ANTIBACTERIAL ACTIVITY OF THE ETHYL ACETATE AND METHANOL EXTRACTS OF CLAUSENA HARMANDIANA AGAINST BACTERIA ISOLATED FROM OTITIS EXTERNA LESIONS OF DOGS

[1]ARINEE CHATCHAWANCHONTEERA, [2]PEERAPOL SUKON, [3]CHAWEE YENJAI, [4]NATTHATHIDA RUENGSRITUNYAKIT, [5]RACHANIDA YINGDON, [6]WICHAYEN ARCHWICHAI

[1]Department of Pathobiology, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, 40002
[2,3]Department of Anatomy, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, 40002
[4]Research Group for Preventive Technology in Livestock, Khon Kaen University, Khon Kaen, 40002
[5]Department of Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen, 40002
[6]Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen, 40002
E-mail: arinee@kku.ac.th

Abstract -
Aim: Otitis externa is a common disease in dogs. Although this disease has multifactorial causes, bacteria play an important role to increase the severity of the disease. In this study, we compared antibacterial activity of the ethyl acetate and methanol extracts of Clausena harmandiana root bark against bacteria isolated from otitis externa lesions of dogs.
Materials and Methods: Clausena harmandiana root bark was collected and ethyl acetate and methanol extracts were prepared. Antibacterial activities of the extracts were tested using the broth microdilution method with 5 replications against 37 samples of bacteria isolated from otitis externa lesions of dogs. The isolated bacteria were 2 Gram-positive bacteria; Staphylococcus aureus, β-hemolytic Streptococcus and 4 Gram-negative bacteria; Klebsiella spp., Pseudomonas aeruginosa, Escherichia coli, and Proteus mirabilis. Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923 were also used as the standard controls. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined for two extracts.
Results: Both ethyl acetate and methanol extracts have comparable MIC and MBC activity against Gram-positive bacteria. In contrast, the ethyl acetate extract showed significantly higher MIC values against Gram-negative bacteria than the methanol extract. As an overall effect of both extracts, MIC and MBC values against Gram-positive bacteria were significantly lower than that against Gram-negative bacteria.
Conclusions: Both ethyl acetate and methanol extracts of Clausena harmandiana root bark have antibacterial activity against bacteria isolated from otitis externa lesions of dogs. They showed stronger inhibitory activity against Gram-positive bacteria than Gram-negative bacteria.

Keywords: Clausena harmandiana, otitis externa, antibacterial activity, MIC, MBC, dogs.

I. INTRODUCTION

Otitis externa, an acute to chronic inflammation of the external ear, is common in dogs accounting for up to 20% in small animal practices [1, 2]. Otitis externa is caused by multifactorial factors that may be classified into the following categories: predisposing (eg., conformational abnormalities, increased moisture and obstruction), primary (eg., ectoparasites, foreign bodies, allergies, and keratinization disorders), secondary and perpetuating factors (eg., bacteria and yeast) [3-5]. Although bacteria are not considered as the primary causes of otitis externa, they are associated with the severity and complication of the disease decreasing the quality of life of dogs. The major bacteria isolated from otitis externa of dogs were Staphylococcus pseudintermedius, Pseudomonas aeruginosa, Proteus spp., Streptococcus spp., Escherichia coli and β-hemolytic streptococci [6-8]. Along with bacterial identification, to find the right choices for antibiotic treatment, antimicrobial susceptibility of the bacterial isolates was also determined [7-10]. Because the extensive use of antibiotics is known to induce drug resistance resulting in treatment failure, alternative treatment methods such as the use of medicinal plant extract has been exploring.

Medicinal plants belonging to genus Clausena have been currently under extensive studies [11-13]. From these studies, many plant species (eg., Clausena lansium, Clausena dentata, Clausena excavate, Clausena wallichii, and Clausena anisata) have been identified as having chemical constituents [11-16]. These chemical constituents, interestingly, were extracted from different parts of the plants for example, the peel [11],the seed [17], the leaf [18, 19], the stem [20, 21], the stem bark [22], the twig [23], and the root [14, 24]. Most chemical constituents identified were carbazole alkaloids and coumarines [11-13, 16, 23-26]. In addition, these chemical constituents have many beneficially biological activities for example, antibacterial [22, 27], antifungal [28, 29], antioxidant [22], anticancer [30], anti-inflammatory [13, 31], antiplasmodial [32], larvicidal [15, 33], and analgesic [32] activities.

The focus of our study was on the species of Clausena harmandiana. This plant is small, evergreen trees or a shrub of about 40-70 cm in height (Figure-1) and is found in the northeast region of Thailand. Like other plant species of the genus Clausena described above, Clausena harmandiana has some

beneficially biological properties for example, antibacterial activity [34], antiplasmodial activity [35], and stimulating glucose uptake[36]. In veterinary medicine, the published report of Clausena harmandiana is rare. Therefore, the objective of this study was to determine the antibacterial activity of ethyl acetate and methanol extracts of Clausena harmandiana root bark against bacteria isolated from otitis externa lesions of dogs.

