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Research Article

Anthelmintic Effects of Betel Nut (*Areca catechu*) Extracts on Roundworms (*Toxocara canis*) and Hookworms (*Ancylostoma caninum*) Isolated from Vietnamese Dogs

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Abstract

Background and Objective: The seeds of the Areca palm (*Areca catechu*), commonly known as betel nuts, are widely used in Vietnam as a traditional treatment for a range of ailments in both humans and animals. However, there has been limited research to investigate a scientific basis for this phytotherapeutic application. In this study the effects of solvent extracts from the betel nut on roundworms (*Toxocara canis*) and hookworms (*Ancylostoma caninum*) freshly isolated from the intestines of Vietnamese domestic dogs was investigated. **Materials and Methods:** Dried betel nut powder was extracted using three different solvents: Ethanol, methanol and hot distilled water. Extracts were then diluted with physiological saline solution to concentrations of 1000, 500, 250 and 125 mg mL^{-1} to perform *in vitro* tests on dog helminths. In order to evaluate their effects, time elapsed between exposure to each extract preparation and the death of 50% (lethal dose 50, LD_{50}) and 100% (lethal dose 100, LD_{100}) of parasites was measured. **Results:** Betel nut extracts showed anthelmintic properties against both roundworms and hookworms. *Areca caninum* was more sensitive than *Toxocara canis* as evidenced by a significantly shorter time to lethality recorded for all tested extracts. Of the three solvents investigated, methanol and aqueous extracts exerted the strongest and weakest effects, respectively. These results indicated that methanol is a suitable solvent to extract anthelmintic-active components from betel nuts. **Conclusion:** This study demonstrated *in vitro* the potent anthelmintic effects of betel nuts on canine ascarids and strongylids, thereby corroborating use in Vietnamese ethnic medicine to treat gastrointestinal worm infestations of humans, to which dogs are companion animals.

Key words: *Ancylostoma caninum*, anthelmintic, *Areca catechu*, betel nut, dog, *Toxocara canis*, Vietnam

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Since benzimidazole became commercially available in the 1960s and the introduction of ivermectin and macrocyclic lactones two decades later, control of parasitic helminth infection of domesticated animals and livestock has been predicated on the use of synthetic anthelmintic drugs[1-4]. However, the sustainability of applying chemical-based 'dewormer' drugs to control helminths has been seriously threatened by the emerging of drug resistance, the incidence of which is increasing worldwide[3-6]. This is primarily because many currently available anthelmintic drugs received regulatory approval several decades ago, such that their widespread long-term misuse has exerted a selection pressure for resistant worms to evolve[7].

Roundworms (*Toxocara canis*) and hookworms (*Ancylostoma caninum*) and are among the most prevalent and important gastrointestinal parasites of the domestic dog (*Canis lupus familiaris*)[8-11]. However, their resistance to licensed veterinary anthelmintic drugs is growing, observed in both adults and larvae[11,12]. Among several strategies that have been proposed as alternatives to synthetic drugs for parasitic disease control, botanical dewormers are recognized as leading candidates[9,13]. Plants provide a rich source of medicinal compounds with diverse chemical structures and novel mechanisms of action, treatment with which can cure various infectious diseases, including those caused by parasites[13-17]. Moreover, such herbal remedies are considered to produce fewer side-effects, less residues, while their veterinary application may generate slower parasite resistance and also induce enhanced growth and survival rates of host animals compared to traditional therapies[18].

The areca nut, commonly known as betel nut and hereafter referred to as such, is the seed of the areca palm (*Areca catechu*). Despite its name, it is not a true nut but rather the seed of a berry. This plant grows in tropical, humid climates across the Pacific and Southeast Asia, including the forests of Vietnam, from where the seeds are cultivated. Betel nuts have long been used in Vietnamese ethnic medicine for their purported anthelmintic effects. Therapies are predicated only on traditional experience, the lack of a scientifically researched, evidence-based rationale until now limits their application to folk medicine.

