Evaluation of some dicotyledonous seed extracts against coconut leaf beetle, *Brontispa longissima* (Gestro) (Coleoptera: Chrysomelidae) and sublethal effect to an endoparasitoid

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ABSTRACT

Aqueous and ethanolic seed extracts of *Annona squamosa* L. and *Mangifera altissima* B. were evaluated in the laboratory on adult coconut leaf beetles, *Brontispa longissima* G. Direct-spray method was employed in delivering the treatments to the test beetles. On the other hand, dip method was used in exposing parasitized pupae to different treatments. Laboratory results revealed that aqueous and ethanolic extracts of *M. altissima* at 15% w/v and 10% w/v, respectively and *A. squamosa* at 2%w/v and 10%w/v, correspondingly were comparable to Thiametoxam™. Percentage mortalities observed for all seed extracts used except the aqueous form of *M. altissima* exhibited a dose-dependent manner. Probit analysis revealed that the trend for LC_{50} for each seed extracts is *M. altissima* aqueous extracts (MAAE) >*A. squamosa* aqueous extracts(ASAE) >*M. altissima* ethanolic extracts (MAEE) >*A. squamosa* ethanolic extracts (ASEE). It was also found that based on recorded mean parasitoid emergence, Thiametoxam™ had greater sub-lethal effect than the botanicals on the natural enemy of coconut leaf beetle, *Tetrastichus brontispae* F. (5.00 ± 3.00). The chemical groups such as phenols, saponins and tannins might be possibly responsible for the observed bioactivities against the hispid beetle. UV-vis analyses of ethanolic fractions further revealed presence of secondary metabolite known to have several biological activities; annonasquacin for *A. squamosa* and mangiferin for *M. altissima*. Proper formulation of these materials can be developed as alternative control to *B. longissima* as they may offer selectivity than conventional insecticide and contain mixture of phytochemicals that can deter development of insect resistance.

Keywords: *Brontispa longissima* G., insect pest, endoparasitoid, *Cocos nucifera* L., seed extracts, insecticidal

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INTRODUCTION

Among the insect pests associated with coconut, *Cocos nucifera* L., the coconut leaf beetle (CLB), *Brontispa longissima* (Gestro) is one of the most serious in Southeast Asia and in the Philippines (Liebregts *et al*. 2006; Chiki *et al*., 2009; Yebron and Batalon 2011; Obra *et al.*, 2020). CLB is a leaf beetle that feeds on young leaves and destructive on both seedlings and mature coconut palms (Lu and Peng, 2017). The egg are brown, flat and are laid singly or in groups of two to four on the still folded heart leaves (Lever, 1979). Eggs are incubated for about 3–7 days and the larvae undergo six larval instars on a period of 23–54 days (Obra *et al.*, 2020). The pupal period is 4–6 days. Its whole life cycle from egg to adult takes 31–67 days (Singh and Rethinam 2005). The adult longevity ranges from two and a half three months on 75-220 days (Tjoa, 1953; Reitham and Singh, 2005).

Severe CLB infestation can lead to defoliation and later to death of the crop (Leibregts *et al.*, 2006). In the Philippines, the pest had affected more than three million coconut trees from August 2007 to March 2011 (Yebron and Batalon, 2011). Therefore, there is still a need to find for alternative control methods which are economical, environment-friendly and compatible to existing biological controls (He *et al*., 2005; Lv *et al*., 2012). Control measures such as use of synthetic insecticides have been long practiced (Food and Agriculture Organization, 2005). However, intensive use of synthetic chemicals can have adverse effects to non-target organisms (Aggarwal and Brar 2006; Desneux *et al*., 2007). Diflubenzuron, carbaryl and carbofuran were found to have secondary exposure toxicity problem (Cruz *et al.*, 2017). In addition, chemical resistanceof CLB is now widespread in coconut-growing countries of Southeast Asia (Food and Agriculture Organization, 2007; Lin *et al.*, 2012; Obra *et al.*, 2020).

