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In vitro larvicidal activity of varying concentrations of madre de cacao (*Gliricidiasepium* Jacq.) Concentrated crude ethanolic extract againts larvae of horn fly (*Haematobiairritans* Linn.)

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Abstract

A study on *in vitro* larvicidal activity of different levels of Madre de Cacao (*Gliricidiasepium* Jacq. Steud) concentrated crude ethanolic extract (CCEE) against hornfly larvae (*Haematobiairritans Linn.*) was conducted. The air-dried leaves of *Gliricidiasepium* were infused in a 1:3 ratio (w/v) using ethanol as a solvent and concentrated in a rotary evaporator (60°C). A total of 120 larvae of *Haematobiairritans* were exposed in various concentrations: 200, 400, 800 and 1000 ppm. Based on the result after 5 hours of exposure, CCE *G. sepium* extract at 200 ppm showed less effect with 30% mortality compared to 400 ppm, 800 ppm and 1000 ppm with 70%, 83% and 100% mortality, respectively. Findings also revealed that CCE of *G. sepium* extract at 1000 ppm, 800 ppm, and commercial larvicide were comparable in causing mortality of *H. irritans* larvae from the first hour up to the fifth hours of exposure. However, in the fifth hour, 400 ppm was also found to be effective. This suggests that the higher the concentration of CCE *G. sepium* extract and the longer the time of exposure, the higher is the percentage mortality of the larvae. Thus CCE *G. sepium* extract can be used as an alternative for commercial larvicide.

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Introduction

Horn flies (*Haematobiairritans*Linn.) is an external parasite considered to be one of the economically important pests infesting cattle. Infestation would result in to decrease in the production (Byford *et al.*, 1992) as these flies will feed on the body tissues including the skin, hair and blood of animals (Kaufman *et al.,* 1995). They also serve as vectors of blood parasites (Fitzpatrick and Kaufman, 2011) and once cattle are infected, it may even lead to the death of the animal (Lovaas, 2008).

The adult hornflies spend most of their life on their host and they swarm on the back and shoulders of the cattle (Cumming and Murray, 2006) causing annoyance and blood loss that results in chronic wasting of calf and decline in cow milk production (Loftin and Corder, 2011).

To combat these flies, most cattle owners utilized synthetic insecticides. However, the improper usage of insecticides may cause the development of resistance against the target pests and can even cause serious human health concerns (Mock, 1997). Thus botanical insecticides derived from plant extracts as an alternative source for chemical control of pests are being looked into (Detablan, 2013). In the Philippines, Madre de Cacao (*Gliricidiasepium*) is the most ideal plant to use as this is abundant in all parts of the country. There are many compounds found in *Gliricidiasepium*. These compounds are reported to have potential antidiarrheic, antidysenteric, antimutagenic, antinephritic, antioxidant, antiradicular, antiviral, bactericide, cancer-preventive, hepatoprotective and viracide activities.

There is no literature regarding the larvicidal activity of Madre de Cacao (*Gliridiasepium*) against horn fly in particular, the effective level of *Gliricidiasepium* CCE extract to be administered as well as the duration of effectivity against Horn fly larvae, thus this study was conducted. The results of this study will be used as a basis and as reference for further study of the potential of Madre de Cacao as an alternative to chemical insecticides.

Materials and methods

Collection and culture of Horn fly (Haematobiairritans) Larvae

Adult Horn flies (*H. irritans*) were collected from three (3) infested cattle at CTU-Barili Large Ruminant Project with the use of an entomological net. Fresh cattle feces that served as culture media for the larvae were also collected and placed inside the plastic bucket and covered to avoid contamination. Five hundred (500) grams of the feces was placed into the rearing cage which is made up of the plastic box. Adult horn flies were transferred to the rearing cage with the media and kept at room temperature (27˚C-30˚C) (Lima *et al*, 2014). Female horn flies were allowed to have a pre-oviposition of 3 days for laying their eggs in the media (Fig. 1). After 18 hours, the eggs hatched and formed into larvae which were then used in the assay (Fig. 2) (Kaufman *et al*, 1995).