Recently, however, therapeutic properties of betel nut for parasitic diseases were demonstrated in part on porcine ascarids and flukes[19]. Yet, there has been no research examining the effect(s) of powdered extracts of betel nut on canine endoparasites. Therefore, our study aimed to investigate their possible anthelmintic properties against canine gastrointestinal helminths, focusing on roundworms and hookworms. The overall purpose was to evaluate for the first time the potential application of a betel nut preparation as a phytotherapeutic drug for the control of canine endoparasitic diseases.

MATERIALS AND METHODS

Collection of dog helminth parasites: Dog intestines were collected as soon as possible after slaughter from abattoirs local to the Vietnam National University of Agriculture, located in the Vietnamese capital city of Hanoi. Following transport to the laboratory, the fresh intestines were dissected to isolate and retrieve endoparasites that remained attached to inner membranes (Fig. 1). Roundworms (*T. canis*) and hookworms (*A. caninum*) were collected (Fig. 2). The identity of each viable specimen was confirmed by morphological examination under light microscopy (Fig. 3). *In vitro* tests with extracts commenced within 2 hrs of parasite collection.

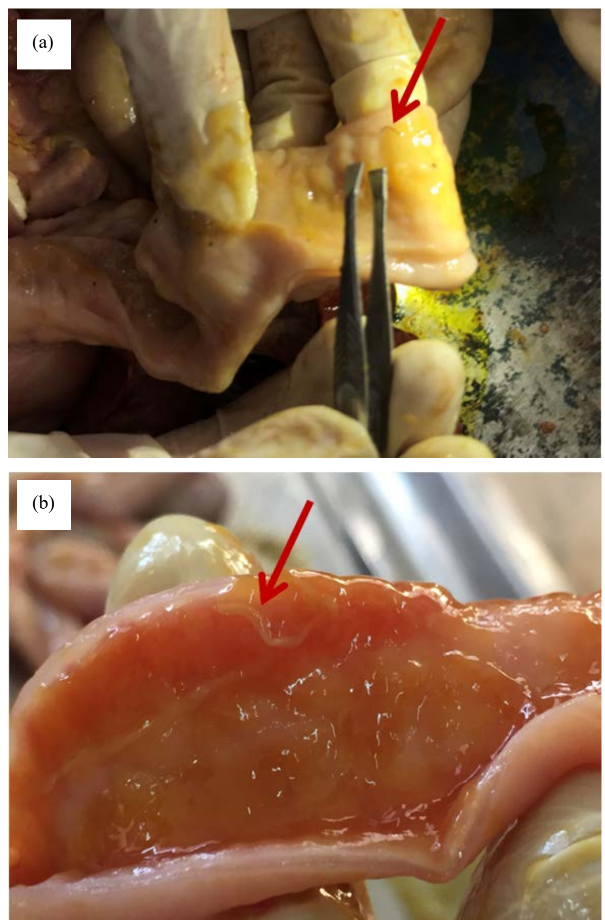

Fig. 1: Hookworms observed on dog intestinal mucosa

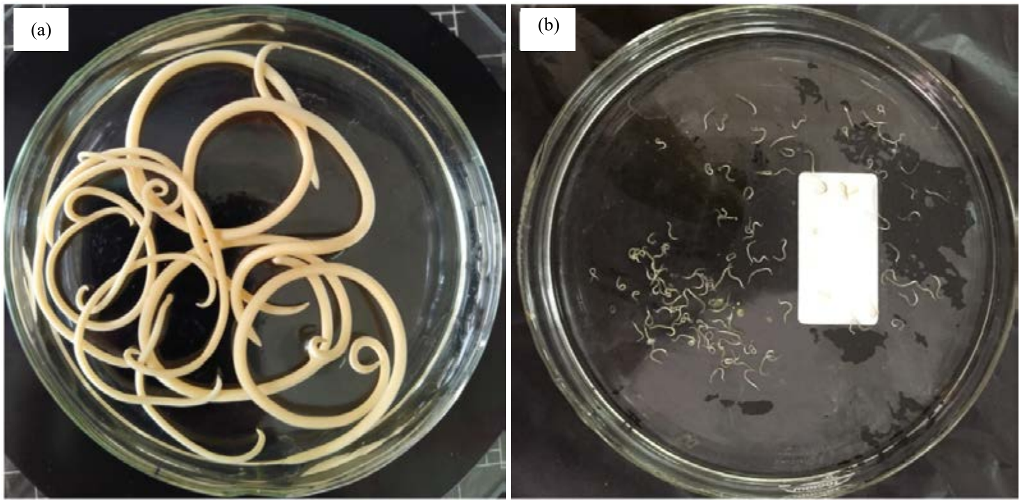

Fig. 2(a-b): (a) Roundworms and (b) Hookworms were isolated from dog intestines

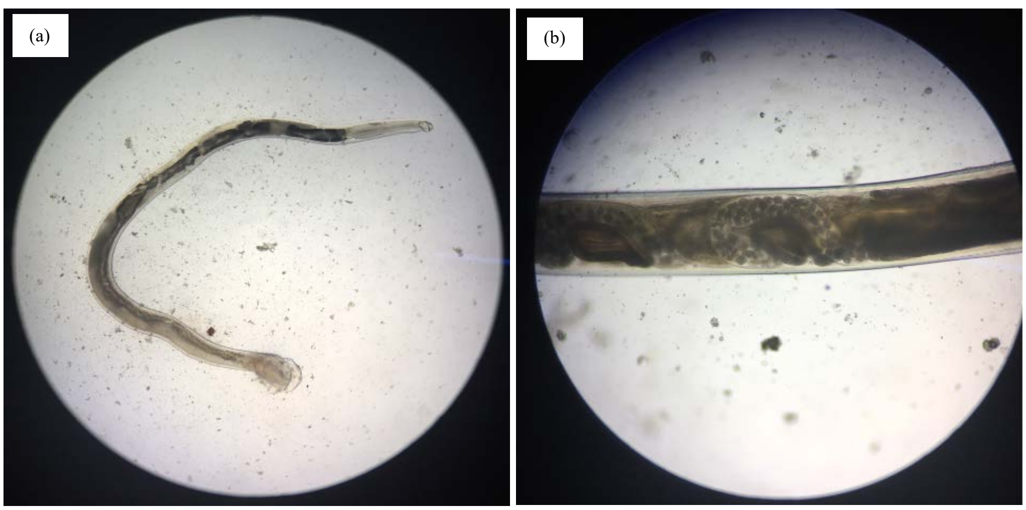

Fig. 3: Confirmation of endoparasite identity by light microscopical examination

