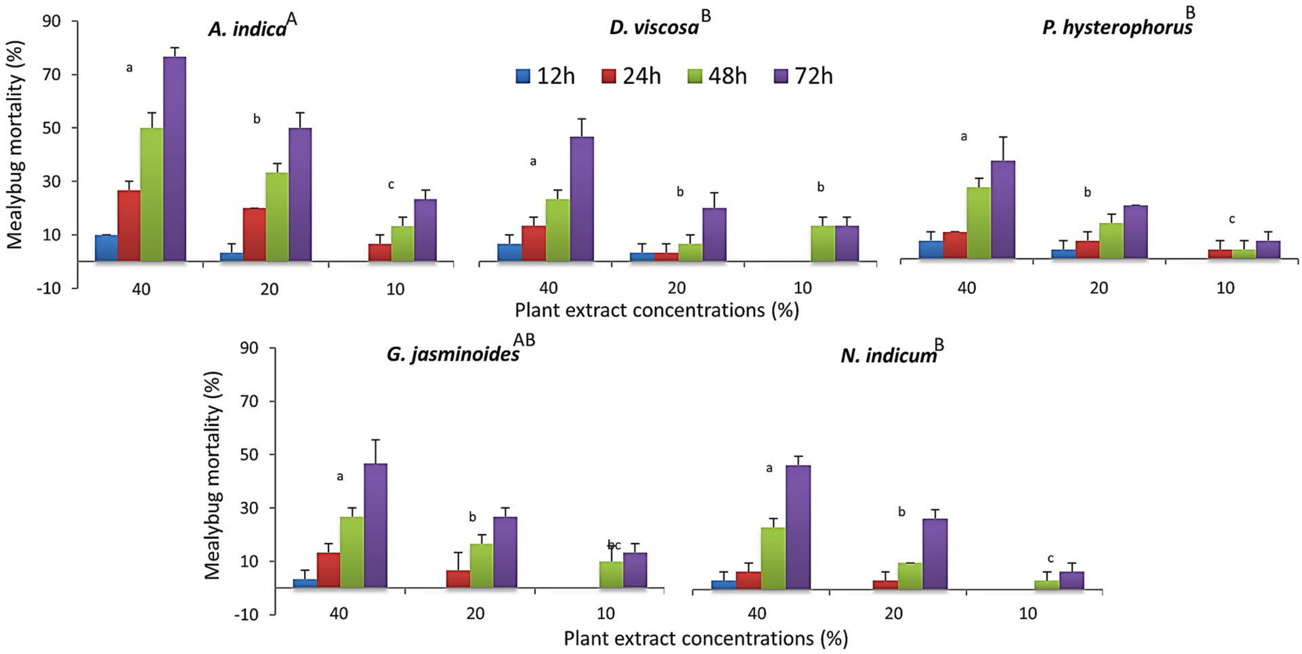

Fig. 1. Percent mortality of 2nd instar nymphs of mealybug *Drosicha mangiferae* exposed to methanolic extracts of different plants. Columns represent average percent mortality of mealybug individuals ± standard error (n=5). For each botanical extract, small alphabets indicate statistical difference among the concentrations (two-way ANOVA; LSD at α = 0.05), while capital alphabets indicate statistical difference among botanical extracts (factorial ANOVA; LSD at α = 0.05).

RESULTS

Toxicity of botanical extracts to mealybugs

Results of toxicity bioassay performed with different concentrations of botanical extracts revealed that all botanical extracts caused significant mortality of mealy individuals ($F_{4, 160}$ = 36.96; p < 0.001) and this mortality response was concentration and time dependent as it augmented along with the concentration of botanical extracts and with different time exposures (Fig. 1). In addition, the interaction of time and concentration had as well a significant impact on the mortality of mealybugs for all botanical extracts (Supplementary Table I).

