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ORIGINAL ARTICLE

Anthelmintic effect of betel nut (*Areca catechu*) and neem (*Azadirachta indica*) extract against liver fluke (*Fasciola* spp.)

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ABSTRACT

Objective: This study aimed to measure the anthelmintic effects of betel nut (*Areca catechu*) and neem (*Azadirachta indica*) leaf extracts against *Fasciola* spp. *in vitro* in comparison with the commercial dewormer, Albendazole, and the negative control, nutrient broth. The study determined the extract concentration that produced the highest efficacy based on the average recorded mean motility time, gross, and microscopic changes of the flukes treated with different concentrations of plant extracts.

Material and Methods: The study consisted of eight treatments. Every treatment consisted of 10%, 20%, and 40% concentrations of both betel nut extract (BNE) and neem leaf extracts, positive control treatment (Albendazole-treated) and negative control treatment (25 ml nutrient broth). The motility of the flukes on all treatments was based on the established motility criteria scoring. The flukes subjected to all treatments were processed for histopathological analysis.

Results: The result of the study revealed that after exposure of *Fasciola* spp. under 10%, 20%, and 40% extract concentrations, betel nut showed higher efficacy having the recorded mean motility time of 0.22, 0.07 min, and no movement upon contact, respectively, than Albendazole which produced mean motility time of 0.38 min. Nevertheless, the flukes treated with 10%, 20%, and 40% neem leaf extracts obtained the average mean motility time of 220, 151, and 98 min, respectively.

Conclusions: The results gathered showed that 40% BNE concentration showed the highest efficacy based on the recorded mean motility time. All treatments of betel nut extract evidently showed marked changes in the gross and microscopic morphology of the flukes. However, the neem extract was ineffective in all concentrations although changes were observed microscopically. Furthermore, the nutrient broth was proven to be effective as a culture medium since the flukes remained active until 8 h of exposure.

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Betel nut; *Fasciola* spp.; motility; morphology; neem leaf

Introduction

Fasciolosis in cattle and buffalo is not uncommon in the Philippines [1,2]. For several years, *Fasciola* spp. has been known to cause serious diseases in livestock, wildlife, and pet animals [3]. Fasciolosis is caused by *Fasciola hepatica* (*F. hepatica*) and *Fasciola gigantica* (*F. gigantica*) commonly in temperate and tropical regions, respectively. *Fasciola gigantica* has been reported in Africa, Asia, and Hawaii [2,4]. *Fasciola hepatica* can be found in more than 50 countries, in all continents of the world, except Antarctica. In general, fasciolosis is more common in animals than in humans. Nevertheless, the number of infected people in the world is thought to exceed two million [4].

This disease was considered to be one of the major threats to livestock production because of its widespread distribution where significant losses may occur [5]. This can be accounted to the confiscated liver, low milk production, reproductive inefficiency, and low meat yield

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[6]. It adversely affects milk production, wool quality, and weight gain of the animal. The effective control of fasciolosis currently includes the strategic and tactile use of anthelmintic medications and proper grazing land management, stock density control, and or rotational grazing. Due to limited available anthelminthic, high veterinary cost, anthelmintic resistance, drug residues in milk and meat, toxicity problems, and not effective intermediate host control measures, the population is dependent on traditional remedies.

Treatment of fasciolosis is more of commercially-manufactured anthelmintic drugs [7], but infrequently with the use of herbal plants [8]. In the Philippines, the use herbal plants in treating diseases have become limited because of the spiraling cost and availability of anthelmintic drugs in the local market [9]. However, there are some serious disadvantages in using chemical dewormers such as development of resistance to anthelmintic, residues and pollution, and long withdrawal period prior to slaughter [10]. Thus, this study aimed to measure the anthelmintic effects of betel nut (*Areca catechu*) and neem (*Azadirachta indica*) leaf extracts against *Fasciola* spp. *in vitro* in comparison with the commercial dewormer, Albendazole, and the negative control, nutrient broth. The study determined the extract concentration that produced the highest efficacy based on the average recorded mean motility time, gross, and microscopic changes of the flukes treated with different concentrations of plant extracts.

