Pakistan J. Zool., vol. 46(4), pp. 1117-1124, 2014.

Effect of Guava Leaf Extract on Citrus Attractiveness to Asian Citrus Psyllid, *Diaphorina citri* Kuwayama

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Abstract.- The effect of guava leaf extract on citrus attractiveness to psyllids, *Diaphorina citri* Kuwayama, was tested with a series of concentrations from 100 mg/L to 10,000 mg/L. Except the lowest concentration, spray application of the extract solutions significantly affected the adult psyllid response to citrus in both cage and Y-olfactometer tests. Significantly highest reductions (44.2% and 50%) in landing of adult psyllids were found on citrus when sprayed with 10,000 mg/L extract solution, followed by 5,000 mg/L extract solution (41.9% and 47.6%) and 1000 mg/L extract solution (32.6% and 35.7%), after treatments of 12 h and 24 h, respectively in choice cage tests. Similar significant effectiveness of all, but 100 mg/L, solutions of guava extract were observed in Y-olfactometer tests where 13.75%, 21.25% and 31.25% female adult psyllids, and 15%, 20% and 32.5% male psyllids moved towards the arm provided with citrus leaves treated with 10,000 mg/L, 5,000 mg/L and 1000 mg/L guava extract, respectively. The response of adult psyllids to guava leaf extract was dosage-dependent. Among three fraction treatments of extract, only petroleum ether exhibited significant effect in reducing psyllid settlement on citrus shoots showing the highest reduction of psyllids (with 1000 mg/L extract, 35.7% and 43.8% and with 500 mg/L extract, 28.9% and 39.5% after treatments of 12 h and 24 h, respectively) in choice cage tests. But in Y-tube response tests, all of three fractions exhibited significant repellence at higher dose (1000 mg/L), compared to control shoots. The repellent effects were petroleum ether fraction (54.7%) > ethyl acetate fraction (37.0%) > n-butyl fraction (31.5%). Our study results reveals that guava leaf extracts may be an effective control option of psyllids in citrus orchards.

Keywords: *Diaphorina citri*, insect behaviour, host selection, *Psidium guava*, leaf extract.

INTRODUCTION

The Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is globally known as the most serious pest of citrus because of its role in carrying the fastidious phloem-limited pathogen of huanglongbing (HLB) disease. The psyllids are host-specific and 27 species within seven genera of Rutaceae family are recognized as suitable hosts of *D. citri* in China (He, 2000). It is supposed to be indigenous pest of the Indian sub-continent (Hollis, 1987) and then spread into the other citrus producing regions worldwide (Halbert and Nunez, 2004; Halbert and Manjunath, 2004). Adults and nymphs of psyllid feed on young leaves causing withering, distortion, and loss of immature leaves and produce irregular shaped canopies. The psyllid causes crop loss mainly by transmitting HLB disease to the orchards. Once the pathogen infects some trees, the disease spreads quickly to other plants in orchard and the infected trees decline within several years (Yang *et al*., 2006). The combined presence of psyllid vector and HLB agent has been the limiting factor in worldwide citrus production. But the fact is that no effective management approaches to control HLB have yet been established (Halbert and Manjunath, 2004; Bove, 2006) and the conventional control of *D. citri* in citrus orchards relies on the use of insecticides (Rogers, 2008) that limits, but does not prevent disease spread (Beattie and Barkley, 2009; Frederic *et al*., 2010). In addition, insecticide use affects populations of natural enemies and may lead to insecticide resistance development to the psyllids. Currently, other management practices are also limited because of lacking in resistant variety, effective biological control agents and cultural control options (Childers and Rogers, 2005; Qureshi and Stanlsy, 2007). To reduce the use of chemicals, limited efforts have been given in mineral oils to slow down the spread of ACP and the HLB pathogen. Some plant based mineral oils have repellent, antifeedant and toxic effects on insects. Neem seed oil and neem seed extract have been

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0030-9923/2014/0004-1117 $ 8.00/0

reported to have toxic effects on female citrus psyllids (Khan *et al*., 2012). However, the issue to control the population of psyllids and the spread of HLB is still in challenge.

