UNIVERSITI PUTRA MALAYSIA

ANTIBACTERIAL EFFECT OF CINNAMON (CINNAMOMUM VERUM) AGAINST BACTERIA ISOLATED FROM CATS WITH OTITIS EXTERNA

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FACULTY OF VETERINARY MEDICINE

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ANTIBACTERIAL EFFECT OF CINNAMON (*CINNAMOMUM VERUM*) AGAINST BACTERIA ISOLATED FROM CATS WITH OTITIS EXTERNA

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It is hereby certified that I have read this project paper entitled "Antibacterial Effect of Cinnamon (*Cinnamomum verum*) against Bacteria Isolated from Cats with Otitis Externa" by Aimi Najwa Binti Mokhtar, and in my opinion it is satisfactory in term of scope, quality and presentation as partial fulfilment of the requirement for the course VPD 4999 – Project.

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DEDICATION

"This project is dedicated especially to my parents, my siblings, my nieces and nephew, my friends and my cats"

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek.

KESAN ANTIBAKTERIA KULIT KAYU MANIS (*CINNAMOMUM VERUM*) TERHADAP BAKTERIA YANG DIASINGKAN DARIPADA KUCING YANG MENGHIDAP OTITIS EKSTERNA

Oleh

Aimi Najwa binti Mokhtar

2016

Penyelia: Prof. Madya Dr. Siti Khairani binti Bejo

Tiga puluh (30) sampel sapuan telinga diambil dari kucing di klinik swasta. Sampel-sampel itu kemudiannya dikultur untuk tujuan pengasingan dan pengenalpastian bakteria. Sifat antibakteria *Cinnamomum verum* dikenalpasti dengan menjalankan ujian sensitiviti antibiotik (AST), inhibasi konsentrasi minima (MIC), bakteriasidal konsentrasi minima (MBC) terhadap bakteria yang diasingkan daripada sampel iaitu *Staphylococcus pseudintermedius, Staphylococcus intermedius, Staphylococcus hyicus, Enterococcus faecalis, Corynebacterium ulcerans, Bacillus* sp. dan *Aggregatibacter actinomycetemcomitans*. Pes kayu manis disediakan dengan mencampurkan serbuk kayu manis dengan air suling yang steril untuk digunakan dalam ujian-ujian tersebut. Kesemua bakteria yang diuji menunjukkan

kecenderungan terhadap pes kayu manis dan beberapa daripadanya menunjukkan rentan terhadap antibiotik yang digunakan secara komersil seperti marbofloxacin, doxycycline dan enrofloxacin. Diameter zon inhibisi (DIZ) bagi setiap bakteria yang diuji dengan antibiotik dan pes kayu manis menunjukkan perbezaan yang signifikan ($p = 0.000$). Hasil ujian MIC menunjukkan kadar aktiviti maksima bagi pes kayu manis adalah daripada 0.03 hingga 1.00g/ml. Hasil ujian MBC pula menunjukkan konsentrasi pes kayu manis yang mampu menghalang pertumbuhan atau membunuh bakteria yang diuji adalah daripada 0.03 hingga 0.67g/ml. Berdasarkan hasil ujian yang diperoleh, ia menunjukkan bahawa *Cinnamomum verum* mempunyai sifat antibakteria terhadap bakteria yang diasingkan daripada kucing yang menghidap otitis eksterna dan ia berpotensi untuk digunakan sebagai rawatan alternatif untuk kes-kes tersebut.

Kata Kunci: *Cinnamomum verum*, kesan antibakteria, otitis eksterna

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Project.

ANTIBACTERIAL EFFECT OF CINNAMON (*CINNAMOMUM VERUM*) AGAINST BACTERIA ISOLATED FROM CATS WITH OTITIS EXTERNA

By

Aimi Najwa Binti Mokhtar

2016

Supervisor: Assoc. Prof. Dr. Siti Khairani binti Bejo

Thirty (30) ear swab samples were taken from cats in a private clinic. The samples were then cultured on blood agar for isolation and identification of bacteria. Antibacterial effect of *Cinnamomum verum* was determined by conducting antibiotic sensitivity test (AST), minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against bacteria isolated from the samples which include *Staphylococcus pseudintermedius, Staphylococcus intermedius, Staphylococcus hyicus, Enterococcus faecalis, Corynebacterium ulcerans, Bacillus* sp. and *Aggregatibacter actinomycetemcomitans*. Cinnamon paste was prepared by mixing cinnamon powder with sterile distilled water for the usage in the tests. All of the bacteria tested showed susceptibility towards the cinnamon paste and some showed resistance towards some of the commercially used antibiotics such as

marbofloxacin, doxycycline and enrofloxacin. There were significant differences in the diameter of inhibition zone (DIZ) of each bacterium tested against different antibiotics and the cinnamon paste ($p = 0.000$). The MIC test result of the cinnamon paste showed its maximum activity values that range from 0.03 to 1.00g/ml. The MBC test shown that the concentration of cinnamon paste that could have inhibited the growth or kill the bacteria tested was ranges from 0.03 to 0.67g/ml. Based on the test results, it suggested that *C. verum* do have antibacterial effect against bacteria isolated from cats with otitis externa and it may be used as the alternative treatment for such cases.

