

Antihelminthic activity of *Leucaena glauca* (Ipil-ipil) seed and leaf extract in an Ascaridae model

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Abstract - Intestinal parasitism remains to be one of the most common infections among children despite the increasing awareness on concepts of hygiene. Commercial medicines abound in the treatment of different types of parasitic infections, however, there is still a continuous search for alternative herbal medicine since they are safer to use and possess less toxicity. Among different herbal plants, certain studies revealed that *Leucaena* spp. have hypoglycemic, anti-diabetic, antimicrobial and antihelminthic properties. This study was carried out to evaluate the antihelminthic activity of *Leucaena glauca* (Ipil-ipil) seed and leaf extract in an Ascaridae model. Aqueous seed and leaf extracts of *L. glauca* was tested against *Ascaris suum* in vitro. Eggs and adult worms were exposed in 5 increasing concentrations of Ipil-ipil seed and leaf extracts. Results of this study revealed an ED50 of 105 mg/ml leaf extract concentration and 47 mg/ml seed extract concentration in egg hatch test. While an ED50 of 102 mg/ml concentration of leaf extract and 96 mg/ml concentration of seed extract in adult motility assay was also obtained. Mann-Whitney U test showed a significant difference (sig. value of 0.037) in the distribution of unfertilized eggs of seed and leaf extracts. It also revealed the similar distribution (sig. value of 0.114) of immotile worms in adult motility test on both extracts. Furthermore, this study presented a dose-dependent antihelminthic activity of Ipil-ipil which provides a new and potential cure against intestinal parasitism.

Keywords - parasitism, *Leucaena* spp., nematode, antimicrobial, Ascaridae

INTRODUCTION

Parasitism is a relationship among two organisms in which one organism, the parasite, is dependent on the other, the host. It oftentimes involves a highly specific relationship which is associated with metabolic dependence of the parasite to its host. Infections caused by parasites usually happen for a long time and may result to death of its host (Gunn and Pitt, 2012).

In the Philippines, according to the Department of Health (DOH, 2011), parasitism persists because of poverty, poor sanitation and hygiene practices especially in areas where there is low economic and human development scale (Agbakoba, 2009). There are three major causes of intestinal parasitism in the Philippines namely: *Ascaris lumbricoides*, *Trichiuris trichiura* and Hookworm.

These worms are collectively known as soil-transmitted helminths (Balolong, 2011).

A. lumbricoides also known as roundworm, is one of the six worms classified by Linnaeus (Ridley, 2012) that is most prevalent parasitic helminths among humans (Araujo, 2006) that might cause serious adverse effects like malnutrition particularly in children (Huang, 2008).

A variety of drugs, such as Albendazole and Mebendazole, are already available against these parasites. However, medicinal plants that were used as raw materials in producing new drugs, like *Atropa belladonna* and *Eucalyptus globules*, are constantly increasing since these plants have innate active components which are used to treat diseases (Okigbo et al., 2008). Such properties are based on the antimicrobial, antioxidant and antipyretic effects of chemicals within the plant (Adesokan et al., 2008). According to Soetan and Aiyelaagbe (2009), research about these plants is considered as one of the leading fields of research worldwide.

Among the different studied plants is *Leucaena glauca* (Ipil- Ipil), commonly known as *Leucaena leucocephala*. It is a herbal plant under the family of Mimosaceae, comprising the tropical and subtropical trees and shrubs and is usually seen in the plains of India (Khare, 2007). It is an essential foliage crop particularly on resource-limited farms in tropical countries (Paengkoum and Traiyakun, 2011). This plant also played a significant role in fodder supply, soil fertility improvement and fuel wood production in many parts of Africa (Dzowela and Otsyina, 2008). Its leaves revealed that it can also be an alternative source of protein in swamp buffaloes (Cherdthong et al., 2011), Thai Brahman cattle (Jetana et al., 2011), goats (Paengkoum, 2010) and laying hens (Atawodi et al., 2008).

Leucaena spp. is rich with crude protein (25-35%) and some other alimentary contents (Ghosh and Bandyopadhyay, 2007). Its seeds and leaves contain 32.16% and 28.75% crude protein, 39.53% and 36.22% carbohydrates, and 55.76% and 51.024% organic carbon respectively (Aijaz et al.).

Though these plants have rich in chemical contents, it also contains toxic substances like mimosine and tannin. The latter reduces digestibility of proteins which results in marked low metabolizable energy (ME) value of *Leucaena* leaf meal in poultry while mimosine and its metabolites are the main obstruction which blocks the usage of the plant as animal feed (Bandyopadhyay, 2007).