A new thought towards the management of HLB in citrus groves has been initiated in 2004 when, in Vietnam, lower psyllid infestation levels and low incidence of HLB have been observed in citrus orchards (cv. King Mandarin) intercropped with guava, *Psidium guajava* L. (Myrtaceae), plants compared to citrus orchards lacking guava (Beattie *et al*., 2006). Previous studies, concerning the mechanism of the guava-citrus intercropping benefits, showed that volatiles from guava leaves may play the role in reducing ACP populations and HLB disease incidences. Zaka *et al.* (2010) reported that the presence of guava shoot significantly reduced psyllids settling on citrus in cage trials. Guava leaf volatiles inhibited attraction of psyllids to normally attractive host citrus volatiles (Onagbola *et al*., 2011) in Y-tube olfactometer tests. Mechanically wounded guava produces toxic and repellent dimethyl disulphides (DMDS), while citrus does not, suggesting these, or other guava compounds may be responsible for the observed deterrence (Rouseff *et al*., 2008). In numerous systems (Coll and Bottrell, 1994; Togni *et al*., 2010; Erickson *et al*., 2012), intercropping has been successfully used in insect pest management. Intercropping is now an important component of integrated pest management in different cropping systems in Kenya implicating push-pull strategies (Cook *et al*., 2007). However, the practice of guava-citrus intercropping is still limited in some areas of China, Vietnam and surrounding regions at a certain scale. One reason may be here that inter-planting of guava may cause the severances of fruit fly damage to citrus (Xie and Zhang, 2005; Xu *et al*., 2005) and occupy the space for citrus. Under these circumstances, use of guava leaf extract may be an option for controlling Asian citrus psyllid infestation and HLB incidence in citrus orchards.

Beneficial effects of guava in different forms have been reported in agriculture systems by some previous studies. Guava leaf powder was found to be toxic to rice weevil, *Sitophilus oryzae* L. and granary weevil, *Sitophilus granaries* L. (Sharaby, 1988). Mixing of 20% guava leaves into the soil was found to reduce the growth of weeds (*Echinochloa colonum* L., a narrow leaved weed and *Portulaca oleracea* L., a broad leaved weed) associated with sunflower cultivation (Dawood *et al*., 2012). Guava leaf aqueous extract affected seed germination and root growth of lettuce, *Lactuca sativa* L. (Chapla and Campos, 2010), guava fruit extracts affected cucumber, *Cucumis sativus*, germination (Bovey and Diaz-Colon, 1968) and guava root exudates affected lettuce germination and the growth of bristly foxtail, *Setaria verticillata*, (Brown *et al*., 1983). These results suggest that guava leaf extract could contribute to an effective integrated pest management program for HLB/citrus psyllid, by reducing citrus attractiveness to them. Application of mineral oil (Poerwanto *et al*., 2012) and plant-based essential oils (Mann *et al*., 2012) to citrus leaves significantly reduced its attractiveness to *D. citri*. However the effect of guava leaf extract on citrus attractiveness to psyllid adults has not yet been tested. Therefore, the present study was designed to investigate the effect of volatiles emanating from guava leaf extracts on the citrus attractiveness to psyllid adults by choice experiments and Y-tube olfactometer response tests.

MATERIALS AND METHODS

Insects

Adults of ACP were collected from 5 year old sweet orange (*Citrus* x *aurantium* L.) trees, grown in the campus of South China Agricultural University. High psyllid populations were maintained by pruning, fertilizing and watering the trees when needed to enhance new growth favoured by the psyllids. Light pruning was done approximately at every 6 weeks. Adults were collected with mechanical aspirators each morning and held in plastic cups for later use in the day. Male and female insects were identified on the basis of their morphology, the tip of the abdomen of adult male of ACP is bent upwards while the abdomen of the female is straight. Orange/yellow colour of female abdomen showed that it contains the eggs (Husain and Nath, 1927; Wenninger and Hall, 2008; Wenninger *et al*., 2009). All the psyllids were preserved after experiment in 70% ethanol and their sexes were reconfirmed under the light laboratory microscope.

