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Research Article

A STUDY ON ANTHELMINTIC AND ANTIBACTERIAL EFFECTS OF EXTRACTS FROM CHINESE HONEYSUCKLE (*QUISQUALIS INDICA* L) SEEDS AND ARECA (*ARECA CATECHU*) NUTS

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ABSTRACT

Objectives: This study aimed to investigate the anthelmintic and antibacterial effects of Chinese honeysuckle (*Quisqualis indica* L) seeds and Areca (*Areca catechu*) nuts, to verify their potentials of applying in practice.

Materials and Methods: Aqueous extracts of plants at different concentrations were used to test with porcine ascarids, porcine flukes, and *Escherichia coli* (*E. coli*) strains isolated from pigs. The time that caused the death of 50% and 100% experimental endoparasites (LD50 and LD100) was used to verify the anthelmintic effects. Diameters of the inhibitory zone induced by extracts investigated with cylinder agar diffusion method were measured to assess their antibacterial effects.

Results and Discussion: Both of Chinese honeysuckle seeds and areca nuts showed anthelmintic and antibacterial effects when tested against ascarides, flukes, and *E. coli* strains. These effects were all exerted in dose-dependent manners. Chinese honeysuckle seed extracts had stronger effects on ascarides, as shown by their significantly shorter LD50 and LD100 time. On the other hand, areca nut extracts were more effective against flukes and bacteria, which was evident by the significantly shorter lethal time values and significantly wider inhibitory zone diameters.

Conclusion: This study demonstrated the anthelmintic and antibacterial effects of Chinese honeysuckle seeds and areca nuts, and therefore partly gives pharmacological basis to explain their traditional use in Vietnamese folk medicine. While Chinese honeysuckle seeds had more potential on ascarids, areca nuts were superior in effects against flukes and bacteria. However, future research that involved *in vivo* experiments is still required to further assess the applicability of these two promissory plants.

Keywords: Chinese honeysuckle seed, Areca nut, Aqueous extract, Anthelmintic effect, Antibacterial effect, Endoparasite, Bacteria.

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INTRODUCTION

There is an increment in resistance of both endoparasites and bacteria to the usually applied chemotherapeutic drugs [1-4], which has made them less effective. Therefore, it is necessary to search and discover new therapies to control such kinds of diseases. Among several potential alternates, medicine plants have been considered as the most promissory candidate [5,6]. Researchers have found that some plants not only induced high treatment effects but also exerted other advantageous characteristics on domestic animal health, such as less side effects, less residues, and in some cases, have the enhancement on growth and reproductivity [7-9]. The spread in resistance and increment in public awareness about synthesis drug draw-backs, such as hazards on human health through residues and the harmful impacts on ecology, have significantly enforced the demands for new and safe alternatives. Because photo-therapies are usually considered to be not only highly effective but also safe; researchers worldwide has been recently focused on them in the effort of searching for new therapies.

Plant materials, such as seeds of Chinese honeysuckle (*Quisqualis indica* L) and nuts of areca (*Areca catechu*), have a long history of application for many pharmacological uses in Vietnam, such as althelmintic and antibacterial drugs applied for both human and domesticated animals [10-12]. However, these therapies are mainly based on traditional knowledge, and lacks of scientific backgrounds have limited their application, especially in a large scale, such as in cases of farm animals. Therefore, our study aimed to investigate these plant pharmacological functions, particularly the anthelmintic and antibacterial properties, so as to evaluate their potentials to use as phytotherapeutic drugs for endoparasitic and bacterial diseases of domestic animals.

MATERIALS AND METHODS

The collection of endoparasites and bacterial strains

Experimental endoparasites, including porcine ascarids (*Ascaris suum*) and porcine flukes (*Fasciolopsis buski*), were collected in local slaughterhouses near the Vietnam National University of Agriculture (such as Vang market slaughterhouse, Da Ton market slaughterhouse, and Trau Quy market slaughterhouse, Gia Lam, Hanoi, Vietnam). Samples were kept in physiological solution (PSS) to bring back to laboratories, and the authentication was performed. The tests of extracts with endoparasites were started within 2 h from the parasite collection.