II. MATERIALS AND METHODS

Clausena harmandiana extract preparation

Clausena harmandiana was collected from Khon Kaen province and was identified by Dr. Pranom Chantaranothai, Faculty of Science, Khon Kaen University, Thailand. A botanically voucher specimen (KK98071790) was deposited in the herbarium of the Thai Forest Herbarium. The root bark was air-dried for 3 hrs and then was extracted with ethyl acetate or methanol (20 L) for 17 hrs. The extracts were filtrated and concentrated by rotary evaporator.

Bacterial suspension preparation

The swab samples were collected from otitis externa lesions of 37 dogs and the causative bacteria were isolated by the conventional culture method. The isolated bacteria were: 10 Staphylococcus aureus isolates, 4 β-Haemolytic Streptococcus isolates, 4 Klebsiella spp. isolates, 10 Pseudomonas aeruginosa isolates, 4 Escherichia coli isolates and 5 Proteus mirabilis isolates. S. aureus ATCC 25923 and E.coli ATCC 25922 were included as standard controls. The isolated bacteria were cultured on blood agar for 24 hrs, and then Mueller Hinton broth for 2-6 hrs. The concentration was adjusted to 0.5 McFarland and diluted to 1: 100 by NSS to make up the final concentration of 10^6 cells /ml.

Antibacterial activity assay

Antibacterial activity was determined as the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). MIC is defined as the least concentration (highest dilution) of the plant extract that can produce no visible bacterial growth (no turbidity when compared with the control tubes). MBC is defined as the least concentration (highest dilution) of the plant extract that can completely kill tested bacteria and is done by sub-culturing the tested dilution on standard plate count agar (Difco™, Becton, USA) and blood agar for 18-24 hrs at 37ºC. To get both MIC and MBC values, the experiment was performed according to National Committee for Clinical Laboratory Standards (NCCLS, 2000).

Statistical analysis

MIC and MBC values were evaluated for their normal distribution with graphical display and Shapiro-Wilk test. Both MIC and MBC values were not normally distributed, so that these values were reported as median (interquartile range). Mann-Whitney U test was used to compare MIC or MBC values of ethyl acetate extraction versus methanol extraction or of Gram-positive bacteria versus Gram-negative bacteria. Data were analyzed with statistical software SPSS version 17 (SPSS Inc., Chicago, Ill). For all comparisons, values of $P<0.05$ were considered significant with 2 tailed-test.

III. RESULTS

The result of this study showed that both ethyl acetate and methanol extracts of Clausena harmandiana root bark had antibacterial activity against bacteria isolated from otitis externa lesions of dogs. Both extracts showed degree of inhibition stronger for the Gram positive than for Gram negative bacteria. For the ethyl acetate extract, MIC values were significantly lower for Gram positive than for Gram negative bacteria (median [interquartile range or IQR], mg/ml, 5.21 [8.47] vs 41.67 [62.50], $P<0.001$); MBC values were also significantly lower for Gram positive than for Gram negative bacteria (median [IQR], mg/ml, 5.21 [8.47] vs 41.67 [41.66], $P<0.001$). For the methanol extract, MIC values were significantly lower for Gram positive than for Gram negative bacteria (median [IQR], mg/ml, 5.21 [5.21] vs 20.83 [20.84], $P<0.001$); MBC values were also significantly lower for Gram positive than for Gram negative bacteria (median [IQR], mg/ml, 10.42 [15.62] vs 41.67 [20.84], $P<0.001$).

MIC and MBC values for Gram positive bacteria were shown in Table 1 and Table 2; those for Gram negative bacteria were shown in Table 3 and Table 4. When compared efficacy of antibacterial activity of the ethyl acetate extract versus of the methanol extract, in over all, no significant differences in the efficacy have been observed between the extracts. However, in some Gram negative bacteria (e.g., P. aeruginosa and E. coli), MBC values were significantly lower in methanol extract than in ethyl acetate extract. However, both extracts showed significantly more antibacterial activity against Gram positive bacteria than Gram negative bacteria (Figure-2).

DISCUSSION

In this study, we found that both ethyl acetate and methanol extracts of Clausena harmandiana had antibacterial activity against bacteria isolated from otitis externa lesions of dogs. In over all, ethyl acetate and methanol extracts were both equally effective in inhibiting the tested bacteria except some Gram negative bacteria (P. aeruginosa and E. coli).