Keywords: *Cinnamomum verum*, antibacterial effect, otitis externa

1.0 INTRODUCTION

Otitis refers to the inflammation of ear canal or pinna regardless what the causes are or the clinical presentation (Jackson and Marsella, 2012). It can be categorized into otitis externa, otitis media and otitis interna. In cats, removal of normal ear canal excretion by using cotton swabs is the most common cause of feline otitis (Kennis, 2013). Cats that are kept in environment with high humidity level and frequently bathed are also to be at higher risk in getting otitis due to canal tissue maceration (Kennis, 2013).

Under normal circumstance, opportunistic bacteria are present in low number and are considered as normal flora. However, once the ear canal is affected, those bacteria will flare up and cause secondary infection. *Staphylococcus spp., Escherichia coli, Corynebacterium spp.,* and *Pseudomonas spp.* are commonly isolated from cases of otitis (Dowling, 1996). In cases of acute otitis related with infections, *Malassezia* or gram positive cocci bacteria; typically *Staphylococcus pseudintermedius* can be seen with or without purulent infiltrate (Jackson & Marsella, 2012).

Aminoglycoside antibiotics such as neomycin and gentamicin are typically used for treatment of otitis externa due to their efficacy against staphylococci and gram negative bacteria (Dowling, 1996). Usage of fluoroquinolones such as enrofloxacin and marbofloxacin in treating cases of otitis has also been practiced nowadays. However, there are adverse effects from the uses of this type of antibiotics and some bacteria start to develop resistant to common antibiotics. Therefore, an alternative medication is needed to overcome the problem.

Cinnamomum verum which is also known as *Cinnamomum zeylanicum* has essential oil as its important characteristic. The essential oil components such as cinnamaldehyde and eugenol are the unique traits of this herb that allows it to act as the antibacterial agent. One of its mechanisms of actions is altering the bacterial membrane permeability when it is used synergistically with other antibiotic against gram negative bacteria (Hemaiswarya and Doble, 2009). However, there is lack of information on the antibacterial properties of *C. verum* sticks against bacteria isolated from cats with otitis externa. Hence the objectives of this study were

1. to isolate and identify the common bacteria present in the ear of cats with otitis externa.
2. to determine the presence of antimicrobial effect of *C. verum* to bacterial isolated from cats with otitis externa.

2.0 LITERATURE REVIEW

2.1 Otitis Externa

Otitis externa is referred to the condition where inflammation affects the external auditory canal starting from the pinna down to the tympanic membrane (Jackson and Marsella, 2012). It is important to differentiate the type of otitis as each of it will be subjected to different treatments approach. According to Jackson and Marsella, (2012), there are several different conditions in cases of otitis externa in cats contributed by the factors which include the onset time of the clinical symptoms, the rate of relapse and/or the clinical presentation. For instance, a case is classified as acute case if the clinical symptoms are presents for less than 7 days, as subacute case if the clinical symptoms are presents for more than 7 days but less than 30 days, and as chronic case if the clinical symptoms are presents for more than 30 days. Recurrent case is identified when it reappear on regular basis even after appropriate treatment. Suppurative case can be seen with various secretions but usually malodorous, in liquid form and associated with head shaking.

High humidity environments, frequently bathed and removal of normal ear canal excretions by using cotton swab are the common predisposing factors of cases of otitis in cats (Kennis, 2013). This eventually leads to traumatic injury to the ear canal and hence led to secondary bacterial infections. The pathogens that are commonly isolated from cases of bacterial otitis are *Staphylococcus spp., Escherichia coli, Corynebacterium spp.,* and *Pseudomonas spp.* (Dowling, 1996). In

addition, enterococci can also be isolated from cats with otitis externa (Hariharan *et al.*, 2006).

Otitis externa resulted when the surface defence mechanisms of the ear is disrupted by cutaneous or systemic diseases. This condition is favourable for the opportunistic bacteria to multiply and produce variety of enzymes and toxins that might harm the animal. One of the functions or mechanisms of the enzymes is of coagulase enzyme which produced by *Staphylococcus pseudintermedius, Staphylococcus aureus* and *Staphyloccocus hyicus*. This enzyme allows for deposition of fibrin on bacterial cells and thus inhibiting their recognition by the host phagocytes (Jackson and Marsella, 2012).