Extraction of plant materials: Betel nuts were purchased from the Binh An medicinal plant supply company (Nghia Trai village, Hung Yen province, Vietnam). Their identity was authenticated by comparison to voucher specimens deposited at the "Vuon Duoc Lieu Thu y" Herbarium, Vietnam National University of Agriculture. The dried material was pulverized to a fine powder of particle size <1 mm prior to extraction (Fig. 4a). This process was performed following the method that described by Nguyen *et al.*[20,21] but with minor modifications. In brief, 10 g of dried material was mixed with 300 mL of solvent, namely either ethanol, methanol or hot distilled water (DW). For the two organic solvents, mixtures were then left for 24 hrs at room temperature to allow for absorption. For DW, after stirring for 30 min mixtures were filtered while the solutions were still hot. This was in keeping with folk experiences in which therapeutic ingredients of plant materials are usually difficult to dissolve in cold, tepid or even warm water. Traditional processing methods, such as decoction (herbal extraction by boiling, then simmering at a moderately hot temperature for several hours) or making tea (steeping in boiling water for a short time) are usually applied to increase the aqueous solubility of the given plant material. Extracts were then filtered through two layers of cheese cloth before being centrifuged at 1800 g for 15 min to remove any residual precipitation. Collected supernatants were filtered again through grade No. 2 qualitative paper (Advantec MFS Inc., Dublin, CA, USA) to eliminate all particles of size > 8 μm. Subsequently, solvents were removed completely using a rotary evaporator set to 37°C and low atmospheric pressure. Before conducting experiments,