Use of botanicals is still regarded as important component of Integrated Pest Management Programme (IPM) (Isman, 2006). Botanicals can reduce cross-resistance (Feng and Isman 1995, Leatemia and Isman 2004b), is target-specific (Du, *et al.*, 2011), environmental-friendly (Vijayaraghavan *et al.*, 2010) and act synergistically (Berenbaum, 1985). There is a vast array of plants which can serve as an endless source of ecologically-sound and safer pest control materials (Javier and Brown 2009, Lv *et al.*, 2012; Mazid *et al.*, 2011). Two potential plant species which may serve such purpose are at is *Annona squamosa* L. (Annonaceae) and *Mangifera altissima* B. (Anarcadiaceae). At is seed extracts have been proven to be toxic to a wide range of insect pests often of economic importance (Ocampo and Ocampo, 2006) such as *Drosophila melanongaster* (Kawazu *et al.*, 1989), *Tribolium castaneum* (Khalequzzaman and Sultana, 2003), *Plutella xylostella* (Leatemia and Isman 2004) and *Culex quinquefasciatus* (Perez-Pacheco *et al*., 2004) but not yet tested to CLB. Meanwhile, there hasn't been any literature so far that documents the potential of *M. altissima* locally known as 'paho' as source of insecticide. Nevertheless, the seeds of this species are extremely bitter and bitterness is an indication that insecticidal compounds such as alkaloids may be present (Harborne, 1982). Moreover, this species belongs to family Anarcadiaceae where in certain species were found to have insecticidal properties (Chopa *et al*., 2006; Oparaeke and Bunmi, 2006; Nascimiento, 2012). It is important to explore the effectiveness of using botanically–derived materials as supplement or alternative to insecticides in *B. longissima* control (Lv *et al*., 2012).

One desirable characteristic of a botanical insecticide is target-specificity (Leatemia and Isman 2004b; Lv, *et al*., 2012). This is one criterion that a plant biocide must satisfy to be accepted by the regulatory agency (Rejesus, 1995). Hence it is integral to determine the effect of bioactive substances on natural enemies (Perera *et al.*, 2000). In this light, the effect of the plant extracts to the eulophid wasp, *Tetrastichus brontispae* (Ferriere), a natural enemy of *B. longissima* was included in the study. *T. brontispae*is a promising pupal parasitoid of CLB (Chen *et al.*, 2010). This study aimed to explore the bio-activity of the seed extracts of *M. altisimma*-B. and *A. squamosal* -L. for controlling adult CLB (*Brontispa longissima* G.) under laboratory regime. Corollary on this, another goal is to investigate on the residual activity of these materials to known bio-control agent of CLB, *Tetrastichus brontispae* F.

MATERIALS AND METHODS

Preparation of Seed Extracts

A.squamosa and *M.altissima* seeds were collected from trees found in Taysan, Legazpi City, Albay, Philippines (13.1205° N, 123.7439° E). Seeds were cleaned, air-dried for 5 days, and powdered using a coffee grinder. The crude aqueous extracts were prepared akin to the method of Chavan *et al*. (2011). Fifty grams of powdered seeds were macerated in 100mL distilled water for 2-days. The mixture was stirred every 24 h using a sterile glass rod and filtered using a clean Muslin cloth. After maceration, the mixture was concentrated overnight on a water bath at 40-45^{0}C. The crude ethanolic extracts of the seeds were made following the procedure of Leatemia and Isman (2004a). One-hundred grams of each sample were extracted using 95% ethanol (5x 200mL) over 5 days by soaking. The extracts were vacuum-filtered (Whatman No. 1) at Bicol University College of Science (BUCS)-Microbiology Laboratory and reduced *in vacuo* using a rotary evaporator (Model RE 47: Yamato Scientific Co., Ltd.) at the Entomology-Epidemiology Laboratory, Philippine Coconut Authority-Albay Research Center (PCA-ARC) in Banao, Guinobatan, Albay. The extracts were dried in an oven at 45-50^{0}C. Different concentrations of each extracts were prepared. Thereafter, extracts were stored at 5^{0}C until the onset of insect bioassays.