2.2 Antimicrobial Therapy

Otitis externa are best treated with topical antimicrobials whereas systemic antimicrobial therapy is only indicated in cases of severe otitis externa, otitis interna or otitis media in which the site of infection cannot be reached (Dowling, 1996). The selection of topical antibacterial agent should be based on the findings of bacterial culture or cytological examination as the incidence of antibiotic resistance is becoming increasingly common (Jackson and Marsella, 2012).

It is best to avoid usage of broad spectrum antibiotic as fluoroquinolones if narrow-spectrum antimicrobial is likely to be effective (Jackson and Marsella, 2012). This is one of the approaches that can be practiced to prevent occurrence of multidrug resistance bacteria. Furthermore, it is noted that if resistance does emerge

to one fluoroquinolone, it is likely to impact all fluoroquinolones (Boothe, 2015). Not all bacteria are susceptible to this group of antibiotic. Obligate anaerobs tend to be resistant to most quinolones, as are most enterococci; which include *Enterococcus faecalis* and *Enterococcus faecium* (Boothe, 2015). Apart from that, the used of high dosage of enrofloxacin in some cats have resulted in occurrence of retinal degeneration and thus its usage to treat cases of otitis in cats should be avoided (Kennis, 2013).

Due to increased risk of ototoxicity (Kennis, 2013) or occurrence of nephrotoxicity if being used systematically (Jackson and Marsella, 2012), usage of antibacterial preparations that contain aminoglycoside antibiotics should be avoided in cats. According to Dowling, (1996), bacteria that are resistant to amikacin will also be resistant towards other aminoglycosides. This statement is correspond with the finding of a study conducted by Hariharan *et al.*, 2006, in which coliforms that appear to be resistant when tested against amikacin were also resistant to gentamycin.

2.3 Cinnamomum verum

Cinnamomum verum is of the family Lauraceae. It is an important traditional herbal medicine which is widely distributed in China, Sri Lanka, Vietnam, Madagascar, Seychelles and India (Li *et al.*, 2012). The components of essential oil of indigenous *C. verum* bark from Colombo, Sri Lanka comprises of 50 to 55% of cinnamaldehyde and 4 to 7% of eugenol (Ranasinghe *et al.* , 2002). Antimicrobial

activity of eugenol and cinnamaldehyde, which are some of the most studied phenylpropenes are contributed by the free hydroxyl group (Nazzaro *et al.* , 2013; Laekeman *et al.*, 1990). Generally, the effects of antibacterial activity of the essential oil of the plant vary and they are influenced by the amount of specific compounds in it. For example, higher concentrations of cinnamaldehyde or eugenol confer antibacterial properties to the essential oil. The mechanisms used by eugenol to act against bacteria is by altering the membrane, affecting the transportation of ions and Adenosine Triphosphate (ATP) and changing the fatty acid profile of the bacteria. Meanwhile, for cinnamaldehyde, it inhibits enzymes that are involved in cytokine interactions at low concentrations, acts as an ATPase inhibitor at higher concentrations and disrupt bacterial cell membrane at lethal concentration (Nazzaro *et al*., 2013)

The antibacterial property of the essential oil is not solely contributed by a single mechanism. Instead, it involves different biochemical and structural mechanisms at multiple sites within and on the bacterial cells (Carson *et al.*, 2002). Those mechanisms include chemical modifications of cell membrane, cytoplasm, enzymes and proteins, complete alteration of the microbial cell conformation and compromisation of the bacterial metabolism through prolonged loss of ions or metabolites due to exposure to the components of essential oil. All of the mechanisms listed will eventually lead to cells death (Burt and Reinders, 2003; Burt, 2004). Most of the previous studies conducted were using the cinnamon against food-related bacteria and no studies were done on *C. verum* against bacteria isolated from cats with otitis externa.

3.0 MATERIALS AND METHODS

3.1 Ear swab collection

Thirty (30) cats in private clinic that are showing signs of ear scratching and having black or brown or yellowish ear wax were selected. Sterile swab was moistened with sterile normal saline and later used to swab the affected ear.

3.2 *Cinnamomum verum* paste preparation

Cinnamomum verum sticks were grounded and filtered to obtain fine cinnamon powder. The *C. verum* powder was mixed with sterile distilled water or nutrient broth at dilution of 1.0g/ml for the usage in antibiotic sensitivity test (AST) or minimum inhibitory concentration (MIC) respectively.

3.3 Bacteria isolation and identification

All of the ear swab samples were cultured on blood agar and incubated at 37°C for 24 hours. Gram staining (Appendix 1) was carried out on each isolates to determine their Gram reaction and cell morphology.