Plants materials

Five-year old sweet orange trees, grown in 25 L plastic pot with loamy soil mixed with leaf compost in the university campus, were used for experiments. These plants were irrigated in need, fertilized fortnightly and pruned approximately at every 6 weeks to enhance new growth and were visually free from any disease and pest. Approximately 5–7 year old guava (*Psidium guajava* L. cv. Pearl, Myrtaceae) trees in the botanical garden of South China Agricultural University, Guangzhou, Guangdong, China were used as the source of leaves for extracts. Fresh new shoots of guava and new strong shoots with fresh leaves of citrus were collected from the field for experiment use.

Preparation of leaf extract

Guava shoots with fresh leaves (10 to 15 cm long) were collected and then dried in oven at 50ºC temperature for 24 h. The dried leaves were ground to powder with an electric grinder and then sieved to avoid unwanted granules from the powder. Fifty gram dried leaf powder was soaked in 500 mL methanol and the mixture was stirred by ultrasound method for 30 min, then it was kept in a dark place for 24 h. The extract solution was filtered and the solid filtration residue was extracted 2nd and 3rd time in the same way. Extract solution was then evaporated by rotary evaporation under reduced pressure at 55°C temperature and the crude extract was preserved at 4°C until use.

The crude extract dissolved in 40% acetone was used for preparation of different concentrations *viz.*, 100, 1,000, 5,000 and 10,000 mg/L for experimental use.

Effect of guava leaf extract solutions on the attractiveness of citrus shoots

The responses of mixed populations of adult male and female psyllids to different concentrations of guava leaf extract solution were observed in choice experiments where 50 adult psyllids (25 male and 25 female) were released into cages (60 cm x 30 cm x 60 cm), each cage with 5 citrus shoots and one from each treatment. One citrus shoot (10–15 cm in length) was placed in a 100 mL flask and the flasks were positioned randomly at equidistance from each other, the shoot was sprayed up to run-off with extract solutions by a hand sprayer and allowed 1 h to dry before placing in the cage. Five treatments (citrus shoots sprayed with 100 mg/L, 1000 mg/L, 5,000 mg/L, 10,000 mg/L extract solutions and only 40% acetone as check) were used for cage trials. Each treatment was replicated 5 times. All the experiments were done under laboratory condition at temperatures of 25±2°C and of 70±5% RH. The number of adults landing on citrus shoots was recorded after 12 h and 24 h of their release in the cage.

Y-tube olfactometer response

Y-tube olfactometer responses to guava leaf extract solutions were measured based on the methods of Horton and Landolt (2007). Each arm of Y-tube was connected to one of the two odour sources with silicone tubing. Response of insects to the odor source emanated from guava-extract treated citrus leaves was compared with that from control leaves (sprayed with 40% acetone). Effectiveness of different concentrations of guava leaf extract against male and female psyllid adults was tested separately. A line was drawn on each of the two arms of the olfactometer at 10 cm from the junction of the two arms. Psyllids crossing either line during 5 min spell were considered to have made a choice. Each test was repeated 8 times, with 10 psyllids on each occasion (80 adults). All the experiments were done under laboratory condition at temperatures of 25±2°C and 70±5% RH. The whole apparatus *i.e.* Y-tube, flask, insect release adapter, silicone tubes, etc. were washed with acetone and then dried before use and it was repeated after each treatment.

