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In vitro anthelminthic activity of aqueous leaf extracts from *Leucaena leucocephala* and *Moringa oleifera* against *Caenorhabditis elegans*

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Abstract
Parasitic infections cause a tremendous burden of disease worldwide and over the years, some helminth species have developed a resistance to the current anthelmintics available in the market. Herbal medicine can therefore be alternative sources of anthelmintics possibly reversing the acquired resistance against some of the conventional drugs. This study aims to determine the anthelmintic properties of aqueous extracts of *Leucaena leucocephala* and *Moringa oleifera* leaves, individually and combined, on helminth model *Caenorhabditis elegans*. Thirty adult *C. elegans* were incubated in the presence of various concentrations (3 mg/mL, 6 mg/mL and 10 mg/mL) of each plant leaf extract, and the combined plant leaf extracts (5 mg/mL), with M9 buffer as negative control, and ivermectin as positive control. All adult worms were counted and assessed for their viability at 20 and 40 hours post-treatment. The aqueous extracts of both plants inhibited the survival of the adult worms in a concentration-dependent manner. Greater efficacy was observed at 10 mg/mL, with mortality rates of 97% for *M. oleifera* and 100% for *L. leucocephala* both at 40 h post-treatment. When combined, the mixed extracts induced adult *C. elegans* mortality with 70% and 94% mortality at 20 and 40 h post-treatment, respectively. This study revealed that both aqueous leaf extracts from *M. oleifera* and *L. leucocephala*exhibited strong *in vitro* anthelmintic activity against the model organism *C. elegans*, which could be potential sources of lead molecules against helminthic infections.

Keywords: herbal medicine, anthelmintic activity, *Leucaena leucocephala*, *Moringa oleifera*, *Caenorhabditis elegans*

1. Introduction
Parasitic infections cause a tremendous burden of disease in both the tropics and subtropics as well as in more temperate climates [1]. Parasitic helminths of humans and other animals pose a major socio-economic importance globally. Parasitic nematodes have a massive, long-term impact on human health, which may range from nontoxic to fatal, and cause substantial suffering, particularly in children. Neglected tropical diseases (NTDs) are diverse communicable diseases that are highly prevalent among populations living in these countries. Populations living in poverty that lack adequate sanitation and in close contact with various infectious vectors are greatly affected. The World Health Organization (WHO) estimates that approximately 1.5 billion people were infected with intestinal worms in 2018, predominantly in disadvantaged communities [2].

For most parasitic infections, control measures involve effective chemotherapy. However, treatment failures have been observed throughout the years due to the intensive use of anthelmintics [3]. Cases of possible drug resistance have been recorded for *Fasciola* sp. with triclabendazole in livestock animals [4] and with benzimidazole and ivermectin for lymphatic filariasis [5]. This remains to be one of the greatest challenges in the control of parasitic diseases. Thus, discovery of possible anthelmintic drugs is very important in assuring the availability of a next line of treatment against these parasites. Plants and natural sources form the foundations of modern medicine with major contributions to the array of available commercial drugs today. Twenty five percent of drugs prescribed worldwide are plant derivatives and about 60% of the world population uses traditional herbal medicine [6]. Therefore, natural sources are useful for the discovery of new lead molecules against parasitic helminths.

The Philippines is a conglomerate of natural resources with many plant species that have been used for medicinal purposes. It is part of the culture of the Filipinos to use almost any plant or herb available as drugs to help alleviate certain illnesses and infections.

Despite this demand for the discovery and development of new alternative anthelminthics, there are still a limited number of studies investigating natural products for their anthelminthic properties. Plants such as *Leucaena leucocephala* and *Moringa oleifera*, locally known as ipil-ipil and malunggay, respectively, have shown anthelmintic properties in previous studies [7-12].

L. leucocephala plant was reported to have worm repellent properties [13]. Traditionally, raw or roasted seeds of the plants are taken as dewormer and even the whole fruit is eaten raw as anthelmintic [7]. A study has shown the detrimental effects of protein extracts from the seeds, shell and cotyledon of *L. leucocephala* on *Haemonchus contortus* eggs [8]. The chloroform-soluble alkaloidal extract from the seeds of this plant has also shown *in vitro* anthelmintic activity against *Ascaris suum* comparable with that of mebendazole at 5mg/mL [9]. A randomized controlled trial to test the efficacy of *L. leucocephala* seeds showed egg reduction of 54% in *Ascaris lumbricoides* and 55% in *Trichuris trichiura* [10].

Considered as one of the most important plants in the Philippines due to its involvement in food preparation and traditional medicine, *M. oleifera* has been known to possess compounds with pharmacological and medicinal values [14-15]. Extracts from the seeds of *M. oleifera* have shown to inhibit larva formation inside the eggs of the ruminant parasite *H. contortus* and render its L3 larvae immobile [11]. *In vitro* ovicidal effect of *M. oleifera* methanolic seed extract has been demonstrated in *Fasciola hepatica* eggs [12] which might be attributed to either the tannins [11]or with the phytochemical benzyl isothiocyanate through suppression of motor systems and metabolic processes of the parasites [16].