Collection and extraction of Madre de Cacao (Gliricidiasepium) leaves

Collection: Fresh Madre de Cacao (*Gliricidiasepium*) leaves were collected at Barangay Cagay, Barili, Cebu (Fig. 3). They were washed and grounded into fine particles, weighed and air-dried at room temperature (until the remaining dried matter was 50% (Fig. 4).

Extraction: 500 grams of air-dried Madre de Cacao leaves were infused in 1500 ml ethanol using a ratio of 1:3 (w/v) for 48 hours. After infusion, the preparation was filtered using a muslin cloth and Buckner funnel with filter paper. The final extract was called Crude Ethanolic Extract (CEE). The CEE was poured into the beaker, covered and stored inside the refrigerator before using it in the experiment (Fig. 5). After filtration, 1320 ml CEE was concentrated using rotary evaporator for 2 hours and 30 minutes at a standard temperature of 60 ºC until the volume was reduced to 120 ml and have achieved a syrup-like consistency (Figure 6). This process separates the ethanol from a purely crude extract of *Gliricidiasepium*and the product was so-called Concentrated Crude Ethanolic Extract (CCEE). The CCEE was transferred in a sterile amber bottle and refrigerated before its use in the assay of the larvae.

Preparation of varying concentrations of Madre de Cacao (Gliridiasepium) CCEE and concentration of commercial pesticide

Preparation of the Varying Concentrations of *G. sepium* CCEE: The volume of the *G. sepium* CCEE was measured using tuberculinesyringe. Volumes used were 200 ppm (T1), 400 ppm (T2), 800 ppm (T3), and 1000 ppm (T4), respectively. These were dissolved in quantum sufficient of distilled water to make 1 ml. The concentration of the *Gliricidiasepium*CCEE was derived from Marquez (1998) and Detablan (2013) using the formula:

$$C = \frac{Weight\ of\ the\ Plant}{Final\ Volume\ of\ the\ Concentrated\ Extract} = \frac{500\ grams}{120\ ml} = 4.2\ g/ml$$

Preparation of Neguvon: 5 g of Neguvon® (Trichlorfon) was dissolved in 1 gallon of distilled water to derive the 0.15% concentration of the solution (where 1 g contains 970 mg of Trichlorfon).This preparation was based on the manufacturer's recommended rate. While the concentration was derived from Detablan (2013) using the formula:

$$Concentration(\%)\frac{Weight\ of\ solute}{Volume\ of\ solution} x\ 100$$

Assay of concentrated crude ethanolic extract (CCEE)

Ten larvae were placed on each petri plates lined with filter paper at the bottom. The varying concentrations of *Gliricidiasepium* CCEE and controls were directly administered using a syringe to the larvae on the Petri plates. The mortality of the larvae was recorded every hour for 5 hours after treatment. The absence of movement upon pricking with the needle indicates death.

Experimental design statistical analysis

The experiment was laid out in Complete Randomized Design (CRD). There were seven treatment groups, replicated three times with ten larvae in each replication. $T_{o(+)}$, 0.15% Neguvon® which served as a positive control; $T_{o(-a)}$, with distilled water; $T_{o(-b)}$, with ethanol and T_1-T_4 were treated with 200 ppm, 400 ppm, 800 ppm, and 1000 ppm of CCEE of *Gliricidiasepium*, respectively. The data were analyzed using one-way ANOVA. The comparison of significant differences among treatments was done using Tukey's Honestly Significant Differences (HSD) using the Statistical Package for Social Sciences (SPSS) version 2.0.