Rearing of Test Insects

Brontispa longissima adults of CLB were collected in several coconut plantations located in Muladbucad Grande, Guinobatan (13°11′N 123°36′E) and Taysan, Legazpi City, (13°12’N, 123°74’E), Albay. These were brought and mass-reared in the BUCS- Animal Room. Insects were placed in plastic rearing boxes (15 x 26cm) with a hole (*c.a* 5 cm in diameter) on the lid covered with fine plastic mesh. Young coconut leaves were cut into pieces (*c.a* 12cm) then placed inside the box. Rearing boxes were double-sealed by placing foam on the sides of the lid. The leaves were replaced every after 2-3 days to prevent cross-contamination of fungus and molds.

Tetrastichus brontispae parasitoids were obtained from mummified CLB pupae from PCA-ARC Biocontrol Laboratory in Banao, Guinobatan, Albay. Mass-rearing was done by exposing 50 day-old unparasitized pupae of *B. longissima* to newly-emerged adult parasitoids. The pupae were placed in tightly-sealed transparent cups (10 cm approx. diameter) with a small hole covered with fine mesh cloth. A piece of tissue soaked in 10% honey solution (Liebregts *et al.*, 2006) was hanged at the center of the container. This served as source of carbohydrates to the parasitoids. The set-up was checked every after 2 days. Resulting mummies were collected and transferred to individual glass tubes after 10-days.

CLB Bioassay

The effect of the crude aqueous and ethanolic seed extracts of *M. altissima* B. and *A. squamosa* L. on CLB adults was assessed in the laboratory in a completely-randomized design (CRD). The concentrations used were based on pre-tests. For aqueous form, the concentrations used were 5, 10, and 15%w/v for *A. squamosa* L. (ASAE) and 7.5, 15, and 30%w/v for *M. altissima* (MAAE). For ethanolic extracts, the concentrations were 2, 4 and 8%w/v for *A. squamosa* L. (ASEE) and 5, 10, and 15%w/v for *M. altissima* (MAEE). An additional treatment was made by emulsifying the second highest concentration of each extract with 0.05 % w/v detergent solution (emuL). Detergent can be used as an emulsifier in order to develop simple preparation using inexpensive, locally available materials that could be used by farmers (Leatemia and Isman, 2004b). Ten adults of CLB were placed in Petri dishes (9cm diameter) lined with filter paper. The insects were directly sprayed thrice using pre-calibrated hand-held spray bottle (UnivTrigger ®) (1spray = ~ 0.5mL) maintaining a distance of 1 ft. There were three replicates for each treatment with distilled water (DWA) as negative control and Thiametoxam-based insecticide (TBI)(ACTARA 25 WG ®) as positive control. Insect mortality was recorded 24 hours after treatment (Khalequzzaman and Sultana 2006, Leatemia and Isman 2004, Seffrin *et al.*, 2010).

Effect on Parasitoids

The sublethal effect of the crude plant extracts to the CLB parasitoid, *T. brontispae* F. was tested in the laboratory based on the protocol of Kheradmand *et al.* (2012). This was carried out by dipping the previously parasitized pupae or mummies of *B. longissima* to the crude extracts for 5 seconds with 4 replications. The mummies were left to dry for 3 hours, and were placed in test tubes to await parasitoid emergence. The total number of parasitoids that emerged from the treated mummies was recorded. Controls of the experiment used were the same as in CLB Bio-assay.

Phytochemical Test

Presence of organic compounds such as phenols, flavonoids, saponins, tannins and triterpenoids on the crude ethanolic and aqueous extracts of *A.*

squamosa L. and *M. altissima* B. was screened according to Himesh *et al.* (2011).

Spectrophotometric Analysis

To further confirm the presence of possible active compounds that may have caused the observed insecticidal effect, crude extracts were analyzed in the UV- Vis spectrophotometer. Two ml of each sample was subjected for analysis. Distilled water and 95% ethanol were used as controls. The standard range for absorption maximum (λ max) used were 218-220 nm for acetogenins in *A. squamosa* extracts (Yong *et al.,* 2012), 202-320 nm for phenolic compounds (Weerasena *et al.*, 1993; Theerasin and Baker, 2009) and 280 nm for mangiferin an important phenols from Anarcadiaceae family (Luo *et al.*, 2012).