The evidence that supports in vitro antibacterial activity of the extracts in this study is MIC and MBC values of the extracts (Table 1-4). In addition to our study reported here, some previous studies reported

antibacterial activity of Clausena harmandiana extracts. In our previous studies reported in a local veterinary journal, the extracts of Clausena harmandiana were shown to have antibacterial activity against pathogenic bacteria isolated from animals such as dogs, pigs and fish [37, 38]. Carbazole alkaloids extracted from Clausena harmandiana twigs had antibacterial activity against pathogenic bacteria such as Salmonella typhimurium and methicillin-resistant Staphylococcus aureus [34]. Carbazole alkaloids, the most chemical constituents extracted from the plant genus Clausena, have also been isolated from the root bark of Clausena harmandiana [39]. Therefore, although we did not know which chemical constituents of the extract were responsible for the antibacterial activity in the present study, we believed that antibacterial activity of the ethyl acetate and methanol extracts of Clausena harmandiana root bark may come from the carbazole alkaloids in the extracts.

In addition to Clausena harmandiana, other plant species of genus Clausena had antibacterial or antifungal or larvicidal activity. The ethanol extract of the stem bark of Clausena hepaphylla, containing flavonoids, alkaloids, saponins and steroids, were associated with antibacterial, antifungal and antioxidant activities [22]. Carbazole alkaloids from the peels of Clausena lansium and from the roots of Clausena wallichii had antibacterial activity against Staphylococcus aureus [12, 14]. The leaf extracts of Clausena anisata had antifungul activity against Aspegillus fumigatus [40]. In addition, a new antifungal coumarin has been isolated from the leaves of Clausena excavate [29]. The leaf extracts of Clausena dentata and the seed extracts of Clausena lansium had larvicidal activity against larvae of some mosquitoes species that act as vectors of dengue and malaria [15, 33]. Moreover, antiplasmodial activity (against Plasmodium spp.) has been observed with the leaf extracts of Clausena anisata[32] and with coumarins and carbazoles extracted from Clausena harmandiana [35].

In this study, we also found that the ethyl acetate and methanol extracts showed better inhibitory activity against Gram positive bacteria than Gram negative bacteria. This finding is interesting because the results from antibiotic susceptibility tests of the bacteria isolated from dogs with otitis externa have shown that both Gram positive and Gram negative bacteria were resistant to the antibiotics tested. For example, more than half of the Gram positive bacteria were resistant to penicillin and ampicillin and all Gram negative bacteria were resistant to penicillin, erythromycin and cephalothin [8]. In one study, beta-hemolytic streptococci resisted to all of the antibiotics tested [9].

As described in the introduction section, otitis externa in dogs is caused by multifactorial factors; therefore, treatment options depend on determining all causative factors. For microorganisms that involved in otitis externa in dogs, not only bacteria infection (commonly Staphyloccocus pseudintermedius, Pseudomonas aeruginosa and Streptococcus spp.) but also yeast infection (commonly Malassezia spp.) can augment the inflammation of the external ear [3]. As a consequence, use of antibiotics alone may not be enough for the successful treatment. In this study, we just discovered that the extracts have antibacterial activity against bacteria isolated from otitis externa lesions of dogs; therefore, it does not mean that the extracts can successfully treat this disease in vivo because as described above many factors may contribute to the disease.

This study has some limitations that can bias the results and further applicability. First, reading MIC values is not easy because of the precipitation of Clausena harmandiana extracts at the bottom of the microplate well. Reading MBC values, in contrast, is quite clear for the inhibitory activity against the study bacteria. Thus, in this study, MBC values are more reliable than MIC values. Second, many factors may associated with MIC and MBC values, for example, source or location of the plant, age of the plant, time of sampling and the extraction methods. These factors may be attributable to effectiveness of the plant extracts. Third, in this study, concentrations of the crude extracts that can kill the tested bacteria are high (in mg/ml); this may cause a problem in a real clinical use. However, this problem may be resolved after the extracts are purified. For instance, a concentration of 0.25 µg/ml of purified carbazole alkaloids extracted from Clausena harmandiana can inhibit methicillin-resistant Staphylococcus aureus [34]. Fourth, although the extracts are clearly shown in inhibiting the tested bacteria in vitro, it does not mean that the extracts can successfully use in vivo because many factors are involved. Therefore, many works need to be done for a real clinical use. For example, the crude extracts would be purified for their active ingredients; then the purified products must be tested both in vitro and in vivo against bacteria isolated from otitis externa lesions of dogs.

In conclusion, both ethyl acetate and methanol extracts can in vitro inhibit both Gram positive and Gram negative bacteria isolated from otitis extrena lesions of dogs. However, for clinical application, many works need to be done.

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Competing interests

All authors have no competing interests for this study.

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Tables:

Table-1: Minimal inhibitory concentration (MIC) values (mg/ml) of Clausena harmandiana root bark extract against pathogenic Gram positive bacteria.