Mimosine provides 14.8% to overall nitrogen content of *Leucaena* seeds. Next only to immature tender leaves, the seeds have higher concentration compared to other parts of the Ipil-Ipil (Chanchay and Poosaran, 2009). The bark and leaves also contain 16.3% and 3% tannin respectively. Furthermore, the leaves also include quercitrin (0.08%). Beta and alpha-aminopropionic acid is

reported to be identical with mimosine (from *Mimosa pudica*). Stachyose is also reported to be present in the seeds (Khare, 2007).

A study on the antihelminthic activity of *Leuceana leucocephala* seed (Ademola, Akanbi and Idowu, 2005) and leaf (Adama et al., 2012) extract against *Haemonchus contortus* ova and larvae has been the major interest of researchers. According to Adama et al. (2012), *Leuceana leucocephala* extract inhibited the egg hatching and larvae development of *H. contortus* although the inhibition efficiency is only more evident on eggs than on larvae.

Given these studies, the researchers aimed to further determine the nematocidal activity of *Leucaena glauca* using an *Ascaris*-swine model (Hall et al., 2011). *Ascaris suum*, the common roundworm of pigs and its life cycle is similar to that of *Ascaris lumbricoides* (Araujo, 2006). Its genome is rich in peptidase linked to the penetration and degradation of host tissues. Therefore, this genome provides a comprehensive resource to the scientific community and underpins the development of new interventions such as drugs, vaccines and diagnostic tests against ascariasis and other nematodiasis (Hall et al., 2011).

MATERIALS AND METHODS

Plant Materials

Leaves and seeds of Ipil-ipil were collected from Agoncillo, Batangas. Voucher specimens of the plant were deposited to the Herbarium of the University of Philippines for authentication.

Parasites

Mature worms of *Ascaris suum* were collected freshly from slaughtered pigs in the abattoirs in Batangas City. The female parasites were selected and dissected in a mortar and pestle to liberate the eggs. The eggs were washed with distilled water and were collected using a 20 µm sieve (Adama et al., 2012).

Preparation of Baldwin and Moyle Solution (Austria and Villapando, 2006)

One hundred fifty one (151) grams of sodium chloride (NaCl), 24.8 grams potassium chloride (KCl), 13.4 grams calcium chloride (CaCl₂), and 20.4 grams of magnesium chloride (MgCl₂) were dissolved in two liters of distilled water. Another 27.2 grams of potassium phosphoric acid (KH₂PO₄) was also dissolved in one liter of distilled water. One volume of KH₂PO₄ and four volumes of NaCl-KCl-CaCl₂-MgCl₂ were mixed and diluted with 35 volumes of distilled water. The pH was adjusted to 7.2 with 1M sodium bicarbonate.

Preparation of Extracts

Aqueous extracts were prepared using hot water extraction method (Chauhan et al., 2008). The 2.5 g of each dry seed and leaf powder were boiled in 100 ml of distilled water with constant stirring for 30 minutes. The solution was allowed to cool down and was filtered through muslin gauze. Filtrate was centrifuged at 3500-5000 rpm for 15 minutes. The supernatant was filtered again using filter paper and stored at 4°C for future use. The procedure was repeated using 5, 10, 15, and 20 grams of dry seed and leaf powder in 100 ml of distilled water to yield the concentrations 25mg/ml, 50mg/ml, 100mg/ml, 150mg/ml, and 200mg/ml respectively.

Egg Hatch Test (EHT)

The EHT was performed using Coles et al. (1992). The 100 µl of fresh macerated eggs was placed in Eppendorf tubes. The tubes were then submitted to different treatments consisting of 5 extract concentrations (25mg/ml, 50mg/ml, 100mg/ml, 150mg/ml, and 200mg/ml of each extract plant). Distilled water was used as a negative control. Three replicates for each concentration of extract and control were performed. These tubes were incubated under humidified condition at ambient temperature (27°C) for 48 hours. Afterhand, three drops of Lugol's iodine solution were added to each well to stop further hatching. All the unhatched eggs (percentage of inhibition) and those in the first stage larvae in each well were counted under a brightfield microscope (Olympus Model BX43). A coulter counter was used to ensure that 100 eggs were counted.

Adult Motility Assay

Ten live worms in each disposable container were suspended in Baldwin and Moyle solution (BaMS). These worms were exposed to different treatments composed of 5 extract concentrations (25mg/ml, 50mg/ml, 100mg/ml, 150mg/ml, and 200mg/ml of each extract plant) in separate containers, in triplicates, at room temperature (25-30°C). The negative control container was added with excess BaMS in order to possess uniform volume (Iqbal et al., 2011).