Biochemical tests (Appendix 2) for identification of Gram positive cocci bacteria included catalase test, coagulase test, blood broth, 6.5% sodium chloride, bile esculin and carbohydrate broths (mannitol, maltose). Biochemical tests such as sulphide indole motility (SIM), Voges-Proskauer (VP), Arginine dihydrolase (ADH)

and carbohydrate broths (mannitol, sorbitol, sorbose, arabinose, raffinose) were implemented for identification of *Enterococcus* sp.

Catalase test, coagulase test, urease test, blood broth, carbohydrate broths (trehalose, sucrose, glucose), nitrate broth, SIM were performed for identification of gram positive rods without endospores. As for gram positive rods with endospores, catalase test was done and followed by motility test to differentiate *Bacillus* sp.

Oxidase test was the first test performs for identification of Gram negative cocci bacterial isolates. Other tests include TSI (Triple sugar iron), SIM, urease test and citrate test. Gram negative rods bacterium were identified using oxidase test, SIM, TSI, urease test, citrate test and followed with carbohydrate broths (glucose, lactose, sucrose) test and Orthonitrophenyl-β-D-galactopyranoside (ONPG) test. All of inoculated test media mention above were incubated at 37°C for 24 hours.

3.4 Antibiotic sensitivity test (AST)

Staphylococcus pseudintermedius, Staphylococcus intermedius, Staphylococcus hyicus, Enterococcus faecalis, Corynebacterium ulcerans, Bacillus sp. and *Aggregatibacter actinomycetemcomitans* that have been isolated from ear swab sample of cats were selected for antibiotic sensitivity test (AST). The concentrations of bacterial inoculums were determined by comparing their turbidity with 0.5 McFarland Standard. Mueller Hinton (MH) agar was used for *Staphylococcus pseudintermedius, Staphylococcus intermedius, Staphylococcus hyicus, Bacillus* sp. and *Aggregatibacter actinomycetemcomitans* whilst blood agar

was used for *Enterococcus faecalis* and *Corynebacterium ulcerans*. The bacterial inoculums were overlaid by using sterile cotton swab on MH or blood agar accordingly. A well of 6.9mm in diameter was cut off from the agar and it was later filled in with cinnamon paste. Blank disc was included in each plate as negative control. In order to determine the susceptibility of these bacteria against the commonly used antibiotic in the private veterinary clinic and in University Veterinary Hospital (UVH), marbofloxacin (5µg), amoxicillin-clavulanic acid (30µg), doxycycline (30µg) and enrofloxacin (5µg) antibiotic discs were used as the reference standard or positive control. The plates were then incubated at 37°C for 24 hours. Six replicates have been made for each bacterium. The antibacterial activity was identified by measuring the diameter of inhibition zone (DIZ) in millimetre (mm).

3.5 Minimum inhibitory concentration (MIC)

Based on the result of antibiotic sensitivity test, bacteria that appear to be resistant to some of the commercially used antibiotic but showed zone of inhibition for the tested cinnamon paste were chosen to proceed with MIC. Broth dilution method using 10ml test tube was performed to determine the MIC for *Staphylococcus hyicus*, *Enterococcus faecalis* and *Bacillus* sp. One gram (1.0g) of cinnamon powder was diluted in 1.0ml nutrient broth. The diluted cinnamon was then serially diluted in two-fold dilution starting from 1.0g/ml until 0.03g/ml in nutrient broth (NB). Bacterial inoculums were prepared by inoculating the bacteria into sterile NB and the turbidity were compared to the 0.5McFarland standard.

Positive control was obtained by adding 10 antibiotic discs (Amoxicillin-Clavulanic Acid and Doxycycline) into 1ml of NB in test tubes whilst negative control tube was only contains NB. One millilitre (1.0ml) of bacterial inoculum was then added into each test tube which containing diluted cinnamon, negative and positive control. Each treatment was done in triplicate. All inoculated test tubes were then incubated at 37°C for 24 hours. Growth inhibitions of the bacteria (level of turbidity) in the test tubes were observed with unaided eyes as to determine the MIC values.

3.6 Minimum bactericidal concentration (MBC)

As the mixture/broth in the MIC tests were too turbid for the MIC value to be read, Minimum Bactericidal Concentration (MBC) was preceded. Ten microlitres (10 µl) of mixture in MIC test tube was withdrawn from each test tube and was then inoculated into blood agar. Inoculated blood agar plates were then incubated at 37°C for 24 hours. Minimum Bactericidal Concentration (MBC) values for the test samples were determined through visualization of area with no bacteria growth that was adjacent to the area with bacteria growth (Appendix 3).