Fractions of the guava extract

In order to determine which chemicals were involved in the repellence against Psyllids, the extract was fractionated as follows: 200 g crude guava extract was taken in a 1000 mL beaker and added little water and stirred to make a paste. First fraction was done with petroleum ether. 500 mL petroleum ether was added to the beaker and stirred for 30 minutes, then allowed to precipitate the solid extract. The upper petroleum ether extract solution was separated carefully from the precipitate by a rubber tube. This process was repeated 3 times. Second and third fractions were done with ethyl acetate and n-butyl solutions, respectively, in the same way. Three fractions of guava extract solution were then evaporated by rotary evaporation under reduced pressure at 40–55°C temperature and the crude extracts were preserved at 4°C until use. Two concentrations (*e.g.*, 500 mg/L and 1000 mg/L solutions) were prepared from each of three fraction crude extracts dissolving in 40% acetone and then repellence tests of these concentrations were done by choice tests and Y-tube olfactometer response tests (as described above). As the main guava extract was found to significantly affect the landing of adult psyllids on citrus shoots at 1000 mg/L concentration, therefore, the highest concentration of different fractions of guava extract was considered 1000 mg/L in choice and Y-tube olfactometer response tests here. Other conditions for these two tests were similar to the above mentioned methods for the main guava extract.

Statistical analysis

Differences between treatments were determined using Statistica (Version 7, StatSoft, Inc.). All data sets were homoscedastic according to Levene's test and means were separated using Tukey's HSD test. T-tests were used to determine whether the number of psyllids moving towards either of the two arms of the olfactometer were significantly different.

RESULTS

Effect of guava-extract solutions on the attractiveness of citrus shoots

Guava-extract solutions reduced the number of adult psyllids landing on citrus shoots. The results (Table I) showed that significantly lower psyllids were found on citrus shoots sprayed with 1000 mg/L and/or more concentrations of guava extract solutions, compared to control shoots. The lowest number of adult psyllids (4.80 at 12 h and 4.20 at 24 h count points) was counted on the citrus leaves when sprayed with 10,000 mg/L extract solution, identically followed by 5,000 mg/L (5.0 at 12 h and 4.40 at 24 h count points) and 1000 mg/L (5.80 at 12 h and 5.40 at 24 h count points), while the highest landing was found on control shoots (8.60 and 8.40 at 12 h and 24 h count points, respectively). Significantly highest reductions (44.2% and 50%) in psyllids landing were found with 10,000 mg/L extract, followed by 5,000 mg/L extract (41.9% and 47.6%) and 1000 mg/L extract solution (32.6% and 35.7%), as compared to control shoots at both count points (after 12 h and 24 h, respectively). The number of adult psyllids landing on citrus leaves sprayed with 100 mg/L guava extract solution did not differ significantly with the control at both counts.

Table I.- Mean number of adult ACP landing on citrus shoots sprayed with guava extract and on unsprayed shoots (Check) in choice cage trials.

Concentration (mg/L)	Mean adult number ± SE (n = 5) 12 h	24 h
100	7.60 ± 0.77 ab	7.00 ± 0.97 ab
1,000	5.80 ± 1.10 bc	5.40 ± 0.96 bc
5,000	5.00 ± 0.75 c	4.40 ± 0.73 c
10,000	4.80 ± 0.64 c	4.20 ± 0.68 c
0 (Ck)	8.60 ± 1.02 a	8.40 ± 0.89 a

Means in the same column having same letter(s) are not significantly different according to Tukey's HSD test at $P=0.05$

Y-tube olfactometer response

The olfactometer responses of adult psyllids to the odor from control citrus leaves and citrus leaves sprayed with guava leaf extract solution at different doses are presented in Figure 1. With both male and female adults, 6.25–12.50% psyllids did not move *i.e.* remained at/near the release point of the Y-tube in different treatments. Fresh citrus leaves and leaves treated with 100 mg/L guava leaf extract solution showed non-significant difference in psyllids response (both male and female). When the dosage reached 1000 mg/L and/or higher concentrations of guava extract solution sprayed on citrus leaves in one arm and control leaves in another arm, highly significant differences between the mean numbers of psyllids in the two arms were observed. Decreased number of adult psyllids (both male and female) was found to make choice towards the arm having increased dosage of guava extract where 13.75% (P<0.000003), 21.25% (P<0.00002) and 31.25% ($P<0.003$) female adult psyllids, and 15% ($P<0.000003$), 20% ($P<0.00003$) and 32.5% ($P<0.004$) male psyllids moved towards the arm provided with citrus leaves treated with 10,000 mg/L, 5,000 mg/L and 1000 mg/L guava extract solutions, respectively, compared to control leaves. The results revealed that the response of adult psyllids to guava leaf extract was dosage dependent and there was little difference in movement of male and female psyllids in different treatments.