Bacteria	Ethyl acetate	Methanol	P-values*
S. aureus (n=10)	5.21 (9.77)	7.82 (7.81)	0.184
β -Hemolytic Streptococcus (n=4)	7.82 (15.95)	5.21 (0)	0.508
S. aureus ATCC 25923 (n=1)	7.82 (0)	7.82 (0)	NA
Over all Gram positive bacteria (n=14)**	5.21 (8.47)	5.21 (5.21)	0.399

Values are medians (interquartile ranges).
*P-values from Mann-Whitney U test
**S. aureus ATCC 25923 was excluded from the calculation.
NA=not applicable

Table-2: Minimal bactericidal concentration (MBC) values (mg/ml) of Clausena harmandiana root bark extract against pathogenic Gram positive bacteria.

Bacteria	Ethyl acetate	Methanol	P-values*
S. aureus (n=10)	5.21 (9.77)	10.42 (15.62)	0.087
β -Hemolytic Streptococcus (n=4)	7.82 (15.95)	7.82 (13.02)	0.765
S. aureus ATCC 25923 (n=1)	7.82 (0)	7.82 (0)	NA
Over all Gram positive bacteria (n=14)**	5.21 (8.47)	10.42 (15.62)	0.101

Values are medians (interquartile ranges).
*P-values from Mann-Whitney U test
**S. aureus ATCC 25923 was excluded from the calculation.
NA=not applicable

Table-3: Minimal inhibitory concentration (MIC) values (mg/ml) of Clausena harmandiana root bark extract against pathogenic Gram negative bacteria.

Bacteria	Ethyl acetate	Methanol	P-values*
Klebsiella spp. (n=4)	62.50 (104.16)	41.67 (46.87)	0.278
P. aeruginosa (n=10)	20.83 (33.86)	20.83 (2.60)	0.442
E. coli (n=4)	83.33 (0.00)	31.25 (20.84)	0.013
P. mirabilis (n=5)	41.67 (10.42)	41.67 (41.67)	0.905
E. coli ATCC 25922 (n=1)	62.50 (0)	41.67 (0)	NA
Overall Gram negative bacteria (n=23)**	41.67 (62.50)	20.83 (20.84)	0.050

Values are medians (interquartile ranges).
*P-values from Mann-Whitney U test
**E. coli ATCC 25922 was excluded from calculation.
NA=not applicable

Table-4: Minimal bactericidal concentration (MBC) values (mg/ml) of Clausena harmandiana root bark extract against pathogenic Gram negative bacteria.

Bacteria	Ethyl acetate	Methanol	P-values*
Klebsiella spp. (n=4)	62.50 (104.16)	41.67 (31.24)	0.405
P. aeruginosa (n=10)	41.67 (15.63)	20.83 (5.21)	0.021
E. coli (n=4)	83.33 (0.00)	41.67 (31.24)	0.040
P. mirabilis (n=5)	41.67 (0.00)	41.67 (31.25)	1.000
E. coli ATCC 25922 (n=1)	62.50 (0)	41.67 (0)	NA
Overall Gram negative bacteria (n=23)**	41.67 (41.66)	41.67 (20.84)	0.104

Values are medians (interquartile ranges).
*P-values from Mann-Whitney U test
**E. coli ATCC 25922 was excluded from calculation.
NA=not applicable

Figure legends:



Figure-1: The mature plant of Clausena harmandiana.

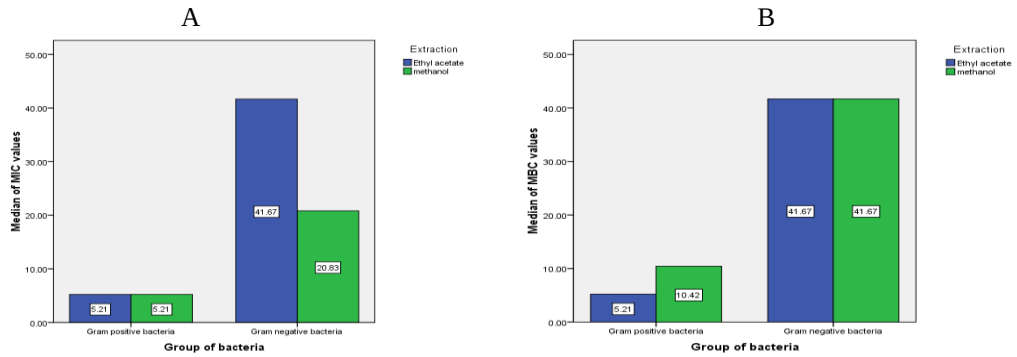


Figure-2: Medians of the MIC and MBC values. Panel A shows medians of the MIC values. For both extracts, MIC values was significantly lower in Gram positive bacteria than in Gram negative bacteria (P<0.001). Panel B shows medians of the MBC values. For both extracts, MBC values was significantly lower in Gram positive bacteria than in Gram negative bacteria (P<0.001).

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