Nevertheless, studies on the anthelminthic activity of the leaves of either plant remain to be very few. The leaves of *L. leucocephala* when juiced and mixed with hot water were used traditionally as treatment concoction for diarrhea and colds [7]. On the other hand, ethanolic leaf extract of *M. oleifera* was shown to inhibit the egg embryonation of *H. contortus* [17]. In this study, individual and combined aqueous extracts of *L. leucocephala* and *M. oleifera* were evaluated for their anthelmintic effect on the free-living nematode *Caenorhabditis elegans*, a very effective and cost-efficient model system in the study of anthelmintic activity [18].

2. Materials and Methods

2.1 Preparation of aqueous extracts of *M. oleifera* and *L. leucocephala* leaves

2.1.1 Plant sampling and identification

Ten kilograms of intact and mature leaves of *L. leucocephala* (Figure 1A) and *M. oleifera* (Figure 1B) were collected from Barangay Pias, General Tinio, Nueva Ecija, the Philippines. The identities of the plant samples were authenticated at Jose Vera Santos Memorial Herbarium, Institute of Biology, College of Science, University of the Philippines Diliman, Quezon City, the Philippines.

Fig 1A: Leaves of *L. leucocephala*

Fig 1B: Leaves of *M. oleifera*

2.1.2 Sample preparation

The leaves of *L. leucocephala* and *M. oleifera* were wiped and thoroughly rinsed with free-flowing clean water and distilled water to eliminate dirt or other contaminants. The cleansed leaves were air-dried for one week and then oven-dried. Presence of molds or deterioration was assessed on the dried leaves before garbling and grinding.

2.1.3 Aqueous extraction

One hundred grams each of powdered *L. leucocephala* and *M. oleifera* leaves were soaked in 1 L of boiled hot water. The resulting mixtures were boiled for more than 30 minutes, filtered using a cheese cloth, transferred into a reagent bottle, and stored at -30ºC for 24-72 hours. Both leaf extracts were freeze-dried using a lyophilizer at the Department of Chemistry, De La Salle University, Manila, the Philippines.

2.2 Maintenance and synchronization of *C. elegans* cultures

2.2.1 Test organism and culture conditions

Cultures of N2 strain of wild-type *C. elegans* (*Caenorhabditis* Genetics Center, University of Minnesota, USA) were maintained at a temperature range of 25 °C ± 2 °C with the Nematode Growth Medium (NGM) plates being refreshed every two days. Microscopic examination was done to monitor the status of the worm. Maintenance of *C. elegans* was performed based on the previously published protocol [19].

2.2.2 Age synchronization of *C. elegans*

Twenty-five gravid adults of *C. elegans* were transferred to each seeded NGM using a micropipette and allowed to lay eggs for the next two hours. After two hours, all adult nematodes were eliminated by washing with a 20% alkaline hypochlorite solution. The hatched eggs were allowed to mature into adult forms after incubating at 25 °C± 2 °C for 3 days.

2.3 Evaluation of the effects of *L. leucocephala* and *M. oleifera* on the viability of *C. elegans*

2.3.1 Mortality assay of adult *C. elegans*

Three concentrations of each plant leaf extract and one concentration of their combination were prepared for the assay (3 mg/mL, 6 mg/mL, 10 mg/mL) based on the previously known median lethal doses for each plant (LD_{50}); 5 ug/mg for *L. leucocephala* [20] and 6 g/kg for *M. oleifera* [21]. Negative control containing the M9 buffer, and positive control containing ivermectin with LD_{50} of 29.5 mg/kg [22] were also prepared. The test was performed using 35 mm x 10 mm petri dishes. In each petri dish, 15 to 50 adult nematodes were brought in contact with the extracts in each concentration to a final volume of 3 mL per petri dish. Each test concentration was performed in triplicates. The plates

were incubated at 20 °C and observations were done after 20 and 40 hours. After incubation, the petri dishes were examined microscopically and all adult worms were counted and determined as either dead or alive through observation and response to touch stimulus. Worms were stroked in a span of 10 seconds using a minuten pin to assess worm survival. They were considered as dead when no movement was observed and as alive when there were at least some tail, head or body movements in response to touch stimulus.

2.3.2 Statistical analysis
Survival rate was calculated using the ratio of the live worms to the total worms used in each test. Data were expressed as mean of replicates ± standard error of the mean (SEM) of different treatments at different concentrations. Significant differences between control and treatment groups used in the assay were determined by one-way analysis of variance (ANOVA) using STATA software. Tukey's Honest Significant Difference Multiple Comparison Test was performed to determine which pairs have statistically significant differences in means. Results were deemed statistically significant if p value < 0.05 at 95% confidence intervals.