Statistical Analysis

Actual numbers observed in the laboratory bioassays were recorded. These were used to compute the percentage mortality using the equation (Gratil *et al*., 2009): % Mortality= N/N_i X *100*, where *N*= Number of dead test insects per treatment and N_i= Initial number of test insects per treatment. The percentage mortality values were converted to arc sine percentage values employing the following formula (Lana, 2009): x= arc sine √*percentage*. The value of 0% is substituted by *1/4n* and the value of 100% by *100-1/4n*, where n is the number of units upon which the percentage data was based. Data from all the tests were grouped and the means were computed. One way analysis of variance (ANOVA) was done and Tukey's HSD test at 0.05 level was used to determine significant differences between treatment means using R Statistical Language and Environment (R Core Team, 2016). Probit analysis was also conducted to determine50% lethal dose concentration (LC_{50}).

RESULTS

Effect on CLB adults in the laboratory

Fig. 1 shows that 15% MAAE had the highest mortality effect on CLB adults followed by 30% and 7.5% MAAE had the lowest. ASAE at as low as 10% have afforded about 70% CLB mortality

comparable to the highest concentration used for MAAE. In addition, for ASAE unlike in MAAE, CLB percentage mortality was dose-dependent. The addition of an emulsifying agent did not increase the lethal effect of both aqueous extracts to the test insects. It was noted that all concentrations tested for both botanicals were at par with the standard CLB insecticide except each of lowest concentration.

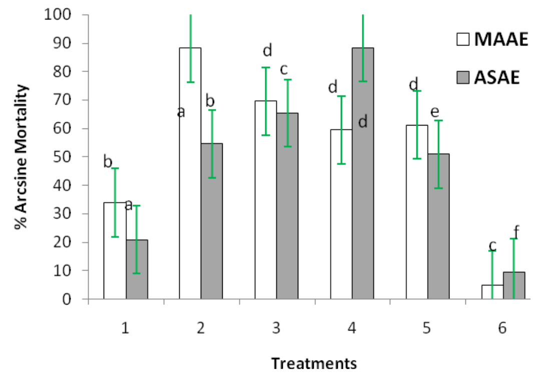

Figure 1. Performance of aqueous seed extracts on CLB under laboratory conditions. Treatments: 1) 7.5 and 5.0%w/v; 2) 10 and 15%w/v; 3) 30 and 15% w/v; 4) 15 and 10 %w/v emulsified with detergent; 5) both Thiametoxam-based insecticide; 6) both distilled water only. Bars with different letter are significantly different at 0.05 significance test by Tukey's HSD.

As shown in Fig. 2, the toxic effect of ethanolic form of the seed extracts was exhibited in a dose-dependent manner. At 5% w/v MAAE had the least mean number of dead test beetles after the experiment while 15% w/v MAEE had the highest. On the other hand, ASEE had the least at 2%w/v and as high as 80% percentage mortality at 8%w/v. The lethal effect of both ethanolic extracts was apparently enhanced by adding an emulsifier at 10% MAAE and 4% ASEE

As presented in Table 1, the least LC_{50} value was recorded for ASEE (0.70) while the highest was on MAAE (10.76). The LC_{50} for both ethanolic seed extracts was least compared to each of the aqueous form. Further as shown in 95% Fiducial limits, the lowest required concentration to achieve 50% CLB mortality in ASEE is 0.20% w/v.

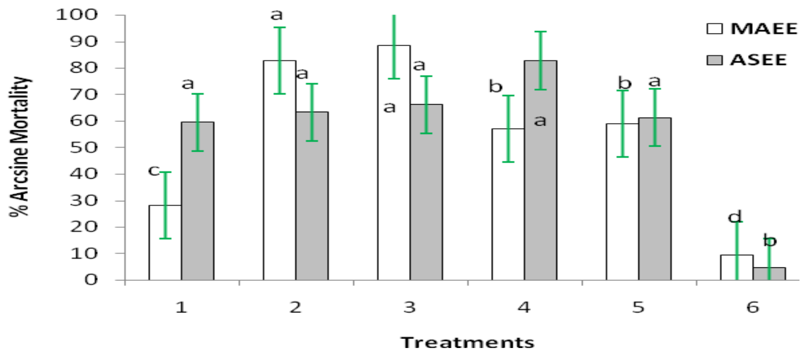

Figure 2. Performance of ethanolic seed extracts on CLB under laboratory conditions. Treatments: 1) 5.0 and 2.0%w/v; 2) 10 and 4 %w/v; 3) 15 and 8 % w/v; 4) 10 and 4 %w/v emulsified with detergent; 5) both Thiametoxam-based insecticide; 6) both distilled water only. Bars with different letter are significantly different at 0.05 significance test by Tukey's HSD.