Ten isolates of *Escherichia coli* (*E. coli*) were isolated from pig feces in Vietnam and preserved in our laboratory (Department of Animal Surgery and Reproduction, Faculty of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi, Vietnam). Their susceptibility and resistance to five common antibiotics, including chloramphenicol, chlortetracycline, nalidixic acid, streptomycin, and sulfadimethoxine had been previously examined following the standards methods [13,14], and the antimicrobial-resistant phenotypes are shown in supplementary data 1. Before the experiments with extracts, all bacterial strains were sub-cultured into Muller-Hinton Agar to obtain fresh colonies, and concentrations of bacteria were adjusted to 10^8 cfu/ml in the tests with plant extracts.

Collection and aqueous extraction of plant materials

Plant materials were purchased from local medicine plant supplying companies. Their identity was confirmed by Dr. Tho Thi Bui based on voucher specimens that had been deposited at Vuon Duoc Lieu Thu y Herbarium, Vietnam National University of Agriculture in Vietnam. Fresh plant materials were washed and preliminary dried in the shadow from 3 to 4 days before being dried at 50°C for 3 h in a constant temperature oven (DK63, Yamayo, Japan). Dried materials were pulverized into a fine powder with the particle size less than 1 mm before extraction. The extraction was conducted followed the traditional decoction method described by Sinh *et al*. and Thuy *et al*. [15,16]. Extraction was performed following the methods described in our previous reports [17,18], and with some modifications. In brief, 100 g of dried material was mixed with 300 ml of distilled water and boil. After boiling in 1 min, the heat was decreased to 60°C and this temperature was kept in 4 h for decoction. Extracts were then filtered through two layers of cheesecloth and adjusted to 100 ml. Filtrates were then centrifuged at 10,000 × g for 30 min before passing through Grade No. 2 qualitative paper (Advantec MFS Inc., Dublin, CA. USA). The extract was then concentrated at 37°C using a rotary evaporator at low atmospheric pressure until 25 ml of the final extract was obtained from each 100 g of crude powder. The extract was centrifuged again at 10,000 × g for 10 min to remove all of the precipitated substances before tests with endoparasites. In the case of bacteria, passing through 0.2 µm pore-sized discs (DISMIC-13JP syringe filter, Toyo Roshi Kaisha, Tokyo, Japan) was applied instead for the purpose of sterilization. The concentrations of these initial extracts were calculated back to the crude powder weight and determined at 4 g plant material/ml (g/ml). They were then diluted (by PSS in case of endoparasites and water in case of bacteria) to make the two-fold dilution range that included the concentrations of 2000 mg, 1000 mg, 500 mg, 250 mg, 125 mg, 62.5 mg, 31.3 mg, and 15.6 mg/ml to per. Extraction was performed 3 times with each plant material to obtain three independent extracts, and all measurements were done in triplicate for each independent extract.

Experiments with endoparasites and the measurement of lethal time

The tests were performed following the methods of Vien [19] and with some modifications described in our previous study [20]. In the experimental groups, each of 10 parasites was put in the Petri dishes that contained extracts at different concentrations. The PSS was used in control group (In preliminary tests, all of the tested parasites were observed to survive for at least 24 h in the PSS). We checked the paralysis of each parasite individually every minute during the 360 min experimental time. Paralysis was recorded at the time that parasites lost their mobility, gave no reaction to the stimulants induced by glass stirring rods and also did not revive when putting in normal PSS. The time that induced the death of 50% experimental parasites called lethal time 50 (LT50) and the time that induced the death of 100% experimental parasites, called lethal time 100 (LT100), were calculated from the linear regression computerized between the time and the percentage of parasites died at that time.