3. Results
3.1 Mortality assay
Table 1 shows the efficacy of the leaf extracts on adult *C. elegans* mortality as compared to ivermectin and M9 buffer. Generally, the aqueous extracts of both plants inhibited the survival of the adult worms in a concentration-dependent manner. No worms died after adding the extracts to the test plates. Significant decrease in survival was seen at 20 hour (h) post-treatment for 3 mg/mL, 6 mg/mL, and 10 mg/mL concentrations of the *L. leucocephala* aqueous leaf extract wherein only 32%, 23%, and 13% of the worms, respectively, were found to be alive. On the other hand, all the worms at 10 mg/mL were dead at 40 h post-treatment. Lower mortality rates were observed in the *M. oleifera* aqueous leaf extracts treatment groups with 58%, 74% and 83% in 3 mg/mL, 6 mg/mL, and 10 mg/mL concentrations, respectively at 20 h post treatment. The highest concentration of *M. oleifera* extract induced 97% mortality rate among the worms at 40 h post treatment. Furthermore, the combined leaf extracts yielded mortality rate lower than that of the individual extract at the highest concentration with 70% and 94% mortality at 20 and 40 h post-treatment, respectively. All the worms survived in the negative control and died in the positive control containing ivermectin.

3.2 Statistical analysis
The mean number of dead *C. elegans* ± standard error of the mean for different treatments at different concentrations for both 20 and 40 h post-treatment were shown in Table 1. Values for mean mortality percentage were found to be significantly different across treatments on one-way ANOVA. Post-hoc pairwise comparisons of means with equal variances using Tukey's HSD test indicated that as compared with the negative control group, there was a statistically significant result on the mortality of *C. elegans* in the ivermectin control group and in various concentrations of both *L. leucocephala* and *M. oleifera* aqueous leaf extracts. This clearly indicates that the death of worms did not happen by chance.

Table 1: Mortality assay results of *C. elegans* in different treatment groups of *L. leucocephala* and *M. oleifera* aqueous leaf extracts

Treatment groups N = 30	20 h Post-treatment		40 h Post-treatment	
	No. of dead individuals*	Mortality rate (%)	No. of dead individuals*	Mortality rate (%)
Negative Control (M9)	0	0	0	0
Positive Control (Ivermectin)	30	100%	30	100%
Leucaena leucocephala				
3 mg/mL	20 ± 1.202	68%	22	73%
6 mg/mL	23 ± 0.577	77%	25 ± 1.667	84%
10 mg/mL	26± 1.732	87%	30	100%
Moringa oleifera				
3 mg/mL	17 ± 1.202	58%	24 ± 0.882	79%
6 mg/mL	22 ± 1.202	74%	26 ± 0.667	88%
10 mg/mL	25 ± 0.577	83%	29 ± 1.000	97%
Combined *L. leucocephala* and *M. oleifera*				
5 mg/mL each	21 ± 2.517	70%	28 ± 1.667	94%

Results were expressed as mean +/- SEM, p value < 0.05, as compared to the positive control

4. Discussion
Currently, there are very few drugs available for treating helminthic infections. Development of new anthelminthic drugs is financially unappealing to private pharmaceutical sector as most parasitic diseases are seen in low- to middle-income countries. For this reason, the use of natural sources, such as plants, opens opportunities for the discovery and development of new forms of treatments against neglected parasitic diseases among humans [23]. In this study, the anthelminthic potential of the aqueous leaf extracts of two locally endemic plants in the Philippines namely *L. leucocephala* and *M. oleifera* was evaluated using the helminth model *C. elegans*.

In general, responses to leaf extracts of *L. leucocephala*, and *M. oleifera* both individually, and in combination were concentration-dependent and were significantly different from the negative control. This study revealed that aqueous extracts of *L. leucocephala* and *M. oleifera* leaves exhibited strong *in vitro* anthelmintic activity against the model organism *C. elegans*, and adversely affected the survival of adult worms. Given that the aqueous extracts of both plants were known to be effective, greater efficacy was observed at 10 mg/mL, with mortality rates of 97% for *M. oleifera* and 100% for *L. leucocephala* both at 40 h post-treatment. Based on these results, a longer duration for its bioactivity might be suggested for both the leaf extracts.

It is recommended that identification of the bioactive compounds responsible for the anthelminthic activities of these plants be done in future studies. This will aid in isolating these compounds and developing new anthelminthic drugs, as well as eliminating substances found in the leaves of these plants toxic to humans such as the alkaloid mimosine in

L. leucocephala. The toxicity of mimosine is a problem found in feeding of *L. leucocephala* in ruminant animals. The bacteria in the rumen of these animals tend to convert the mimosine into 3-hydroxy-4-(1H)-pyridone [24] which causes low weight gain, neonatal death, enlargement of pituitary gland, low conception rate, alopecia, thyroid gland hypertrophy and other associated breeding problems in animals like cattle [25-26]. Identification of the bioactive compounds is also crucial in determining the mode of action responsible for their anthelminthic properties.

As this is a pilot study on the effects of these leaf extracts on *C. elegans*, further researches on locomotion, thigmotaxis, and nociception should be done to determine their effects on the motor and somatosensory behaviours of the worm. *In vivo* and toxicity studies should likewise be performed so as to further evaluate the anthelminthic potentials of *L. leucocephala* and *M. oleifera* leaves.

5. Conclusion

This study concludes that aqueous extracts of *L. leucocephala* and *M. oleifera* leaves have shown strong *in vitro* anthelmintic activity against the model organism *C. elegans*. However, additional evaluation is required such as phytochemical analysis and identification of the active anthelmintic compound.

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