Materials and Methods

Collection of samples

Live *Fasciola* spp. were collected directly from the liver of buffaloes bought from the slaughter house. The samples were transported to the laboratory for further processing. The liver was cut on the bile duct part where the flukes were found and collected using a non-traumatic thumb forceps. Right after collection, the flukes were immediately exposed to the different treatments. Only flukes with normal gross morphologic tegument and exhibit motility by visual inspection were selected. The flukes were weighed using a digital pocket weighing scale system. The motility of the flukes was recorded.

Betel nut extract (BNE) preparation

Fresh mature betel nuts were collected. The fresh fruits were halved and the nuts were separated from the shell using a knife and were scooped using a teaspoon then placed in a bottle container. The fresh seeds were air-dried for 5–7 days. The dried seeds were ground into powder form using mortar and pestle [3]. The powdered material of dried betel nut seeds weighing 500 gm was mixed with 1 l of 80% ethanol for 48 h to get the concentrated extracts. Using a muslin cloth and Whatman No. 1 filter paper, the extract was double filtered and was concentrated using rotary evaporator machine. The extract was stored at 4°C until use.

Neem leaf extracts preparation

Fresh mature neem leaf samples were collected and washed gently with tap water. After washing with tap water, the fresh leaves were air-dried for 5–7 days. Using mortar and pestlel the dried leaves were pulverized [3]. The powdered material of dried mature neem leaves weighing 500 gm was mixed to 1 l of 80% ethanol for 48 h to get the concentrated extracts. Using a muslin cloth and Whatman No. 1 filter paper, the extract was double filtered and was concentrated using rotary evaporator machine. The extract was stored at 4°C until use.

Experimental design

Motile flukes weighing from 1 to 2 gm (±0.2 gm) were included in the study and separated into eight groups. Two different liver tissues containing flukes were used. One liver was used for the first four treatments (T1–T4) and another for the remaining treatments (T5–T8) (Table 1). The flukes after collection on the liver using a non-traumatic thumb forceps were carefully and individually placed on the digital pocket weighing scale. The flukes that fit with the accepted weight range were kept in petri dishes containing the different plant extract concentrations. Each treatment contained five flukes. The experimental set-up was as follows:

Criteria for motility

Motility scores were obtained using the scoring criteria: Score 3—whole body moving; Score 2%–50% of body parts moving; Score 1—grossly immobile but microscopically alive; Score 0—dead [11]. All flukes in every treatment were scored individually at 15 min interval according to the motility criteria established.

To be able to interpret the scores, the efficacy of the herbal preparations were based on the following indicators:

Table 1. Experimental design of the treatments and concentrations.

Group	Concentration	Description
T1	10% BNE	2.5 ml BNE/25 ml nutrient broth
T2	20% BNE	5 ml BNE/25 ml nutrient broth
T3	40% BNE	10 ml BNE/25 ml nutrient broth
T4	10% NLE	2.5 ml NLE/25 ml nutrient broth
T5	20% NLE	5 ml NLE/25 ml nutrient broth
T6	40% NLE	10 ml NLE/25 ml nutrient broth
Positive control	10% Al	2.5 ml Al/25 ml nutrient broth
Negative control	Nutrient broth*	25 ml of nutrient broth

BNE = betel nut seed extract; NLE = neem leaf extract; Al = Albendazole.

Table 2. Mean scores for the interpretation of the flukes' motility in minutes to mortality.

Mean score	Interpretation
<0.273	Highly effective
0.274–0.494	Effective
>0.495	Ineffective

95% confidence interval.

Table 3. Summary of effect of betel nut seed extract and neem leaf extract on liver flukes based on the recorded motility time.