Table 1. Probit Analysis for the different seed extracts against CLB.

Seed extracts	LC_{50}* (%w/v)	95% Fiducial Limits		Regression equation	$(X^2)^+$
		Lower	Upper		
MAAE	10.76	6.48	17.88	*Y = 2.76 + 2.17X*	0.00
MAEE	6.84	5.44	8.60	*Y =- 0.08 + 6.12X*	0.61
ASAE	9.71	7.15	13.20	*Y = 1.35 + 3.70X*	0.77
ASEE	0.70	0.20	2.49	*Y = 5.14 + 0.89X*	0.95

* Lethal concentration of seed extracts needed to kill50% of CLB after24-hours; +Chi-square value.

Effects to parasitoid emergence

A significant reduction in the number of emerging progenies of the parasitoid, *T. brontispae* from the previously parasitized pupae of *B. longissima* was recorded in all treatments except in ASAE ($F_{5,18}$=3.14; p=0.0327) (Fig. 3). The ethanolic extracts of both plant materials have drastically reduced the number of emerging parasitoids from CLB mummies compared to the control. There was no significant difference with the effects of ASEE, MAAE, and MAEE on parasitoid emergence. However, synthetic-insecticide, Thiametoxam had the least average number of emerged *T. brontispae* adults.

Screening for phytochemicals

The result of phytochemical screening indicated the presence of some compound groups in the crude extracts used in CLB bioassays (Table 2). It revealed that phenols were present in ASEE, MAAE MAEE. Saponins were only detected in ASEE. Tannins were also present in ethanolic extracts but not in the aqueous extracts of both plant materials while flavonoids and triterpenoids were not detected. A range of 236.50 nm-297.91 nm was recorded on the ethanolic extracts of *M. altissima* (Fig. 4a).

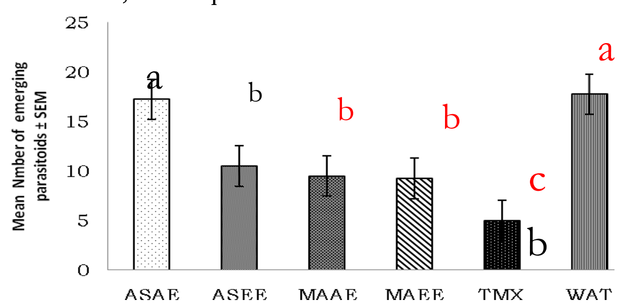

Figure 3. Effect of the best botanical concentrations *T. brontispae* emergencein ambient laboratory conditions. Bars with different letter differ significantly at 5 % significance level by Tukey's-HSD.

Acetogenins was present only in the ethanolic extracts of *A. squamosa* (Fig. 4b). It was indicated by the recorded λ max of 218 nm. This is in agreement with the data from Yong *et al.* (2012) for annosquacins *A*, *B*, and *D*. These values were within the range stated for phenolic compounds in the literature (Weerasena *et al.*, 1993; Theerasin and Baker, 2009). Phenolic derivative such as cathechin was also found inboth *M. altissima* and *A. squamosa* crude ethanolic extracts. The same extract of *M. altissima* contains mangiferin as indicated by the λ max of 280 nm (Luo *et al.*, 2012).