Fig. 4(a-b): Pulverized powder of dried betel nuts (a) and extracts of this plant material dissolved in different solvents at a concentration of 1000 mg mL^{-1} (b)

condensed extracts were re-dissolved in a physiological salt solution (PSS) to obtain extracts at four concentrations: 1000, 500, 250 and 125 mg mL^{-1}. This two-fold dilution range was calculated based on the weight of dried material used in extraction (Fig. 4b). The PSS was used in this experiment with ascarids and strongylids as preliminary tests showed no observable adverse effect on viability *in vitro* upon bathing either endo-parasite in this solution.

Experiments on endoparasites and measurement of lethal time: Tests were performed following the methods described by Nguyen *et al.*[21] but with minor modifications. In experimental groups, parasites were placed in 10 cm petri dishes containing 10 mL solutions of extract at different concentrations. The PSS alone was used in the control group (in pilot tests, roundworms and hookworms isolated from dog intestines survived for at least 10 hrs when bathed in PSS).

The paralysis of each parasite was tested individually once a minute over a period of 360 min. Paralysis was recorded when parasites were observed to no longer show motility and gave no detectable response to the stimulation of gentle touching with a glass rod. The time elapsed between exposure to an extract solution and death of 50% of parasites was called lethal time 50 (LT_{50}). Similarly, the time to death of 100% of parasites was called lethal time 100 (LT_{100}), based on 30 experiments performed independently of each other, results were evaluated to compare the effect on parasitic helminths of exposure to a range of extract concentrations.

Statistical analysis: Data were expressed as Mean$\pm$Standard error (Mean$\pm$SE). Data were analyzed using Excel v.16.0 software (Microsoft, Redmond, WA, USA) with the Statcel 4 add-in analysis package (OMS Ltd., Saitama, Japan). The effects of different concentrations of each extract were compared using One-way ANOVA followed by the *post hoc* Bonferroni test. Student's t-test was utilized to compare the LT_{50} or LT_{100} of different extracts. When a probability level was less than 5% it was considered significant ($p<0.05$).

RESULTS

In order to assess the effects of dried powder of betel nuts extracted using three different solvents on dog parasitic helminths, their LT_{50} and LT_{100} time values over a concentration range were measured. The results with roundworms and hookworms were shown in Table 1 and 2, respectively.

Effects of solvent extracts from betel nuts on roundworms: Betel nut extracts exerted an observable effect on roundworm viability *in vitro*, which was dose-dependent. Hence, there was an inverse correlation between the treatment dose and the recorded LT_{50} value (Table 1). For example, when the concentration was halved from 1000 to 500 mg mL^{-1}, LT_{50} values for the ethanol extract increased significantly, from 270$\pm$10.4 to 310$\pm$11.2 min. Similar results were attained for the same doses of methanol and DW extracts (from 218$\pm$8.6 to 277$\pm$8.4 min and from 307$\pm$7.9 to 350$\pm$12.3 min, respectively). Of the three preparations, the

Table 1: LT_{50} and LT_{100} values for roundworms (*Toxocara canis*) isolated from dog intestines following *in vitro* exposure to different solvent extracts of areca nut seeds

	LT_{50} (min)			LT_{100} (min)		
Concentration (mg mL^{-1})	Ethanol	Methanol	DW	Ethanol	Methanol	DW
1000	$270\pm10.4^{a,B}$	$218\pm8.6^{a,A}$	$307\pm7.9^{a,C}$	315 ± 8.9^{B}	250 ± 10.1^{A}	$(70\pm9.8)^{\#}$
500	$310\pm11.2^{b,B}$	$277\pm8.4^{b,A}$	$350\pm12.3^{b,C}$	$(70\pm10.8)^{\#}$	$(90\pm11.2)^{\#}$	$(60\pm9.8)^{\#}$
250	-	348 ± 9.8^{c}	-	-	$(75\pm10.7)^{\#}$	-
125	-	-	-	-	-	-
Control	Parasite death not detected (0%)					