At 12 h post-exposure, maximum mortality of mealybugs was exhibited by 40% extract of *A. indica* (10%), followed by *D. viscosa* (6.67%) and *P. hysterophorus* (6.67%), while *N. indicum* and *G. jasminoides* caused minimum mortality (Fig. 1). Extracts of *A. indica* was more effective with maximum mortality (26.67%) against mealybugs according to observation at 24 h post-treatment, followed by G. *jasminoides* and *D. viscosa* (13.33%). In the same way, maximum average mortality of mealybugs was exhibited by *A. indica* (50%) followed by *G. jasminoides* (46.67%) and *P. hysterophorus* (26.67%). Similar trend of mortality was recorded for 48 and 72 h time intervals. Minimum mortality was recorded for 10% extracts varying from zero at 12 h post-exposure for *N. indicum*, *D. viscosa* and *G. jasminoides* to 23.33% for *A. indica*, followed by *G. jasminoides* observed at 72 h post-treatment (13.33%; Fig. 1). Nevertheless, factorial analysis and LSD test revealed that, on overall basis, extracts of *A. indica* and *G. jasminoides* were the most effective and toxic against 2nd instar *D. mangiferae* nymphs showing significantly higher mortality than other three extracts (Fig. 1).

Moreover, probit analysis corroborated the same trend of toxicity of botanical extracts against mealybugs. According to probit regression, at 48 h post-exposure, *A. indica* was the most effective followed by *P. hysterophorus* with LC_{50} values of 38.20 and 81.80%, respectively. Similarly, the extract of *A. indica* showed minimum LC_{50} value (20.00 %) at 72 h post-treatment, followed by the extract of *G. jasminoides, N. indicum* and *P. hysterophorus* (Table II). Similar trend was found in case of median lethal time (LT_{50}) values (Table III). For 20% concentrations, minimum LT_{50} values were recorded for the extracts of *A. indica* (68.91 h) and *N. indicum* (92.65 h), while maximum ones were found for the extracts of *P. hysterphorus* (124.45 h) and *D. viscosa* (121.30 h). For 40% extracts, *A. indica* (47.97 h) and *G. jasminoides* (71.26 h) were most effective with minimum LT_{50} values followed by *N. indicum and D. viscosa* (Table III).

Response of mealybugs to plant essential oils

According to the results, all essential oils exhibited a significant mortality of mealy individuals ($F_{2, 120}$ = 6.42; p = 0.002) and this mortality response was concentration and time dependent as it increased along with the concentration of essential oils and exposure time (Fig. 2). In addition, the interaction of time and concentration had as well a significant impact on the mortality of mealybugs for all essential oils (Supplementary Table II).

At 12 and 24 h time intervals, maximum mortality of mealybug individuals was recorded by 2.00% essential oil of *D. alba* (6.67 and 23.33%, respectively) and *S. aromaticum* (6.67 and 16.67%, respectively), while minimum mortality was given by 0.50% concentrations ranged from zero for all essential oils at 12 h to 3.33% for *S. aromaticum* and *C. citratus* at 24 h (Fig. 2). In the similar way, maximum average mortality of mealybugs was exhibited by *D. alba* (30.01%), followed by *S. aromaticum* (23.33%) and *C. citratus* (16.67%). Similar trend of mortality was recorded for 72 h time interval.

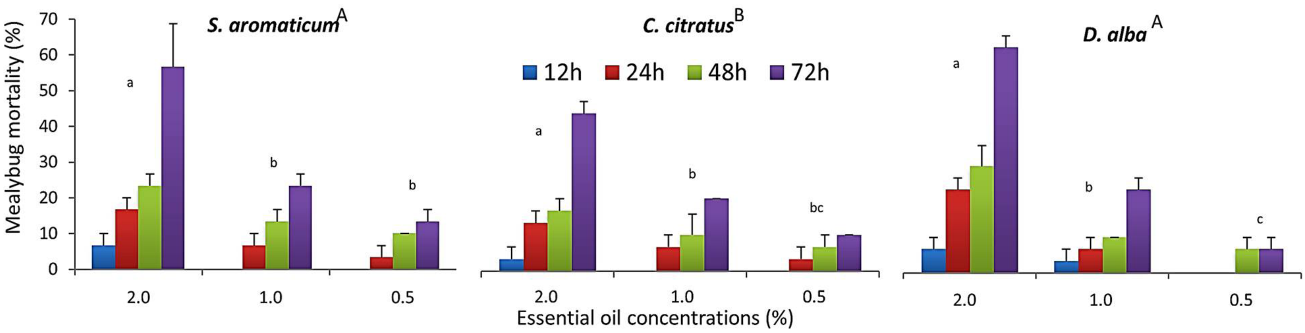

Fig. 2. Percent mortality of 2nd instar nymphs of mealybug *Drosicha mangiferae* exposed to different plant essential oils. Columns represent average percent mortality of mealybug individuals ± standard error (n=5). For each essential oil, small alphabets indicate statistical difference among the concentrations (two-way ANOVA; LSD at α = 0.05), while capital alphabets indicate statistical difference among essential oils (factorial ANOVA; LSD at α = 0.05).