4.0 RESULTS AND DISCUSSION

Eleven isolates were isolated from the 30 samples collected and majority of them were *Staphylococcus* sp. (Table 1 and Figure 1). Thirty percent (30%) of the samples were positive for non-pathogenic staphylococci. This finding was corresponds with previous study conducted by Hariharan *et al.,* 2006, which shown 75 out of 103 isolates (30%) were non-pathogenic staphylococci.

Malassezia or gram positive cocci bacteria particularly *S. pseudintermidius* can be seen with or without purulent infiltrate in cases of acute otitis associated with infections (Jackson and Marsella, 2012). This is reflected in the results of present study in which 30% of the samples taken positive for *S. pseudintermedius*. *Staphylococcus hyicus* was usually present in skin infections in pigs and cattle. The organism was also isolated from milk of cows (Songer and Post, 2005). In this present study, 20% of the samples taken were positive for *S. hyicus*. This suggests that *S. hyicus* is not only found in pigs and cattle, but it can also be found in cases of otitis externa in cats. The remainder consisted of small amount (6%) of isolates which include *S. intermedius, Bacillus* sp., and *E. durans*. Other bacteria isolated (3% each) were *E. faecalis, Chromobacterium, C. ulcerans, Dermatophilus* sp. and *Aggre. actinomycetemcomitans*.

Test samples which shown diameter of inhibition zone (DIZ) of above 7mm will be taken as positive result (Prabuseenivasan *et. al*, 2006). In this present study, the test sample was *C. verum* paste. The result shown, seven bacteria tested (*S. pseudintermedius, S. intermedius, S. hyicus, E. faecalis, C. ulcerans, Bacillus* sp. and *Aggre. actinomycetemcomitans)* were sensitive to *C. verum* paste (Table 2).

Statistical analysis reveals that, there were significant differences (p = 0.000) on the DIZ of each bacterium tested against the *C. verum* paste and the commercial antibiotics. Of the 6 Gram positive bacteria tested, *S. hyicus* (DIZ=19.17mm) was the most susceptible to the *C. verum* paste. This was followed by *S. pseudintermedius* (19.13mm), *S. intermedius* (18.67mm), *Bacillus* sp. (17.83mm), *E. faecalis* (10.94mm) and *C. ulcerans* (8.41mm). One Gram negative bacteria namely *Aggre. actinomycetemcomitans* showed susceptibility to the *C. verum* paste with DIZ of 15.40mm. Complete results of the AST were shown in Appendix 4.

The results of *C. verum* paste were consistent with the findings of the study conducted by Prabuseenivasan *et al.* , 2006, in which the sample tested was found to be equally effective against both gram negative and gram positive bacteria. According to Yap *et al.* , (2015) and Borges *et al.* , (2013), acidifying and denaturation of protein of the bacterial cell membrane occur due to accumulation of the components essential oil there. This leads to irreversible cell membrane damage. In addition to that, the disrupted cell membrane will eventually lead to extensive leakage or exit of important molecules and ions from bacterial cells and thus led to cells death (Prabuseenivasan *et al.* ,2006; Denyer and Hugo, 1991).

Based on the result of AST, *S. hyicus*, *E. faecalis*, and *Bacillus* sp. were selected to proceed with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests as they were the representative for *Staphylococcus* group and opportunistic bacterium apart from being the common bacteria isolated from the samples respectively.

The MIC and MBC value of *C. verum* against *E. faecalis* was the lowest (MIC=1.00g/ml; MBC=0.66g/ml ± 0.16) compared to *Bacillus* sp. (MIC=0.29g/ml ± 0.11; MBC=0.14g/ml ± 0.05) and *S. hyicus* (MIC= 0.03g/ml; MBC= 0.03g/ml). This might be due to emergence of resistance by this bacteria towards many antibiotics as it posses both intrinsic and acquired resistance trait as stated by Fraser *et al.* , 2015. Meanwhile, the presence of endospore in *Bacillus* sp. might be the reason that causes the MIC and MBC value of *C. verum* against this bacterium to be lower than *S. hyicus.* As stated in the website of Microbiology Department of Cornell University, (2016), endospore is a resistant differentiated cell produced under stressful conditions that make the bacteria to not readily kill by many antibiotics. This suggests that higher concentration of *C. verum* is needed to act on *E. faecalis* and *Bacillus* sp. than on *S. hyicus*.