Where, less than 100%, ($^{\#}$n) indicates the % of experimental parasites recorded as dead after 360 min observation, -: Indicates that no experimental parasites were recorded as dead after 360 min observation. Values in the same column marked with different superscript lowercase letters (^{a,b,c}) are significantly different (p<0.05) by One-way ANOVA and *post hoc* Bonferroni test. LT_{50} or LT_{100} values in the same row marked with different superscript uppercase letters (^{A,B,C}) are significantly different (p<0.05) by One-way ANOVA and *post hoc* Bonferroni test

Table 2: LT_{50} and LT_{100} values for hookworms (*Ancylostoma caninum*) isolated from dog intestines following *in vitro* exposure to different solvent extracts of areca nut seeds

	LT_{50} (min)			LT_{100} (min)		
Concentration (mg mL^{-1})	Ethanol	Methanol	DW	Ethanol	Methanol	DW
1000	$70\pm6.3^{a,B}$	$58\pm4.5^{a,A}$	$87\pm5.8^{a,C}$	$86\pm9.7^{a,B}$	$69\pm8.8^{a,A}$	$98\pm10.2^{a,C}$
500	$108\pm9.1^{b,B}$	$87\pm7.9^{b,A}$	$137\pm11.3^{b,C}$	$140\pm7.9^{b,B}$	$120\pm8.9^{b,A}$	$185\pm11.2^{b,C}$
250	$243\pm10.2^{c,B}$	$168\pm9.1^{c,A}$	$287\pm11.4^{c,C}$	$307\pm9.2^{c,B}$	$217\pm8.4^{c,A}$	$347\pm10.9^{c,C}$
125	-	257 ± 12.4^{d}	-	-	$(80\pm9.6)^{\#}$	-
Control	Parasite death not detected (0%)					

Where, less than 100%, ($^{\#}$n) indicates the % of experimental parasites recorded as dead after 360 min observation, -: Indicates that no experimental parasites were recorded as dead after 360 min observation. Values in the same column marked with different superscript lowercase letters (^{a,b,c}) are significantly different (p<0.05) by One-way ANOVA and *post hoc* Bonferroni test. LT_{50} or LT_{100} values in the same row marked with different superscript uppercase letters (^{A,B,C}) are significantly different (p<0.05) by One-way ANOVA and *post hoc* Bonferroni test

Fig. 5: Contraction response of dog roundworms (*Toxocara canis*) following exposure to a methanol extract of betel nuts at 1000 mg mL^{-1}

methanol extract produced the strongest effect as its LT_{50} values were shortest for both 1000 and 500 mg mL^{-1} concentrations (218±8.6 and 277±8.4 min, respectively). Furthermore, contraction responses of roundworms after being wetted were most profound using methanol extract (Fig. 5). In contrast, the DW extract exerted the weakest effect as shown by its significantly lower LT_{50} values (307±7.9 min in 1000 mg mL^{-1}, 350±12.3 min in 500 mg mL^{-1}). Exposure to the DW extract did not kill all tested roundworms, even at 1000 mg mL^{-1}, whereas, ethanol and methanol extracts at that highest concentration were 100% lethal to tested roundworms, with an LT_{100} value of 315±8.9 and 250±10.1 min, respectively.