Experiments with bacteria and the measurement of inhibitory zone diameters

The antibacterial activity of the crude extracts against bacteria was determined by the agar diffusion method that applied cylinders as a reservoir for antimicrobial substances [21,22]. We used stainless steel cylinders with an outside diameter of 8 mm (±0.1 mm), an inside diameter of 6 mm (±0.1 mm), and a length of 10 mm (±0.1 mm). Cylinders were put on Muller-Hinton Agar plate inoculated with bacteria and 100 µl of extracts at different concentrations, such as 2000 mg, 1000 mg, 500 mg, 250 mg, 125 mg, 62.5 mg, 31.3 mg, and 15.6 mg/ml, were filled in each cylinder. All plates were then left in the refrigerator for 24 h to allow the diffusion of extracts from cylinders to agar [23], and after that, they were incubated at 37°C for 18–24 h. Diameters of inhibitory zones induced by extracts were then measured to evaluate their antibacterial effects, and interpreted as follows: diameter ≥30 mm: good inhibition, 15 mm ≤diameter <30 mm: intermediate inhibition, and diameter <15 mm: weak inhibition [24].

Statistical analysis

Data were expressed as mean ± standard error (mean ± standard error of mean). Data were analyzed using the Statcel software (Yanai Hisae, Laboratory of Mathematics, Faculty of Science, Saitama University, 1998). One-way ANOVA followed by *post hoc* Bonferroni test was used to compare the effects of different concentrations of each extract and paired *t*-test was used to compare the effects of two different extracts at the same concentrations. Significance was established when the probability levels were <5% ($p < 0.05$).

RESULTS

Althelmintic of aqueous extracts from Chinese honeysuckle seeds and Areca nuts on veterinary endoparasites

To evaluate the anthelmintic effects of extracts on porcine ascarids (*A. suum*) and porcine flukes (*F. buski*), we measured and calculated the LT50 and LT100 of different concentrations. The results on porcine ascarids are shown in Table 1 and Fig. 1, while those on porcine flukes are shown in Table 2 and Fig. 2.

From Tables 1 and 2, we see that both of extracts exerted anthelmintic effects on the two tested endoparasites, and these effects were in dose-dependent manners, because following the decrement in concentrations, their LT50 and LT100 values were significantly increased. With porcine ascarids, Chinese honeysuckle seeds showed stronger anthelmintic effects, because, at the same concentrations, their LD50 and LT100 were always shorter than those from areca nuts, and significant differences were observed at the 1000 mg/ml and 500 mg/ml doses, with both LD50 and LD 100 values (Table 1). In addition, extracts of Chinese honeysuckle seeds were able to kill

Table 1: The LT50 and LT100 of extracts of Chinese honeysuckle seeds and areca nuts on porcine ascarids (*Ascaris suum*)

Concentration (mg/ml)	LT50 (min)		LT100 (min)	
	Chinese honeysuckle *seeds*	*Areca nuts*	Chinese honeysuckle *seeds*	*Areca nuts*
2000	20±3.4^{a}	31±4.6^{a}	35±3.1^{a}	41±4.2^{a}
1000	**29±5.2^{ab,*}**	47±6.6^{ab}	**55±7.2^{b,*}**	78±6.8^{b}
500	**60±5.9^{b,*}**	89±7.6^{b}	**78±8.5^{bc,**}**	106±9.4^{c}
250	**177±8.3^{c,***}**	-	**250±13.5^{c,***}**	-
125	**280±14.2^{cd,***}**	-	($^{\#}$80 ± 5.8)	-
62.5	-	-	-	-
31.3	-	-	-	-
15.6	-	-	-	-
Control	No parasites were death in the control group			