G. *Data Gathered*

The Percent Efficacy of *G. sepium* CCEE was computed using Reik and Keith (1957) formula.

$$\%\ Efficacy = \frac{Number\ of\ dead\ larvae\ per\ treatment}{Total\ Number\ of\ Larvae\ Exposed}\ x\ 100$$

Gathered results were classified into <70% efficacy, the plant extract is said to be non-effective; 70-81% efficacy, the plant is said to be effective; and 81-100% efficacy, the plant is said to be highly effective.

$$Mortality\ rate = \frac{T1\ (M1) + T2\ (M2) + T3\ (M3) + T4\ (M4)}{Total\ Number\ of\ Larvae\ Exposed}\ x\ 100$$

$$= \frac{T1\ (9) + T2\ (21) + T3\ (25) + T4(30)}{120} x100$$

$$= \frac{85}{120}\ x100$$

$$Mortality\ rate = 70.83$$

Results and discussion

The effect of the first hour of exposure of the different treatments to the horn fly larvae showed that Treatment 0 or commercial insecticide had the highest larval mortality, followed by T3 and T4. The percentage of mortality was above 81-100% for To (Positive control) and T4; which indicates their high affectivity against the horn fly larvae. Treatment 3 indicates that 800 ppm of the CCE *Gliricidiasepium* extract showed that it was also effective. Analysis of variance showed no significant difference among the treatment means, suggesting that all of the three treatments have the same efficacy. Treatment 1 and T2 showed less effect compared to the negative controls such as water and 10% ethanol. These

treatments showed a highly significant difference from To (Positive control). This means that since the percent mortality for T1 and T2 was below 70% means this suggests that they were not effective. The result indicated that the higher levels of CCE *Gliricidiasepium*extract specifically at 1000 ppm and 800 ppm, were comparable with commercial insecticide in the efficacy against Horn fly larvae.

Table 1. Mean percent mortality of Horn fly larvae exposed after 5 hours with varying concentrations of *G. sepium* CCEE.

Treatments	Number of hours exposure				
	1 hour (%)	2 hours (%)	3 hours (%)	4 hours (%)	5 hours (%)
To$_{+}$ (Neguvon)	86.67^{a}	86.67^{a}	86.67^{a}	100.00^{a}	100.00^{a}
To$_{-a}$ (Water)	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{c}	3.37^{d}
To$_{-b}$ (10% ethanol)	0.00^{b}	10.00^{b}	20.00^{b}	26.67^{b}	30.00^{c}
T1 200 ppm	0.00^{b}	0.00^{b}	3.33^{b}	10.00^{bc}	30.00^{c}
T2 400 ppm	3.33^{b}	6.67^{b}	10.00^{b}	13.33^{bc}	70.00^{b}
T3 800 ppm	76.67^{a}	76.67^{a}	80.00^{a}	80.00^{a}	83.33^{ab}
T4 1000 ppm	83.33^{a}	93.33^{a}	96.67^{a}	100.00^{a}	100.00^{a}

Means having the same letter designation are not significant based on Tukey's HSD.

The effect of exposure of horn fly larvae to the different treatments for two (2) hours is shown in Table 1. Results revealed that T4, followed by To (Positive control) and T3 had the highest percentage of larval mortality. Treatment 4 or the 1000 ppm of CCE *Gliricidiasepium* extract and the use of a commercial insecticide (To) is highly effective in causing mortality to horn fly larvae. Analysis of variance (ANOVA) however showed that there were no significant differences among treatment means, suggesting the comparable effect of higher levels of CCE *Gliricidiasepium* extract with commercial insecticide. Treatment 1 and Treatment 2 showed no significance from the negative controls such as water and 10% ethanol but it showed a highly -significant difference from To (Positive control) suggesting that since T1 and T2 were below 70% mortality, these levels were effective.

The effect of exposing the different treatments to the horn fly larvae for three (3) hours showed the same trend with exposure for one and two hours. In four (4) hours of exposure of the horn fly larvae to the varying concentrations of *G. sepium* CCEE, results revealed that T4 and To (Positive control) caused a 100% mortality, followed by T3 at 80% and this indicated that the three concentrations are highly effective against horn fly larvae. Statistical analysis showed that there were no significant differences among the three treatment means, suggesting a comparable effect. On the other hand, T1 and T2 were highly significant from To (Positive control) and it is comparable to the effect of the negative controls. Since they cause below 70% mortality, this also suggests that these concentrations are not effective in causing mortality to the horn fly larvae after four hours of exposure.