Effects of solvent extracts from betel nuts on hookworms: As shown in Table 2, betel nut extracts also exerted a noticeable, dose-dependent effect on hookworm viability *in vitro*. Thus, there was a significant inverse correlation between treatment dose and recorded LT_{50} and LT_{100} values. Of the three investigated preparations, the methanol extract showed the strongest effect as it induced the lowest LT_{50} and LT_{100} values at 1000, 500 and 250 mg mL^{-1} (58±4.5, 87±7.9, 168±9.1 min and 69±8.8, 120±8.9, 217±8.4 min, respectively). Moreover, contraction responses of hookworms were most distinct upon wetting with methanol extract (Fig. 6).

Comparative anthelmintic effects of solvent extracts from betel nuts: In order to compare the sensitivity of roundworms and hookworms to betel nut extracts, their LT_{50} and LT_{100} values were inspected (Fig. 7 and 8). The LT_{50} values of

Fig. 6: Contraction response of dog hookworms (*Ancylostoma caninum*) following exposure to a methanol extract of betel nuts at 1000 mg mL^{-1}

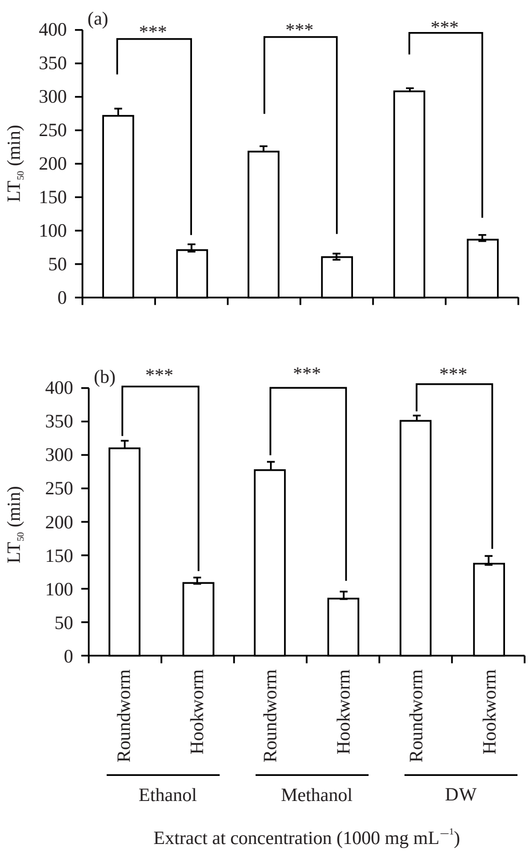

Fig. 7(a-b): LT_{50} of ethanol, methanol and DW extracts on roundworms and hookworms at concentrations of (a) 1000 and (b) 500 mg mL^{-1}

Values marked with a superscripted asterisk indicate significantly different LT_{50} by student's t-test (***p<0.001)

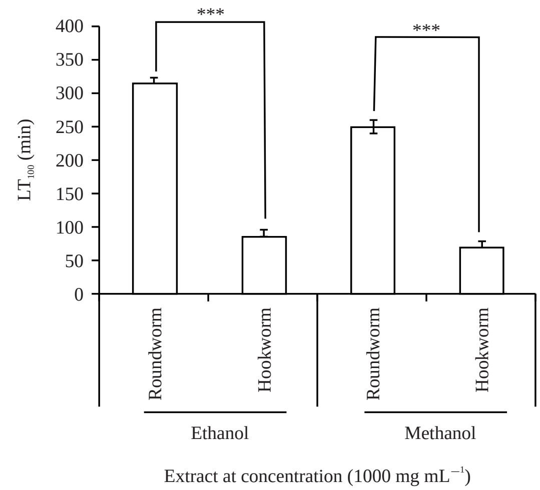

Fig. 8: LT_{100} of ethanol and methanol extracts on roundworms and hookworms at a concentration of 1000 mg mL^{-1}

Values marked with a superscripted asterisk indicate significantly different LT100 by Student's t-test (***p<0.001)

hookworms were significantly shorter than those of roundworms, for all three tested extracts at the two highest concentrations of 1000 and 500 mg mL^{-1} ($p<0.001$, Fig. 7a and b). Similar results were attained for LT_{100} using ethanol- and methanol-derived extracts at 1000 mg mL^{-1} concentration ($p<0.001$, Fig. 8).