Table 1: Total Number of Animals According to the Type of Bacteria Isolated

Bacteria Isolated	No. of Animals
Non pathogenic Staphylococcus	9
S. pseudintermedius	9
S. hyicus	7
S. intermedius	2
Bacillus sp.	2
E. durans	2
E. faecalis	1
Chromobacterium	1
C. ulcerans	1
Dermatophilus sp.	1
Aggre. actinomycetemcomitan	1

Figure 1: Total Number of Animals According to the Type of Bacteria Isolated

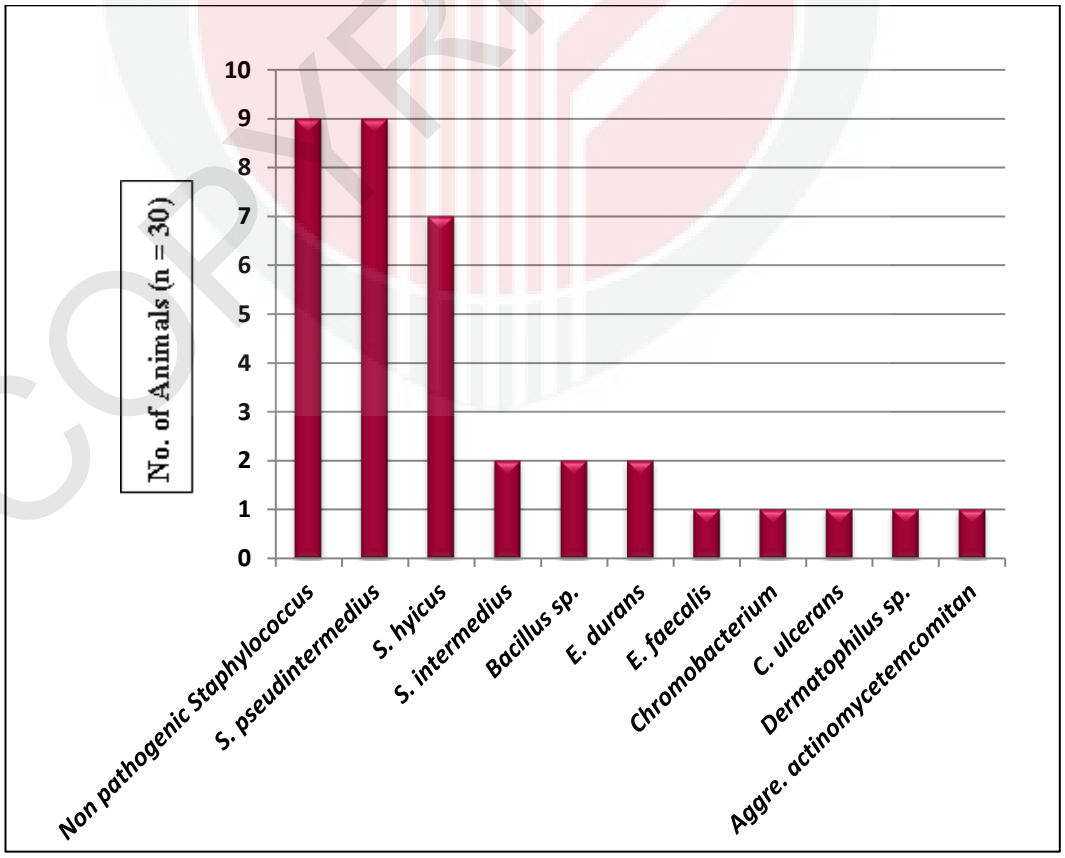

Table 2: The Sensitivity of Bacteria Isolated towards Commercial Antibiotics and the *C. verum* Paste

Antibiotics / Isolates	Marbofloxacin	Amoxicilin-Clavulanic Acid	Doxycycline	Enrofloxacin	*C. verum* Paste
S. pseudintermedius	Sensitive	Resistant	Sensitive	Sensitive	Sensitive
S.intermedius	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive
S. hyicus	Resistant	Sensitive	Sensitive	Sensitive	Sensitive
E. faecalis	Resistant	Sensitive	Resistant	Resistant	Sensitive
C. ulcerans	Sensitive	Resistant	Sensitive	Sensitive	Sensitive
Bacillus sp.	Resistant	Sensitive	Resistant	Resistant	Sensitive
Aggre. actinomycetemcomitans	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive

5.0 CONCLUSION AND RECOMMENDATIONS

In conclusion, from this study, the most common bacteria isolated from cats with otitis externa were *Staphylococcus* spp., *Bacillus* sp. and Enteroccoci. It was also found that *C. verum* do have antibacterial effect against *S. pseudintermedius, S. intermedius, S. hyicus, E. faecalis, C. ulcerans, Bacillus* sp. and *Aggre. actinomycetemcomitans*. The MIC and MBC value of *C. verum* paste against *S. hyicus* was the highest compared to *Bacillus* sp. and *E. faecalis*. *Cinnamomum verum* has the potential to be used as alternative treatment for otitis externa due to bacterial infection. Further research need to be done in order to determine the risk of toxicity of *C. verum* when being used on cats (in vivo). Besides, referring to previous studies conducted, usage of *C. verum* extraction oil against bacteria results in higher MIC value, thus usage of *C. verum* oil extraction is recommended for better distribution of the chemical composition of the essential oil. Other than that, using *C. verum* synergistically with commercial antibiotic on the isolated bacteria is also recommended to see whether or not it can help to potentiate the activity of the antibiotic.