Fig. 1. Eggs of *Haematobiairritans (10x).*

Upon five (5) hours of exposure, the data showed that To and T4 caused 100% mortality to the horn fly larvae. This is followed by T3 at 83.33%. Statistical analysis showed that To (Positive control), T3 and T4

were not significantly different from each other suggesting a high effectivity and a comparable effect against horn fly larvae.

Fig. 2. Larva of *Haematobiairritans (10x).*

The data further showed that T2 and T3 are comparable in their effectivity. The results is in consonance with the standard criteria by Riek and Keith (1957) that volumes greater than 400 ppm were highly effective. The efficacy of treatment 1 is comparable to the effect of negative (-) b control (10% ethanol) but it showed significant to negative (-) a control (water). Mortality of the larvae found in a negative (-)a control (water) after five (5) hours of exposure may be due to starvation. However, these treatments are highly significant from treatment 0 (Positive control) indicating that this concentration is not effective in causing mortality against the horn fly larvae.

Fig. 3. Leaves of Madre de cacao (*Gliricidiasepium*).

The results of exposure of horn fly larvae imply that the longer the time of exposure, the higher is the percentage of mortality. Besides, the higher the concentration of CCE *G. sepium*extracts, the higher is its effectivity in causing mortality of the horn fly larvae. Although profiling of the compounds present in *G. sepium* was not conducted; however several pieces of literature support the results of the study.

Fig. 4. Chopped air-dried leaves of Madre de cacao (*Gliricidiasepium*) leaves.

The efficacy of high concentrations of CCE *G. Sepium* extract at concentration 1,000 ppm and 800 ppm in causing high and even 100 percent mortality to the horn fly larvae are supported by Ciccio and Cahverri (2015) and Sinha (2013) that the insecticidal properties of *G. sepium* are attributed to the presence of volatile compounds such as terpenoids in particular monoterpenoids (Pare and Tumlinson, 1999) which are specifically toxic against pest insect due to their neurotoxic mechanism. Another compound in *G. sepium,* Linalool (Ciccio and Cahverri, 2015) has been demonstrated to act on the nervous system as well. Furthermore, Walitwitiya (2009) reported that α-pinene, eugenol and thymol compound showed high larvicidal activity against multiple larval stages of *Aedes aegypti.*

Also saponin in *G. sepium* also gives rise to increased mortality levels, lowered food intake, weight reduction, and retardation in development in pest insects (DeGeyter*et al.,* 2007). Another compound in *G. sepium* that shows larvicidal activity is the presence of rotenone isoflavonoid which reduces chances survivability of larvae (Sinha, 2013). Thus, the higher the level of concentration of CCE G. sepium extract and the longer that the horn fly larvae

are exposed to the insecticidal properties/ compounds found in the extract, the higher is the larvicidal effectivity.

Fig. 5. Crude ethanolic extract (CEE) of *Gliricidiasepium*.

On the other hand, the active ingredient that is found in commercial insecticide (Neguvon) results in immediate mortality to the horn fly larvae due to the action of trichlorfon that is inhibitors of acetylcholine esterase (AChE), the neurotransmitter responsible for nerve impulses between nerves and their receptors. Inhibition of cholinesterase leads to an accumulation of acetylcholine, causing disruptions in the central nervous system that lead to the death of an insect (Canadian Council of Ministers of the Environment, 2012).

Fig. 6. Concentration of *G. sepium* extract using rotary evaporator (60˚C).

The larvicidal activity of *Gliricidiasepium*CCEE was dependent on the concentration, the percent mortality increases as the *G. sepium* becomes more concentrated. The longer the time of exposure, the higher the mortality of *Haematobiairritans* larvae. The *Gliricidiasepium* CCEE was highly effective at 800 ppm and 1000 ppm, indicating that ethanol extracted effectively the phytochemicals present in *Gliricidiasepium*.

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