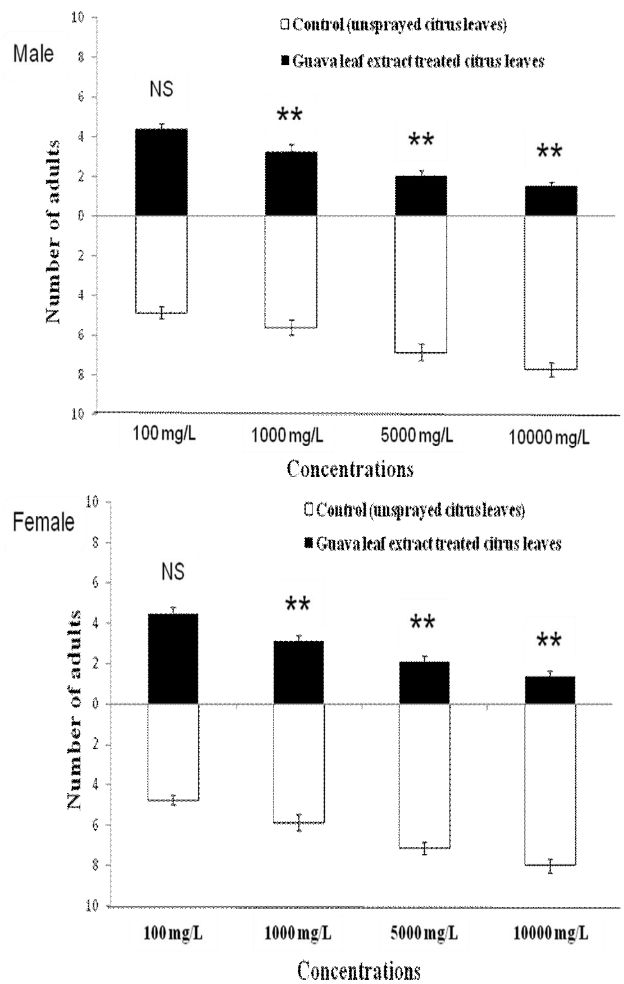

Fig. 1. Choice response of male and female adult psyllids to volatiles emanating from fresh citrus foliage and citrus foliage treated with different concentrations of guava leaf extract solutions. ****** denotes significant differences between control and treatment at *P<0.01*; NS denotes a non-significant difference.

Effect of fractionated guava extract solutions on the attractiveness of citrus shoots

The methanol extract was separated into 3 fractions using petroleum ether, ethyl acetate and N-butyl, which were tested in a choice experiment at two different concentrations (500 mg/L and 1000 mg/L). Petroleum ether fraction extract significantly reduced the landing of psyllid adults and showed the lowest number with both concentrations at two count points (with 500 mg/L extract, 5.4 and 5.6 and with 1000 mg/L extract, 5.4 and 5.2 at 12 h and 24 h count point, respectively), compared to all other treatments and control shoots (Tables II and III). Ethyl acetate extract significantly reduced the landing of psyllids with both concentrations only at 24 h count point, not at 12 h count point, compared to control shoots. N-butyl extract solution only with the higher dosage (1000 mg/L) showed significant reduction in psyllid landing at 24 h count point (Table III), compared to control shoots. There was no significant difference in reducing landing of psyllids on citrus shoots when sprayed with ethyl acetate and n-butyl extracts at both concentrations and at both count points and these two extract was statistically identical with the control shoots at both concentrations at 12 h count point.