At 72 h, maximum mortality was exhibited by 2.00% extracts of *D. alba* 63.33%), followed by *S. aromaticum* (56.67%) and *C. citratus* (43.33%). Nevertheless, according to factorial analysis and LSD test, on overall basis, the essential oil of *D. alba* and *S. aromaticum* were the most effective and toxic against 2nd instar *D. mangiferae* nymphs showing significantly higher mortality than *C. citratus* (Fig. 2).

Table II.- Median lethal concentration (LC_{50}) values of different plant extracts and essential oils bioassayed against 2nd instar nymphs of mealybug *Drosicha mangiferae* Green.

Treatment	Observation time (h)	LC_{50} (%)	Lower and Upper 95% Fiducial Limits (%)	X^2 (df = 7)*	P
Botanical extracts					
A. indica	48	38.20	29.74 – 60.56	18.24	0.011
	72	20.00	17.13 – 23.35	15.45	0.031
D. viscosa	48	646.87	Incalculable	-	-
	72	48.45	32.97 – 151.49	34.05	< 0.001
G. jasminoides	48	148.94	Incalculable	-	-
	72	42.19	31.87 – 111.98	28.06	< 0.001
N. indicum	48	99.61	55.98 – 885.56	24.43	0.001
	72	44.70	33.40 – 61.12	18.19	0.011
P. hysterophorus	48	81.80	49.20 – 513.87	28.31	< 0.001
	72	59.20	39.32 – 202.74	30.42	< 0.001
Essential oils					
S. aromaticum	48	6.31	2.92 – 43.41	9.98	0.190
	72	0.90	0.65 – 1.98	51.64	< 0.001
C. citratus	48	12.25	Incalculable	-	-
	72	1.27	1.07 – 1.63	4.02	0.778
D. alba	48	2.16	1.24 – 18.67	28.51	< 0.001
	72	0.80	0.69 – 0.98	20.91	0.004

*Since the significance level is less than 0.15, a heterogeneity factor is used in the calculation of confidence limits.

Table III.- Median lethal time (LT_{50}) values of different plant extracts and essential oils bioassayed against 2nd instar nymphs of mealybug *Drosicha mangiferae* Green.

Treatment	Botanical Concentration (%)	LT_{50} (h)	Lower and Upper 95% Fiducial Limits (h)	X^2 (df = 10)*	P
Botanical extracts					
A. indica	20	68.91	59.64 – 84.71	41.01	< 0.001
	40	47.97	43.81 – 52.61	20.89	0.022
D. viscosa	20	121.30	86.46 – 432.45	74.24	< 0.001
	40	76.93	66.58 – 95.26	32.75	< 0.001
G. jasminoides	20	94.85	74.53 – 175.64	83.16	< 0.001
	40	71.26	63.47 – 93.33	48.27	< 0.001
N. indicum	20	92.65	79.50 – 121.96	38.52	< 0.001
	40	74.92	65.85 – 87.97	37.29	< 0.001
P. hysterophorus	20	124.45	93.28 – 245.32	35.89	< 0.001
	40	86.21	71.80 – 117.72	37.34	< 0.001
Essential oils					
S. aromaticum	1.0	102.47	83.47 – 153.41	39.50	< 0.001
	2.0	68.58	57.86 – 89.07	65.63	< 0.001
C. citratus	1.0	112.35	85.78 – 225.96	54.51	< 0.001
	2.0	81.34	69.14 – 105.80	43.16	< 0.001
D. alba	1.0	115.69	89.42 – 202.95	38.12	< 0.001
	2.0	61.30	53.46 – 73.14	43.74	0.003

*Since the significance level is less than 0.150, a heterogeneity factor is used in the calculation of confidence limits.