Group	Mean motility time	Interpretation
T1	0.22a (± 0.0997)	Highly effective
T2	0.07a (± 0.0235)	Highly effective
T3	No movement upon contacta	Highly effective
T4	220 (± 40.3556)	Ineffective
T5	151 (± 40.2758)	Ineffective
T6	98 (± 42.7534)	Ineffective
Positive control	0.38a (± 0.2003)	Highly effective
Negative control	362 (± 63.0986)	Ineffective

$p < 0.0001$.

Motility and morphology analysis

Motility analysis

The motility time of all flukes exposed to different treatments was recorded. Any motility observations were noted based on the established motility criteria (Table 2). The flukes that showed no movement visually were placed on a slide and were viewed and examined using a light microscope to confirm for any movement before subjected to formaldehyde.

Morphological analysis

After the observations, three flukes from each treatment were placed in a 10 ml vial containing 10% formaldehyde. The fixed flukes were submitted to a laboratory for further processing of the fluke tissues. Only the longitudinal section of one fluke and a cross section (anterior and posterior part) of each of the two flukes on every treatment were processed. The processed tissues on the slides were examined under a light microscope for deviations from normal fluke cross section and pictures were taken.

Statistical analysis

Statistical analysis was done on all treatment using analysis of variance for completely randomized design (CRD) and factorial in CRD. Duncan's multiple range test was used for the mean comparison of the treatment.

Results and Discussion

The effect of betel nut and neem leaf extracts from morphology and motility of the liver flukes exposed to both extracts were recorded. The mean motility scores of the different treatments were calculated and was the basis of the efficacy interpretation. Changes in the microscopic structure of the flukes' tegument were described in comparison with normal structure. This was done in all representative samples of the different treatments.

Table 3 shows the recorded mean motility time of flukes in all treatments. In 10%, 20%, and 40% concentrations, BNE was found to be highly effective against *Fasciola* spp. with mean motility time average of 0.22, 0.07 min, and no movement upon exposure, respectively. The results showed that 10%, 20%, and 40% betel nut concentration had no significant difference with that of the Albendazole-treated group. All flukes were observed to have sudden paralysis then became immobile and suddenly stopped moving upon exposure to the BNE extract treatments. In addition, it was observed that a significant increase in efficiency of BNE as the concentration is increased based on the motility of the flukes. At 40% BNE extract concentration, the flukes became immobile and non-motile upon exposure. This treatment had the highest efficacy based on meat motility time. Statistical analyses showed no significant differences at 10%, 20%, and 40% BNE concentrations. In the study of Jeyathilakan et al. [3], revealed that the betel nut extract is effective *in vitro* at 1%, 2.5%, and 5% concentrations against adult *Fasciola* with the recorded motility time of 2 min, 30 sec, and 30 sec, respectively. Furthermore, Tangalin [12] revealed that the powdered form of betel nut has showed better results and an *in vivo* anthelmintic effect at 30 gm/20 kg body weight against roundworms and tapeworms of small ruminants and native chickens. Peng et al. [13] reported that water extracts of the betel nut can effectively kill tapeworms. In addition, Orazaga and Orazaga [14] reported that at 6 gm/kg bwt of sun-dried betel nut has 41.48% reduction in epg of roundworms after 28 days of treatment.

Arecholine, active ingredient found in betel nut, inhibits the gamma amino benzoic acid that causes paralysis of the helminths [14]. Peng et al. [13] stated that the betel nut, with its diverse pharmacologic properties, has a future for treating parasitic and other diseases.