The worms were observed after 24 hours. Finally, the treated and negative control worms were exposed to heated inoculating loops to fully validate for motility. Immotile worms were assessed as dead and the number of live and dead worms in each container was counted and recorded (Derrain et al., 2012).

Statistical Analysis

Data from EHT and adult motility assay were computed using one-way analysis of variance (ANOVA). The means of extract concentrations were submitted to the non-parametric test of Mann-Whitney U and Kruskal-Wallis. All analyses were made with the Statistical Package for Social Sciences (SPSS) version 18 for Windows at a significance level of 5%. For EHT and Adult motility assay, effective dose (ED50) and Lethal Concentration (LC50) was calculated as the concentration of extracts producing 50% effect and inhibition of eggs

hatching and motility by probit-analysis. ED50 and LC50 were known to have the same value since the tests used the number of incubation time.

RESULTS AND DISCUSSION

Figure 1 presents the average number of immotile worms out of 10 live worms using different Ipil-ipil leaf and seed extract concentrations. For the leaf extract, the average numbers of immotile worms were 0.67, 2.67, 3.67, 5.67 and 8.33 in 25mg/ml, 50mg/ml, 100mg/ml, 150mg/ml and 200mg/ml leaf concentration respectively. On the other hand, the seed extract showed the average numbers of 1, 2.33, 5.33, 6.33 and 8.67 in 25mg/ml, 50mg/ml, 100mg/ml, 150mg/ml and 200mg/ml leaf concentration respectively. These results denote that the inhibition of Ipil-ipil leaf extract to the motility of adult worms follow a concentration-dependent manner. This is similar to the study of Iqbal et al. (2011) which presents the dose dependent antihelminthic effect of the herbal formulation of *T. ammi* seeds, flowers of *C. procera*, and leaves of *A. indica* and *N. tabacum*.

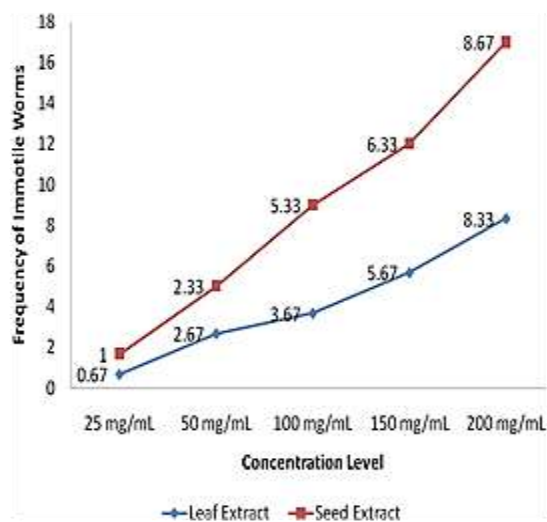


Figure 1
Frequency distribution of immotile worms in relationship with extract concentrations

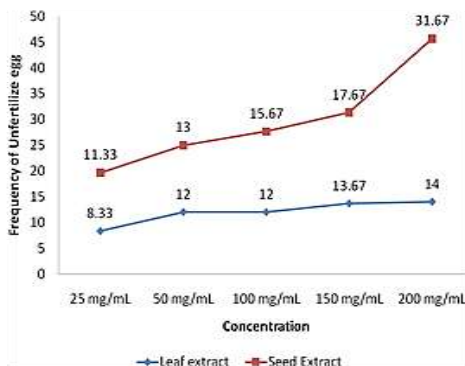


Figure 2
Frequency distribution of unhatched eggs in relationship with extract concentration

Figure 2 shows the frequency of EHT using different Ipil-ipil leaf and seed extract concentrations. This test indicates the average number of unfertilized egg out of 100 eggs counted in every extract concentration. For the leaf extract, the average numbers of unhatched eggs were 8.33, 12, 12, 13.67, and 14 in 25mg/ml, 50mg/ml, 100mg/ml, 150mg/ml and 200mg/ml leaf concentration respectively. While the seed extract concentration of 25mg/ml, 50mg/ml, 100mg/ml, 150mg/ml and 200mg/ml showed 11.33, 13, 15.67, 17.67, and 31.67 average numbers of unfertilized eggs respectively. These results revealed that the number of unfertilized eggs increases in a minimal amount as the concentration increases. This is related to the study of Adama et al. (2012) that shows inhibitory effects of *L. leucocephala* and *Gliricidia sepcium* leaf extract on *Haemonchus contortus* ova. This is also similar to the study of Ademola, Akanbi and Idowu (2005) which shows the nematocidal activity of *L. leucocephala* seed extract against sheep nematodes in a dose-dependent manner by chromatographic fraction.