($^{\#}n$) means that there was only n % of experimental parasites died after 360 min of observation. (-) means that there were no experimental parasites died after 360 min observation time. Values in the same column with different superscripts (a, b, c, d) are significantly different ($p<0.05$) by one-way ANOVA and *post hoc* Fisher's least significant test. LT50 or LT100 values in the same row marked with a superscripted asterisk are significantly lower than LT50 or LT100 of another extract by paired *t*-test (*$p<0.05$, **$p<0.01$, ***$p<0.001$) and are represented in bold letters

Table 2: The LT50 and LT100 of extracts of Chinese honeysuckle seeds and areca nuts on porcine flukes (*Fasciolopsis buski*)

Concentration (mg/ml)	LT50 (min)		LT100 (min)	
	Chinese honeysuckle *seeds*	*Areca nuts*	Chinese honeysuckle *seeds*	*Areca nuts*
2000	63±4.8[a]	**43±6.2[a,*]**	75±5.1[a]	**63±7.3[a,*]**
1000	97±7.1[ab,*]	**67±7.4[ab,**]**	145±9.3[b]	**94±10.4[b,**]**
500	153±8.3[b,*]	**109±9.3[b,*]**	202±11.5[c]	**152±12.2[c,*]**
250	209±11.2[c]	**215±11.8[c,*]**	([#]62 ± 4.1)	305±14.3[d]
125	-	**238±13.5[c,***]**	-	**330±15.4[d,***]**
62.5	-	**321±12.2[d,***]**	-	70±8.8[#]
31.3	-	-	-	-
15.6	-	-	-	-
Control	No parasites were death in the control group			

([#]*n*) means that there was only n % of experimental parasites died after 360 min of observation time. (-) means that there were no experimental parasites died after 360 min of observation time. Each value represents the mean±SE for three groups, in which each group contained 10 experimental individuals. Values in the same column but with different superscripts (a, b, c, d) are significantly different (p<0.05) by one-way ANOVA and *post hoc* Fisher's least significant test. Values in the same row but marked with a superscripted asterisk indicate the significantly lower LT50 or LT100 time by paired *t*-test (*p<0.05, **p<0.01, ***p<0.001) and are represented in bold letters. SE: Standard error

Table 3: The inhibitory zones of different concentrations of Chinese honeysuckle seeds and areca nuts aqueous extracts on *Escherichia coli* bacterial strains

Concentration (mg/ml)	Diameter of inhibitory zone (mm)	
	Chinese honeysuckle *seeds*	*Areca nuts*
2000	**31.4±2.8[a]**	**41.4±3.1[a,**]**
1000	27.2±1.9[ab]	**37.3±2.2[ab,**]**
500	21.7±1.7[b]	**33.5±1.7[b,*]**
250	15.5±1.4[c]	27.8±2.1[c,*]
125	10.1±0.9[cd]	19.6±1.6[cd,*]
62.5	8.1±0.7[d]	15.2±1.5[d,*]
31.3	-	9.1±0.9[e,***]
15.6	-	8.3±0.6[e,***]
Control	No inhibitory zone	

(-) means that there were no inhibitory zones. Each value represents the mean±SE for 10 *Escherichia coli* bacterial strains. Values in the same column with different superscripts (a, b, c, d) are significantly different (p<0.05) by one-way ANOVA and *post hoc* Fisher's least significant test. Values in the same row marked with superscripted asterisk indicate that these inhibitory zones were significantly wider when compared with those of other extracts by the paired *t*-test (*p<0.05, **p<0.01, ***p<0.001). The bold letter indicates the good inhibitory effects (with diameter≥30 mm). SE: Standard error

100% of tested ascarids at 250 mg/ml concentration (LT100: 250 ± 13.5 min), while areca nut extracts at this concentration induced no death on these parasites. In contrast, areca nuts exerted stronger effects on porcine flukes. At same concentrations, LD50 and LT100 of this extract were always shorter than those from Chinese honeysuckle seeds, and significant differences were observed at the concentrations of 2000, 1000, 500, 250, 125, and 62.5 mg/ml, in either LD50 or LD 100 or both values (Table 2). In addition, an extract of areca nuts had the ability to kill 100% of tested porcine flukes at the concentration of 125 mg/ml (LT100 = 330 ± 15.4 min), while at this concentration, Chinese honeysuckle seeds did not induce any death on the tested parasites.