Table II.- Mean number of adult ACP landing on citrus shoots sprayed with fractionated guava extract (500 mg/L) and on unsprayed shoots (Check) in choice cage trials.

Treatment	Mean adult number ± SE (n = 5)	
	12 h	24 h
Petroleum ether extract	5.40 ± 1.29 b	5.60 ± 1.26 c
Ethyl acetate extract	8.00 ± 1.20 a	8.60 ± 1.29 b
N-butyl extract	7.80 ± 1.04 a	9.20 ± 1.44 ab
Citrus shoot treated with acetone (solvent) *i.e.* Control	8.40 ± 1.21 a	9.80 ± 1.53 a

Means in the same column having same letter(s) are not significantly different according to Tukey's HSD test at *P=0.05*

Y-tube olfactometer response test with different fractions of guava extract

The olfactometer responses of adult psyllids (mixed population) to different fractions of guava leaf extract odour at two different doses are presented in Figure 2 and Figure 3. In all the experiments, 3.75–12.50% psyllids did not move *i.e.*

Table III.- Mean number of adult ACP landing on citrus shoots sprayed with fractionated guava extract (1000 mg/L) and on unsprayed shoots (Check) in choice cage trials.

Treatment	Mean adult number ± SE (n = 5)	
	12 h	24 h
Petroleum ether extract	5.40 ± 1.13 b	5.20 ± 1.01 c
Ethyl acetate extract	7.40 ± 1.05 a	7.60 ± 0.85 b
N-butyl extract	7.00 ± 0.35 a	7.40 ± 0.58 b
Citrus shoot treated with acetone (solvent) *i.e.* Control	7.60 ± 0.77 a	8.60 ± 1.19 a

Means in the same column having same letter(s) are not significantly different according to Tukey's HSD test at *P=0.05*

remained at/near the release point of the Y-tube in different treatments. With 500 mg/L solution, only the petroleum ether extract exhibited significant difference (*P*= 0.0166) between the mean number of psyllids in the two arms (Fig. 2), compared to control. The repellent effect of other two fractions was non-significant at 500 mg/L solution with control treatment. All the fractions had significant repellence at 1000 mg/L concentration over control treatment (Fig. 3). The highest repellent effect on adult psyllids was observed when citrus shoots were treated with petroleum ether extract (psyllid movement 29%, *P*=0.0003), followed by ethyl acetate extract (psyllid movement 34%, *P*=0.0072) and n-butyl extract (psyllid movement 37%, *P*=0.0185), compared to control shoots (64%, 54% and 54%, respectively). The results revealed that the response of adult psyllids to different fractions of guava leaf extract was dosage dependent.

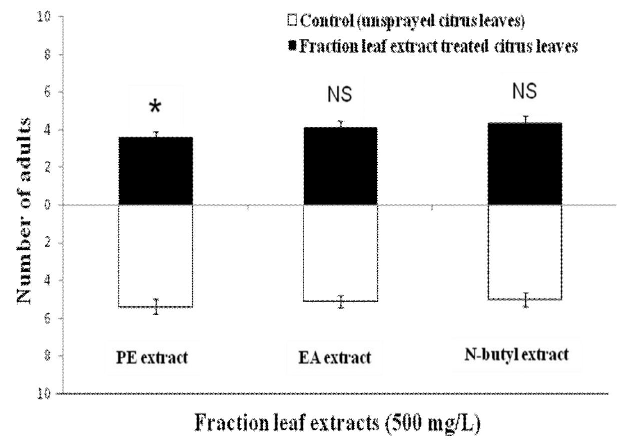

Fig. 2. Choice response of adult psyllids to volatiles emanating from fresh citrus foliage and citrus foliage treated with different fractions of guava leaf extract at 500 mg/L concentration. *****denotes significant differences between control and treatment at *0.01<P<0.05*; NS denotes non-significant differences; PE = Petroleum ether and EA = Ethyl acetate.