All concentrations of neem leaf extracts were found to be ineffective based on the established mean motility score interpretation criteria with the average recorded mean motility time of 220, 151, and 98 min, respectively. The flukes upon exposure to neem leaf extract remained to be active for about 3 h and 45 min, but then showed a marked decreased in motility then afterward became immobile; however, microscopically alive. Amin et al. [15] reported that 10% neem leaves water extracts was moderately effective against the gastrointestinal parasites of sheep when administered orally. In addition, Radhakrishnan

et al. [16] stated that goats fed with neem leaves was effective in lowering the worm count of abomasum from 262.25 to 139.25. In sheep, neem water extracts was 53.72% effective against *Haemonchus contortus* [17]. Likewise, Chandrawathani et al. [18] revealed that the neem treated groups had a lower fecal egg count of 1,160–3,733 epg compared with control group which yielded 2,140–11,117 epg; however, this is not significant.

Larvicidal activity of neem against *Lymnaea acuminata* and *F. giganitca* had been reported by Sunita and Singh [19] and Sunita et al. [20]. In addition, 1% azadirachtin (active ingredient of neem) extracted from seeds was 68.3% effective against *Haemoncus contortus* [21]. Rabiu and Subhasish [22] reported that aqueous neem leaves extract was effective against *Pheretima posthuma, Racinoa spiralis*, and *Ascaridia galli.* In addition, neem alcoholic extract was found effective against *F. gigantica* [23,24]. However, in this study, the anthelmintic property of neem leaf extract was not effective as compared with betel nut extract and albendazole based on mean motility time.

Grossly, the flukes in all treatment of BNE showed shrinkage and deformation of the body shape and shrunken edges of the tegument were noticed. At 10% BNE concentration, the fluke showed mild separation of the tegument and vacuolations on the parenchyma were observed microscopically. At 20% BNE concentration, vacuolations on the parenchyma of the fluke were evident and there was also severe separation of the tegument. Finally, at 40% BNE concentration, there was a massive and severe damage on the tegument and vacuolation of the parenchyma (Fig. 1). On the study conducted by Jeyathilakan et al. [3], betel nut extract caused severe damages on the flukes at 1%, 2.5%, and 5% concentrations including the separation of the tegument and vacuolations on the parenchyma microscopically.

The flukes under neem leaf extracts (NLE) treatments showed normal structures and did not show any marked effect or malformations on the body grossly. However, flukes exposed in 10% NLE showed marked separation of the tegument and mild vacuolations on the parenchyma, while at 20% NLE-treated flukes, parenchymal vacuolation became evident and few separation on the tegument. At 40% NLE-treated flukes, severe parenchymal vacuolation and disruption on the tegument were prominent (Fig. 2).

Similar to the results of BNE, flukes treated with Albendazole at 10% concentration (2.5 ml Albendazole) yielded an average mean motility time of 0.38 sec which was considered to be highly effective based on the established recorded mean motility time. Microscopically, the control fluke showed massive damages including vacuolations on the parenchyma, disruption, and separation of the tegument (Fig. 3).

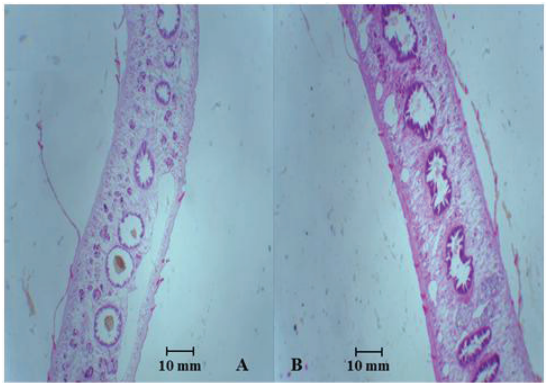

Figure 1. (A) Longitudinal section and (B) cross section of *Fasciola* sp. exposed to 40% BNE showing massive separation of the tegument and vacuolations of parenchyma (40 × magnification).