Antibacterial effects of aqueous extracts from Chinese honeysuckle seeds and Areca nuts on *E. coli* strains

Inhibitory zones induced by aqueous extracts from Chinese honeysuckle seeds and areca nuts on *E. coli* strains are shown in Table 3 and Fig. 3.

We observed that both of the two extracts exerted antibacterial effects on *E. coli* strains, because they induced inhibitory zones when tested with the cylinder plate diffusion method. These effects were in dose-dependent manners, because the following decrement in extract concentrations, the inhibitory zone diameters were significantly decreased (Table 3). In addition, areca nut extracts showed stronger antibacterial effects, because, at similar concentrations, their induced inhibitory zones were always wider than those induced by extracts of Chinese honeysuckle seeds, and the significances were observed with all tested concentrations. Furthermore, areca nuts started to exert inhibitory zones from the concentration of 15.6 mg/ml (diameter: 8.3 ± 0.6 mm), while Chinese honeysuckle seeds started to induce inhibition zones at the four-time higher concentration (62.5 mg/ml, diameter: 8.1 ± 0.7 mm).

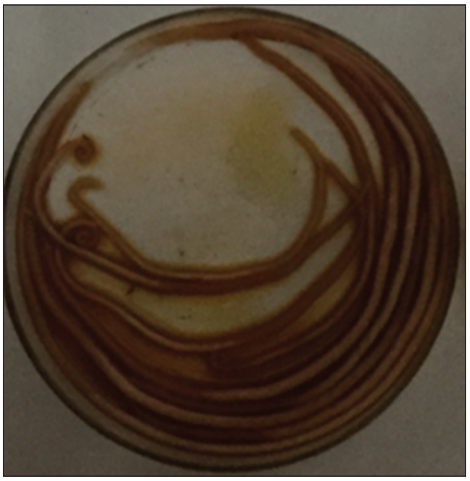

Fig. 1: Paralysis of porcine ascarids (*Ascaris suum*) under effects of 500 mg/ml aqueous extract from Chinese honeysuckle seeds

Fig. 2: Paralysis of porcine flukes under effects of 500 mg/ml aqueous extract from Areca nuts

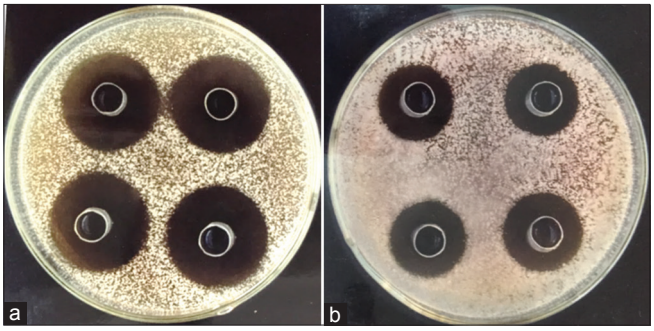

Fig. 3: Inhibitory zones induced by aqueous extract from (a) areca nuts and (b) Chinese honeysuckle seeds at the concentration of 500 mg/ml