Table 2. Phytochemical screening for *A. squamosa* and *M. altissima* seed lextracts**.**

Chemical group	*A. squamosa*		*M. altissima* B.	
	Aqueous	*Ethanolic*	*Aqueous*	*Ethanolic*
Phenols	--	+	+	+
Flavonoids	--	--	--	--
Saponins	+	+	--	--
Tannins	--	+	--	+
Triterpernoids	--	--	--	--

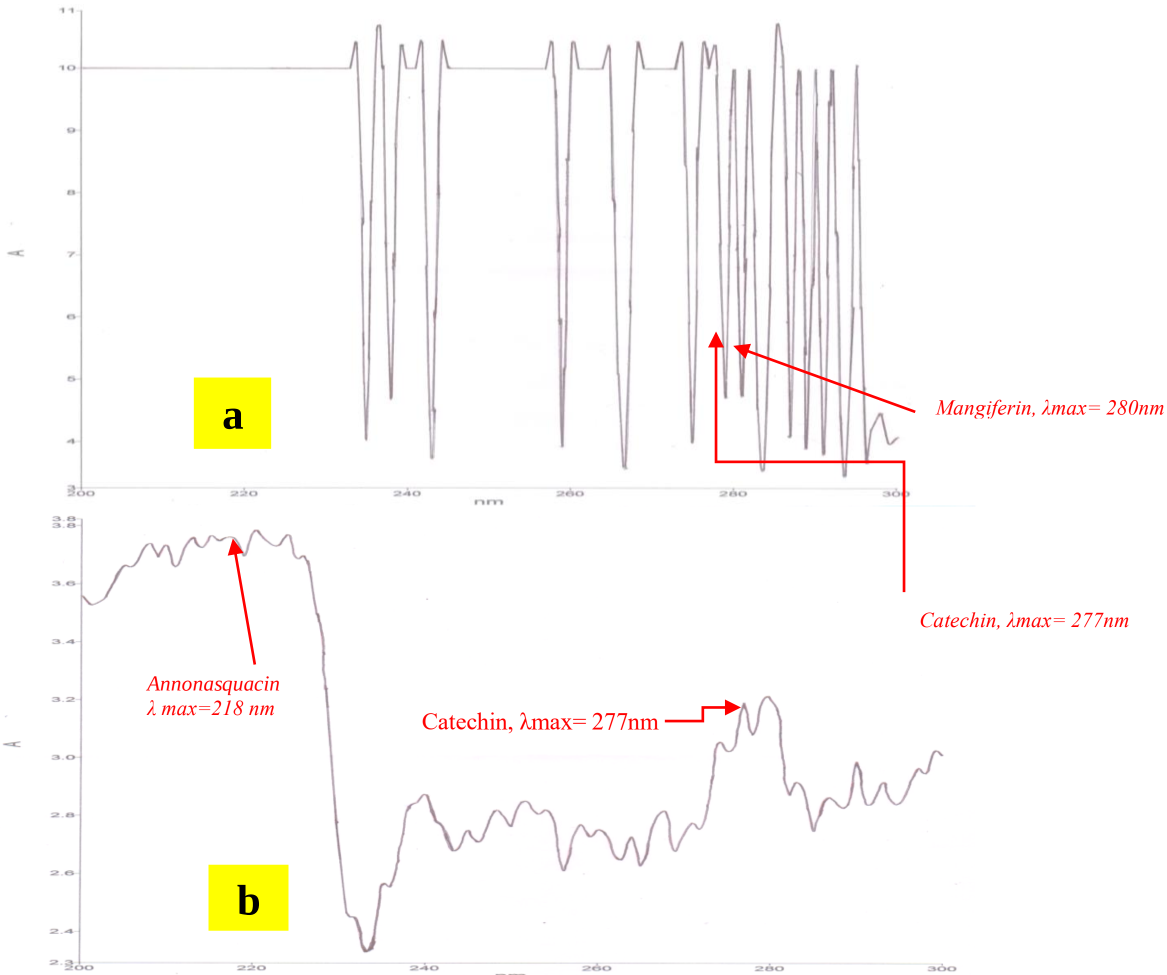

Figure 4. Spectrophotometric profile of the ethanolic crude seed extracts of *Mangifera altissima* B. (a) and *Annona squamosa* L. (b) with peaks of few probable active chemical constituents shown.