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APPENDIX

Appendix 1: Gram staining

i. The isolates were smeared on glass slide and let to dry.

ii. Flood the slides with Crystal violet for 1 minute and washed with distilled water.

iii. Flood the slides with Lugol's iodine for 1 minute and washed with distilled water.

iv. The slides were decolorized with Acetone for 2 to 3 seconds and immediately washed with distilled water.

v. Flood the slides with diluted Carbol Fuschin for 1 minute and washed with distilled water and dried.

Appendix 2: Biochemical Tests

Catalase test

i. Inoculate bacteria colonies on glass slides containing Catalase reagent. Observe for bubbles formation.

Coagulase test

i. Inoculate a loopful of bacteria colonies on a glass slide with normal saline.

ii. Add a drop of rabbit plasma and observe for agglutination within 30 seconds. Proceed with tube coagulase test if the slide coagulase test came out negative.

iii. In tube coagulase test, add 0.3ml of Rabbit plasma into empty test tubes.

iv. Inoculate loopful of colonies into the test tubes.

v. Incubate the test tubes at 37°C for 4 hours. Observe for clotting.

Haemolysin test

i. Inoculate a loopful of colonies into blood broth (tryptose broth containing defibrinated blood)

ii. Incubate at 37°C for 24 hours. Observe for signs of haemolysis.

6.5% Sodium Chloride test

i. Inoculate a loopful of colonies into 6.5% Sodium Chloride(NaCl) broth.

ii. Incubate at 37°C for 24 hours. Positive reaction is when the broth changes its colour from purple to yellow.

Bile esculin test

i. Streak the slant surface of the bile esculin agar with colonies by using inoculating needle.

ii. Incubate at 37°C for 24 hours. Observe for changes of colour from yellow to black.

Carbohydrate broths test

These include mannitol, maltose, glucose, lactose, sorbitol, trehalose, sucrose, sorbose, arabinose and raffinose.

i. Inoculate a loopful of colonies into the carbohydrate broths.

ii. Incubate at 37°C for 24 hours. Observe for change of colour into yellow indicating acid production.

Nitrate reduction test

i. Inoculate loopful of colonies into nitrate broth and incubate it at 37°C for 24 hours.

ii. Add 5 drops of Reagent 1 (containing sulfanilic aid and acetic acid) and Reagent 2 (containing N,N-Dimethyl-l-naphthylamine and acetic acid) and shake the tube for 1 minute.

iii. Observe for immediate change of colour into red. If negative, let it stand for 15 minutes and observe again.

Voges-Proskauer (VP) test

i. Inoculate loopful of colonies into Voges-Proskauer (VP) broth.

ii. Incubate at 37°C for 24 hours.

iii. Add 6 drops of α–naphthol and 2 drops of potassium hydroxide solution into the broth.

iv. Mix well for 30 seconds and if there is no reaction let to rest for 30 minutes. Observe for change of colour from yellow to pink or red.

Arginine dihydrolase (ADH) test

i. Inoculate loopful of colonies into Arginine dihydrolase (ADH) broth.

ii. Overlay the broth with sterile mineral oil and incubate at 37°C for 24 hours.

iii. Notified change of colour from yellow to purple as positive result.

Oxidase test

i. Inoculate bacteria colonies on filter paper containing Oxidase reagent. Observe for dark blue or purple colour development.

Triple Sugar Iron (TSI) test

i. Stab the butt of the Triple Sugar Iron (TSI) agar and streak the slant surface with colonies by using inoculating needle.

ii. Incubate at 37°C for 24 hours. Observe for changes of colour and presence of gas at the butt and slant of the agar.

Urease test

i. Streak the slant surface of Christensen's urea agar with colonies by using inoculating loop.

ii. Incubate at 37°C for 24 hours. Observe for change in colour from yellow to red.

Sulfide Indole Motility (SIM) test

i. Inoculate Sulfide Indole Motility (SIM) medium with colonies by a single clean stab using inoculating needle.

ii. Incubate at 37°C for 24 hours.

iii. For the result of motility test, observe for turbidity surrounding the stabbed area.

iv. For the result of indole test, overlay the medium with Kovac's reagent and observe for immediate development of pink to red colour.