Table 1
Kruskal Wallis Test

Null Hypothesis	Sig. Value	Decision
The distribution of unfertilized eggs is the same across categories of concentrations	0.285	Retain null hypothesis
The distribution of dead worms is the same across categories of concentrations	0.071	Retain null hypothesis

Table 1 illustrates the significant difference (P-value <0.05) between distribution of unhatched eggs and immotile worms and the different

concentration used. Kruskal Wallis test revealed the statistically insignificant values of 0.285 and 0.071 in EHT and adult motility assay respectively. Both of these results showed that the distribution of unfertilized eggs and immotile worms do not vary in any category of Ipil-ipil concentration. This is contradictory to the study of Peter and Deogracious (2006) which showed that the ascaridal effect of *T. riparia*, *C. papaya*, *C. occidentalis* and *M. foetida* leaf extracts increases with the increasing concentration and incubation time.

Mann-Whitney U test presented the significant values of 0.037 and 0.114 in EHT and adult motility assay respectively. These results indicated that the statistically significant difference between the seed and leaf extract was only observed on egg hatch test. This is parallel to the calculated ED50 (Table 3) of EHT which are 105 mg/ml and 47 mg/ml concentration of leaf and seed extract respectively. While the ED50 (Table 3) of 102 and 96mg/ml were observed in the adult motility assay. These concentrations would entice 50% of eggs not to hatch and 50% of the adult worms to be immotile. This also present that the seed extract is better in EHT (ED50= 47mg/ml) since it could already inhibit 50% of egg hatching in a minimal amount of concentration as compared to the leaf extract (ED50= 105mg/ml). These results correspond to the study of Ademola, Akanbi and Idowu (2005) which observed the rich fractions of alkaloids on *Leucaena* seeds. This also supports the study of Soetan et al. (2011) which showed that the *Parkia biglobosa* seed extract have more antihelminthic potential on nematode eggs than the leaf extract.

Table 2
Mann-Whitney U test

Null Hypothesis	Sig. Value	Decision
The distribution of unfertilized eggs is the same across categories of extract	0.037	Retain null hypothesis
The distribution of dead worms is the same across categories of extract	0.114	Retain null hypothesis

Table 2 presents the significant difference (P-value <0.05) between the distribution of unhatched eggs and immotile worms and the kind of extract used (seed or leaf). The effective and lethal dose is presented in Table 3.

Table 3
Effective Dose 50 and Lethal Dose 50

	ED50/LC50	
	Leaf extract	Seed extract
EHT	105 mg/mL	47 mg/mL
Adult Motility Assay	102 mg/mL	96 mg/mL

CONCLUSION

The results of this study showed the anthelmintic activity of Ipil-ipil seed and leaf extracts against *Ascaris suum*. These are quite similar to the reported ovicidal and larvicidal effect of *Leucaena leucocephala* seed extract against *Haemonchus contortus* ova (Adama et al., 2012) as well as the effect of leaf extract against gastrointestinal sheep nematodes (Ademola, Akanbi and Idowu, 2005) which presents the increase number of efficacy as the concentration increases.

Anthelmintic activity of Ipil-ipil is bound to the presence of alkaloids, flavanoids and tannin (Ademola, Akanbi and Idowu, 2005). However, this study showed that there is no significant difference (sig. value of 0.114) between the seed and leaf extract on adult motility test. However, there is a significant difference (sig. value of 0.037) between the seed and leaf extract concentration on the egg hatch test. According to Adama et al. (2012), these findings may be due to the active substances present in the extracts that would cross more easily in the shell of eggs than the cuticles of larva. It was also found out that the seeds are more ovicidal than the leaf extract, as assessed in the ED50 of 47 mg/ml. This result was probably because Ipil-ipil seeds have higher concentration of mimosine, an active alkaloid but toxic component which results to impaired growth and alopecia on ruminants (Bandyopadhyay and Ghosh, 2007), compared to other parts of the Ipil-ipil (Chanchay and Poosaran, 2009). According to Ademola, Akanbi and Idowu (2005), the most active fraction of the *Leucaena* seed are polyphenols, namely flavonoids and tannin, which in the absence of alkaloids, means that the most potent anthelmintic principles of the seed can be obtained without the risk of mimosine toxicity.

The parasite used in the study was chosen due to its considerable similarity in protein profile and morphology with *A. suum* (Alba et al., 2009). Based from the findings of the present study, it is safe to assumed that Ipil-ipil seed and leaf extract may also have probable effects on the most common human infecting nematode, *A. lumbricoides*. The researchers therefore conclude that Ipil-ipil seed and leaf extract can be used as an anthelmintic agent in regulated concentration due to the acceptable results on the selected parasite.

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