Probit analysis showed similar trend of toxicity of plant essential oils against mealybugs as depicted by analysis of variance. According to probit analysis, the essential oil of *D. alba* was the most effective at 48 h, followed by *S. aromaticum* with LC_{50} values of 2.16 and 6.31%, respectively, while the essential oil of *C. citratus* revealed maximum LC_{50} value (12.25%). At 72 h post-treatment, essential oil of *D. alba* and *S. aromaticum* were more toxic (LC_{50} = 0.80 and 0.90%, respectively) than oil of *C. citratus* (LC_{50} = 1.27%; Table II). Similar trend was found in case of median lethal time (LT_{50}) values (Table III). For 1.00% oil concentrations, minimum LT_{50} values were found for the essential oils of *S. aromaticum* (102.47 h), while maximum was recorded for the essential oil of *D. alba* (115.69 h). For 2.0% essential oil concentrations, *D. alba* essential oil was most effective with minimum LT_{50} value of 61.30 h, followed by *S. aromaticum* (68.58 h) and *C. citratus* (81.34 h; Table III).

DISCUSSION

D. mangiferae is a damaging pest of different agricultural and horticultural crops including citrus. It has become problematic to control with synthetic insecticides, most probably due to reduced penetration of pesticides and field-acquired resistance (Franco *et al*., 2004; Sreerag *et al*., 2016). Therefore, it is imperative to seek out new control tactics for mealybug control on citrus plants which would be more biorational and environment-friendly than conventional synthetic chemicals. The present study was aimed to screen out some plant-based chemicals for their toxicity against *D. mangiferae* nymphs.

Results revealed a significant effect of botanical extracts and plant essential oils on the mortality of mealybug nymphs as compared to control and this mortality increased along with the treatments (concentration and time). However, no considerable mortality of mealybug nymphs was observed at 12 and 24 h post-treatment for all and even till 48^{th} h for the extracts of *D. viscosa* (sanatha) and *N. indicum* (kaner) and for all three essential oils. This delayed toxicity might result from the slow uptake of botanical constituents by the mealybugs as manifested in case of ineffectiveness of chemical insecticides against most of the mealybugs (Mani and Shivaraju, 2016; Sreerag *et al*., 2016).

Among the tested botanical extracts, *A. indica* (neem) and *G.* jasminoides (gardenia) were found to be the most effective ones against 2^{nd} instar *D. mangiferae* mealybugs. These findings are in conformity with the work of many previous researchers. For instance, seeds and leaves extracts of *A. indica* have been shown to exhibit excellent insecticidal, repellent and antifeedant properties against a number of insect pest species (Mourier, 1997; Benelli *et al*., 2017). Likewise, Prishanthini and Vinobaba (2014), Badshah *et al*. (2017) and Majeed *et al*. (2018) demonstrated the effectiveness of *A. indica* extracts against cotton mealybug (*Phenococcus solenopsis*). Moreover, the methanolic extract of *G. jasminoides* has been found effective against many sucking pests including *Myzus persicae*, *Tetranychus urticae* and *Aphis gossypii* (Kim *et al*., 2005; Ahmed and Din, 2009). Similarly, Li and Fang (2010) showed high contact toxicity of petroleum ether extracts of *G. jasminoides* to 3^{rd} instar nymphs of brown planthopper *Nilaparvata lugens* causing ≥ 80% mortality.

Among three essential oils tested in this study, *D. alba* (dhatura) and *S. aromaticum* (clove bud) were the most effective ones against *D. mangiferae* nymphs. Many previous researchers have reported the detrimental effects of extracts and essential oils of *D. alba* on mealybugs (Lanjar *et al*., 2015), stored grain pests (Ali *et al*., 2012), aphids (Kuganathan *et al*., 2008), subterranean termites (Ahmed *et al*., 2005) and mosquitos (Mehdi *et al*., 2012). Different alkaloids, particularly tropane alkaloids, are the major bio-constituents of genus *Datura* plants which are responsible to exert anti-insect effects (Fang, 2009; Monira and Munan, 2012).

CONCLUSION

Based on the results of this study, it is concluded that although lacking quick knockdown effect, indigenous plant extracts and essential oils can be effectively employed against sucking insect pests such as *D. mangiferae.* Botanical extracts of *A. indica* (neem) and *G. jasminoides* (gardenia) and essential oils of *D. alba* (dhatura) and *S. aromaticum* (clove bud) appeared to be the most effective against 2^{nd} instar *D. mangiferae* individuals with minimum LT_{50} and LC_{50} values, hence, are suggested to be incorporated in future biorational management programs for mealybugs, particularly in horticultural and green-house crops. Moreover, the bioactive constituents of these phyto-extracts responsible for the observed mealybug mortality should be focused for their potential characterization in future research.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: http://dx.doi.org/10.17582/journal.pjz/2019.51.5.1815.1822

Statement of conflict of interest

The authors declare no conflict of interest.

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