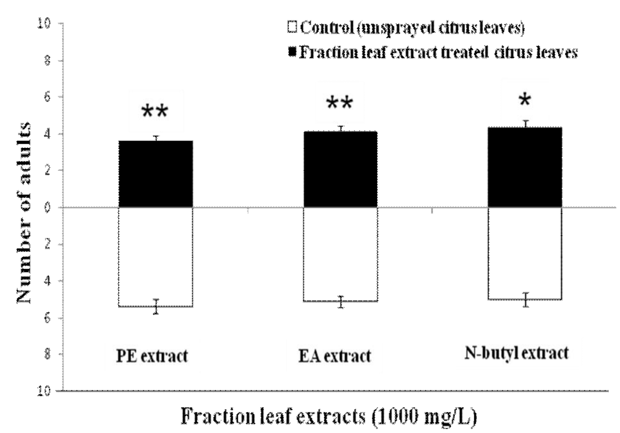

Fig. 3. Choice response of adult psyllids to volatiles emanating from fresh citrus foliage and citrus foliage treated with different fractions of guava leaf extract at 1000 mg/L concentration. ******denotes significant differences between control and treatment at *P<0.01*; *****denotes significant differences between control and treatment at *0.01<P<0.05*; PE = Petroleum ether and EA = Ethyl acetate.

DISCUSSION

The results in the present study revealed that psyllids alightment on citrus shoot that was sprayed with guava leaf extracts was significantly reduced, compared to unsprayed citrus shoots- the mechanism can be explained in two ways. First, the volatiles emanated from guava extracts directly repelled psyllids, and the second is masking citrus emissions or changing volatile profile emitted from citrus shoot, disrupting psyllids host finding cues. Zaka *et al*. (2010) reported that the presence of guava shoot significantly reduced psyllids settlement on citrus shoots in cage trials and the repellent effect was dose dependant. Sulphur volatile compounds produced from crushed guava leaves repel *D. citri* (Rouseff *et al*., 2008). The aqueous extract from Myrtaceae family has been reported to have larvicidal and repellent activity against mosquitoes (Cheng *et al*., 2009). The leaf extract of guava was reported to be toxic against *Tribolium castaneum* Herbst (Mostafa *et al*., 2012) and an excellent repellent for blister beetles in the field (Sami and Shakoori, 2007). These results corroborate with the present study, suggesting that guava extracts reduce citrus attractiveness to psyllids either by direct repellent effect or indirectly affecting the emissions of host finding cues from citrus shoots. However, these results don't confirm the indirect effect of guava leaf extract on citrus attractiveness to psyllids.

In Y-tube olfactometer response test, the result reveals that the presence of guava leaf extract affects host odour finding by psyllid adults resulting fewer movement towards the arm with guava extract-sprayed citrus shoots, compared to unsprayed shoots and the effect was dose dependant. The adults of *D. citri* generally rely on olfaction and vision for detection of host cues (Wenninger *et al*., 2009). In T-olfactometer test, sulphur volatiles from *Allium* spp. affected Asian citrus psyllid response to citrus volatiles (Mann *et al*., 2011). With reference to fractions, only petroleum ether extract significantly reduced psyllids alightment and Y-tube responses to the citrus shoots, compared to untreated and even treated by other two fraction extract solutions, indicating that most of the volatiles responsible for affecting citrus shoot attractiveness to psyllids were isolated with petroleum ether solvent. Fractionating method followed in this study may have less accuracy, but the results obtained here showed that guava leaf extract affects citrus attractiveness to psyllids.

Our results suggest that guava extract may directly repel psyllids, volatiles from guava extract may mask citrus emissions or change volatile profile emitted from citrus, and thereby affects psyllids host finding volatile cues.

ACKNOWLEDGEMENTS

The present research was done as a part of PhD work. Thanks are due to BARC, NATP: Phase-1, Bangladesh, for the Fellowship of PhD study. The research has been financed by the project (GJHZ1140) of the Department of Education of Guangdong Province and project (31071712) of the Natural Science Foundation of China (NSFC).

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(Received 22 May 2014, revised 30 May 2014)