DISCUSSION

Our results demonstrated the *in vitro* anthelmintic and antibacterial properties of Chinese honeysuckle seeds and areca nuts on both of animal endoparasites and bacteria, and therefore partly confirm they are traditional apply to treat diseases associated with endoparasite and bacterial infection. Similar to our investigation, the concurrent effects on both endoparasites and bacteria have been previously observed with several plants [25-28]. The mechanisms that by which a plant exerts multi-therapeutic functions have not yet been fully established and might be various depending on each plant, but researchers have suggested that the presence of several, and not one, active components is responsible for this mode of action [18,25,29]. The capacity of Chinese honeysuckle seeds and areca nuts to simultaneously target many pathogens in digestive tracts, including ascarides, flukes, and bacteria might fulfill a multiple target strategy that has been proposed for future drugs [18,25,29,30]. However, because our study has just demonstrated their *in vitro* effects, the *in vivo* experiments are still required to confirm their treatment efficiency, and also to assess their safety and side effects on host animals [31,32]. These future researches would provide more evidence and support the use of these herbs as cheap and safe natural remedies for both antiparasitic and antibacterial diseases [33].

Based on our observations, Chinese honeysuckle seeds were superior in the effects on ascarides, while areca nuts exerted stronger inhibitions on flukes and bacteria. These results suggested that depending on infected pathogens, the selection of suitable medicine plants is necessary to produce high treatment effects. In addition, following the traditional knowledge and experiences, it is more common for a therapeutic plant to be used in mixtures with other materials than applied alone [34]. Synergistism induced by mixtures of several plants might be explained through the presence of various photocomponents, which at the same time concurrently enhance effects on one pathogenic factor, or stimunously target multiple factors involved in the diseases [35]. Chinese honeysuckle seeds and areca nuts have been traditionally applied as both of a singular therapy and as an ingredient of phytomaterial mixtures [10-12]. There are also folk therapies in which these two plants are concurrently applied to treat the diseases of endoparasites and digestive disorders [36,37]. In our opinion, it is worth to investigate their functions when being applied in different forms, so as to verify the advantages of each way and propose the appropriate use in different treatment. These background researches will help to provide further insight into their therapeutic potentials and broaden their apply, both as anthelmintic and antibacterial phytodrugs.

CONCLUSION

This study demonstrated the *in vitro* anthelmintic and antibacterial effects of Chinese honeysuckle (*Q. indica* L) seeds and Areca (*A. catechu*) nuts on animal endoparasites and bacterial strains, and therefore partly explains the traditional therapeutic uses of these medicine plants. While Chinese honeysuckle seeds showed better effects on porcine arcarides, areca nuts exerted stronger inhibition on both of porcine flukes and *E. coli* bacteria. However, further researches are necessary to identify their active components and verify their *in vivo* effects in the experimental models.

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We are grateful to Dr. Tho Thi Bui for the identification of plant materials and the authentification of experimental endoparasites.

CONFLICTS OF INTEREST

None of the authors have any competing interests to declare.

AUTHORS' CONTRIBUTIONS

This study was performed in the collaboration between all authors. Hai Thanh Nguyen and Thanh Van Nguyen were the main investigators of the study. Other authors, including Miyamoto Atsushi and Ha Thi Thanh Nguyen participated in the extraction of medicinal plants and assisted the first two authors in all *in vitro* experiments. Thanh Van Nguyen designed the study, wrote the manuscript and is the corresponding author of this research.

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SUPPLEMENTARY DATA

Supplementary data 1: Phenotypes of antimicrobial resistance of 10 tested *Escherichia coli* isolated from pig feces

Phenotypes of resistance	Number of resistant isolates (%)
CHL-CTC-STR-NAL-SDM	1 (10.0)
CLH-CTC-STR-SDM	2 (20.0)
CLH-CTC-NAL-SDM	3 (30.0)
CLH-CTC-SDM	3 (30.0)
SDM	1 (10.0)
Resistant from 1 to 5 antibiotics	10 (100)

(a) CHL: Chloramphenicol, CTC: Chlortetracycline, STR: Streptomycin, NAL: Nalidixic acid, SDM: Sulfadimethoxine. (b) Breakpoints suggested by the Clinical and Laboratory Standards Institute were used for determination of resistance to CHL (32 μg/ml), CTC (16 μg/ml), NAL (16 μg/ml), and SDM (512 μg/ml). The breakpoint of STR was 32 (μg/ml)