Citrate test

i. Streak the slant surface of the Simmons Citrate agar with colonies by using inoculating loop.

ii. Incubate at 37°C for 24 hours. Observe for change in colour of the medium from green to blue.

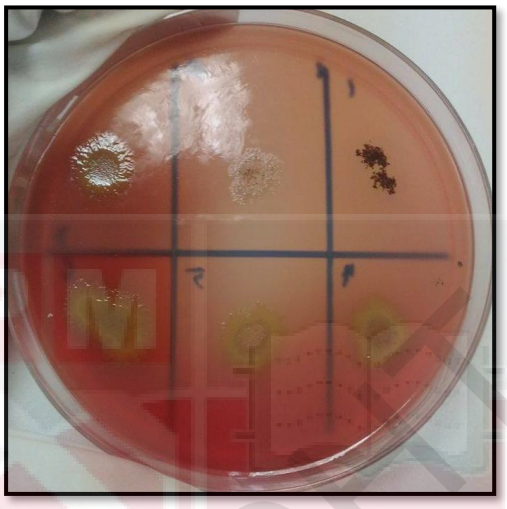
Orthonitrophenyl-β-D-galactopyranoside (ONPG) test

i. Inoculate loopful of colonies into test tube containing nutrient broth.

ii. Add Orthonitrophenyl-β-D-galactopyranoside (ONPG) disc into the test tube.

iii. Incubate at 37°C for 24 hours. Observe for change in colour of the broth from colourless to yellow.

Appendix 3: Minimum Bactericidal Concentration (MBC) – *Bacillus* sp. on blood agar plate inoculated with *C. verum.*



Appendix 4: Antimicrobial activity of commercially used antibiotics and *C. verum* paste against *S. pseudintermedius, S. intermedius, S. hyicus, E. faecalis, C. ulcerans, Bacillus* sp. and *Aggre. actinomycetemcomitans*.

Bacteria	Treatment	DIZ (mm)	Standard error	p value
S.pseudintermedius	Blank disc	1.16E-14	0.659	
	Marbofloxacin	25.325	0.659	
	Amoxicilin-Clavulanic Acid	26.178	0.659	
	Doxycycline	32.603	0.659	
	Enrofloxacin	27.997	0.659	
	Cinnamon paste	19.135	0.659	0.000
S. intermedius	Blank disc	6.22E-15	0.659	
	Marbofloxacin	26.207	0.659	
	Amoxicilin-Clavulanic Acid	41.307	0.659	
	Doxycycline	33.683	0.659	
	Enrofloxacin	28.718	0.659	
	Cinnamon paste	18.677	0.659	0.000

S. hyicus	Blank disc	8.88E-15	0.659	
	Marbofloxacin	20.86	0.659	
	Amoxicilin-Clavulanic Acid	42.342	0.659	
	Doxycycline	43.493	0.659	
	Enrofloxacin	28.82	0.659	
	Cinnamon paste	19.173	0.659	0.000
E. faecalis	Blank disc	0.000	0.659	
	Marbofloxacin	1.42E-14	0.659	
	Amoxicilin-Clavulanic Acid	31.6	0.659	
	Doxycycline	10.723	0.659	
	Enrofloxacin	1.78E-14	0.659	
	Cinnamon paste	10.948	0.659	0.000
C. ulcerans	Blank disc	5.33E-15	0.659	
	Marbofloxacin	29.433	0.659	
	Amoxicilin-Clavulanic Acid	1.658E+01	0.659	
	Doxycycline	32.05	0.659	
	Enrofloxacin	30.7	0.659	
	Cinnamon paste	8.417	0.659	0.000
Bacillus sp.	Blank disc	5.33E-15	0.659	
	Marbofloxacin	7.752	0.659	
	Amoxicilin-Clavulanic Acid	37.967	0.659	
	Doxycycline	11.1	0.659	
	Enrofloxacin	9.262	0.659	
	Cinnamon paste	17.83	0.659	0.000
Aggre. actinomycetemcomitans	Blank disc	1.78E-15	0.659	
	Marbofloxacin	32.703	0.659	
	Amoxicilin-Clavulanic Acid	47.857	0.659	
	Doxycycline	45.13	0.659	
	Enrofloxacin	34.727	0.659	
	Cinnamon paste	15.403	0.659	0.000

Note: The acceptable range of DIZ of Gram positive and Gram negative bacteria against antibiotics tested respectively; marbofloxacin= 24-30mm, 29-37mm, amoxicillin-clavulanic acid=28-36mm, 18-24mm, doxycycline=23-29mm, 18-24mm, enrofloxacin=27-31mm, 32-40mm. For *C.verum* paste, DIZ above 7mm is considered as positive result.