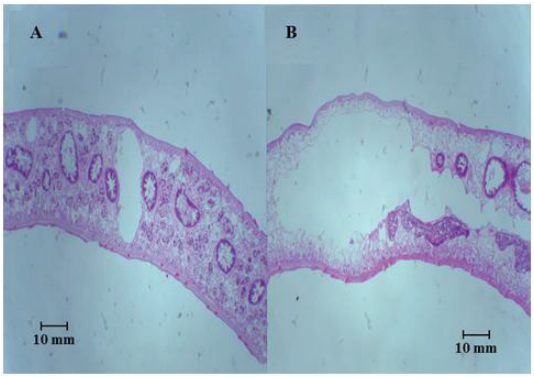

Figure 2. (A) Longitudinal section and (B) cross section of *Fasciola* sp. exposed to 40% NLE showing disruption of the tegument and vacuolations of parenchyma (40 × magnification).

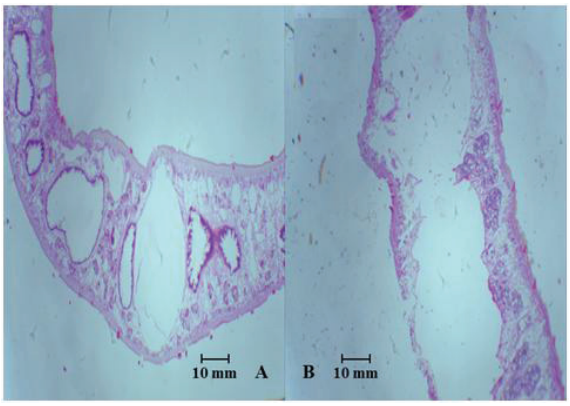

Figure 3. (A) Longitudinal section and (B) cross section of *Fasciola* sp. exposed to 10% Albendazole showing mild separation of the tegument and vacuolations of parenchyma (40 × magnification).

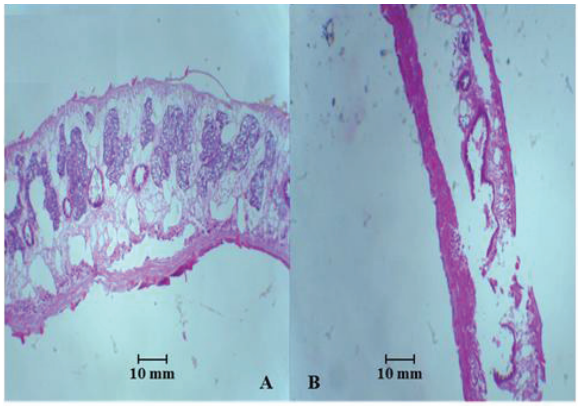

Figure 4. (A) Longitudinal section and (B) cross section of Fasciola sp. in untreated group showing the tegument and mild vacuolation on the parenchyma (40 × magnification).

The flukes under the negative control group remained active until 8 h of exposure to the nutrient broth. The morphology of the flukes was not altered grossly although few vacuolations on the parenchyma of the fluke were observed microscopically while the tegument remained intact on the body (Fig. 4).

Conclusion

The results gathered showed that 40% BNE concentration showed the highest efficacy based on the recorded mean motility time. All treatments of betel nut extract evidently showed marked changes in the gross and microscopic morphology of the flukes. However, neem extract was ineffective in all concentrations although changes were observed microscopically. Furthermore, the nutrient broth was proven to be effective as a culture medium since the flukes remained active until 8 h of exposure. Finally, this study provided a future for betel nut in treating Fasciolosis.

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Conflict of Interest

The authors declare that there is no conflicting interest concerning the publication of this manuscript.

Author contribution

Elnalyn C. Yamson, Victoria V. Viloria, and Claro N. Mingala designed the experiment. Elnalyn C. Yamson and Claro N. Mingala collected the data and conducted the analysis. Elnalyn C. Yamson, Gabriel Alexis S. P. Tubalinal, and Claro N. Mingala drafted the manuscript. Elnalyn C. Yamson, Gabriel Alexis S. P. Tubalinal, Victoria V. Viloria, and Claro N. Mingala critically reviewed the article and Claro N. Mingala finally approved for publication.

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