DISCUSSION

The results of the study reported herein demonstrate the *in vitro* anthelmintic effects of solvent extracts from betel nuts on roundworms and hookworms isolated from dog intestines. Based on these findings we propose that *A. catechu* seeds be considered as candidate phytotherapy for the infection of dogs with these gastrointestinal parasites. Resistance to anthelmintics is of growing veterinary concern, posing a major threat to the welfare of domestic pets and to the production of farmed animals throughout the world[13]. It has been suggested that in order to achieve sustainable control of parasitic worms an integrated approach incorporating many strategies, including the discovery of new drugs, is necessary, so as to minimize the pressure of parasitic adaptation[2]. Effects of medicinal plants on endoparasites have been observed previously for several species including *Acacia* spp., *Allium sativum*, *Artemisia* spp., *Balanites aegyptiaca*, *Calendula micrantha officinalis*, *Commiphora molmol* (myrrh), *Cucurbita pepo* (pumpkin seeds), *Curcuma longa* (turmeric, curcumin), *Nigella sativa* and *Peganum harmala*[13-17]. Interestingly, there was also a report of synergistic anthelmintic effects between a plant material (from *Citrus aurantifolia* swingle seeds) and a synthetic drug (mebendazole), resulting in better treatment efficacies for animals[9]. This research suggests that betel nuts may now be added to the list of promising botanical dewormers for the control of parasitic helminths that have shown prior resistance to first-line synthetic drugs.

In the present study, the observed effects of the betel nut varied depending on the helminth species tested. Therefore, so that it may be applied in veterinary practice when a given endoparasitic species that is infecting an animal is identified, suitable doses and treatment regimens need to be established. As evident from our results, roundworms appear to be more resistant than hookworms, indicating that higher doses would be required to treat *T. canis* infections. However, as we have examined *in vitro* effects only, follow-up research that includes *in vivo* experiments is required to confirm betel nut treatment efficiency as well as to assess its safety and potential side-effects on host animals.

Current results show that the nature of the extracting solvent plays an important role in determining anthelmintic properties because preparations derived using water and different alcohols as solvents exerted varying effects. The diverse antimicrobial activities of plant extracts attained with various solvents are explained by differences in their degrees of solubility for a range of phytochemicals[22]. We observed that methanol was superior to ethanol and water in the extraction of anthelmintic properties from powdered betel nuts, suggesting that this solvent should be investigated in more detail. Further studies are needed to identify the active components of *A. catechu* and to determine their solubility in each of the solvents used herein as well as to fully explain the varying anthelmintic effects induced.

CONCLUSION

This study demonstrates the *in vitro* anthelmintic effects of solvent extracts from betel nuts on gastrointestinal parasitic helminths of domestic dogs. In so doing, it provides partial scientific evidence to explain the longstanding use of this plant material in traditional medicine to control endoparasitic diseases. In Vietnam, this alternative, non-prescribed practice includes the alleviation of roundworm and hookworm infections in both dogs and humans. However, in order to pursue its application in veterinary medicine to treat diseased dogs, follow-up research is necessary, not only to verify the effects *in vivo* but also to establish suitable therapeutic doses and treatment regimens.

SIGNIFICANCE STATEMENT

Domestic dogs frequently suffer from gastrointestinal parasitic worm infections. As these are recurrent, an animal should be ‘dewormed’ regularly to stay healthy. In many Asian countries including Vietnam, preparations of the betel nut, the seed of the areca palm, are used as an inexpensive veterinary therapy. Yet, detailed scientific support for traditional treatments is often lacking-including to expel worms. This study looked at the effect various doses of different betel nut extracts had on two species of worm that commonly infect the guts of dogs. The findings show the potential application of a betel nut preparation as a drug to control canine worm infections. Further research is required to determine how the drug works and to optimize the dosage.

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