DISCUSSION

In the present study, contact toxicity of crude plant extracts to CLB adults was observed in the laboratory. The crude seed extracts of *M. altissima* showed insecticidal effects to adults CLBlwhen applied topically (Figs. 1a and 2a). Phytochemicals were detected from the phytochemical screening and UV-Vis Analysis on *M. altissima* seed extracts which are mostly phenolics (Table 2 and Fig. 4a). Phenolics synthesized primarily from products of the shikimic acid pathway, have several important defensive role in the plants (Mazid *et al.*, 2011). In fact, it is one of the three main chemical classes associated with insecticidal and fungicidal activities (Park, 2000; Boulogne *et al.*, 2012, Kaushik *et al.,* 2019). Tannins were also detected on *M. altissima* extracts from the phytochemical test (Table 2). This chemical group occurs as secondary metabolites in plants and has been known to exhibit insecticidal property due to their ability to form complex proteins (Ibanez *et al.*, 2012). The toxic effect of *M. altissima* extracts to CLB might be due to the presence of mangiferin (Fig 3a). It was earlier reported that mangiferin exhibit many biological activities, such as antifungal and anti-inflammatory (Singh *et al.*, 2009). Mangiferin are ubiquitous in Anarcadiaceae family and in fact extends to other plant families such as *Anemarrhena* (Lao *et al.*, 2012). Researchers have found that botanical formulations of several species from Anarcadiaceae family are potent to wide array of insect pests (Abolghasemi *et al.*, 2018; Tyagi *et al.*, 2019).

Another botanical extracts tested was of *A. squamosa* and were found to be potent also to adults of *B. longissima* (Figs. 1b, 2b). It was observed that the performance of *A. squamosa* seed extracts in high concentrations (%w/v) were comparable to the conventional insecticide for CLB, thiametoxam (Actara 25 WG ®). Similar to Kempraj and Bhat (2014) a dose-dependent contact toxicity of the seed extract of *A. squamosa* was also shown. In the present study, the highest CLB mortality was exhibited at 4% w/v *A. squamosa* ethanolic extracts. This was slightly higher to the findings of Suhasini and Arivudainambi (2020) wherein at 2% w/v *A. muricata* crude extracts was observed to cause significant mortality on rice stem borer, *Scirpophaga incertulas* (Walker). However, it was slightly lower with the results of Kumar *et al.*, (2010) who found that 5% w/v ethanolic extract of *A. squamosa* was effective against *Sitophilus oryzae*. Similar with *M. altissima* the insecticidal efficacy of *A. squamosa* seed extracts was widely investigated (Ravaomanarivo *et al.*, 2014; Kulkarni, 2017). In the current study, the occurrence of acetogenins specifically annonasquacin in *A. squamosa* crude ethanolic seed extracts was confirmed by spectrophotometric analysis (Fig. 4b). Bioactivity of *A. squamosa* crude seed extracts to CLB can be attributed to the presence of bioactive substances, particularly acetogenins (Cheng-Yao *et al.*, 2019). Furthermore, the effects of *A. squamosa* seed extracts were intensified upon emulsification of 0.005% w/v detergent solution and hence potentially it may serve as cheaper insecticides. The same results wereas shown in the previous study of Sathya *et al.* (2019) wherein the extracts was further improved by mixing with the organic mixture called panchagavya which showed promise against *Spodopter litura* Fab.

Aside from the efficacy of the seed extracts against CLB, selectivity to its natural enemies such as its parasitoid, *T. brontispae* was also tested by examining parasitoid emergence. It was shown that crude seed extracts hasve less pronounced effect than the control on the parasitism potential of the CLB parasitoid (Fig. 3). Probably the reason behind is that the residues were less toxic than the control with thiametoxam as active ingredient. Blibech *et al.* (2019) showed that pesticide residues can affect parasitism viability of parasitoids. The side-*effects* of thiametoxam to some beneficial insects usually those utilized as biological control agents have been widely documented (Costa *et al.*, 2014; Beloti *et al.*, 2015; de Paiva *et al.*, 2018; Wang *et al.*, 2019). These results suggest that selected concentrations of *M. altissima* and *A. squamosal* seed extracts used in the study are toxic against CLB but are less potent to its endoparasitoid relative to the control; hence has the potential to be integrated to existing biological control programs. Confirmatory field efficacy test in of these seed extracts against CLB is hereby recommended.

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