Antibacterial activity of *Myrsine seguinii* leaf crude extract against selected bacterial isolates

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Abstract

Plants have many antibacterial properties as secondary metabolites such as ligans, quinones, phenolic acid, flavonols, and anthocyanin. The bioactive components in plants are essential for the role of development of modern drugs. In this study, the antibacterial activity of M. seguinii leaf extract which belongs to the family of Primulaceae, was screened using paper disc diffusion method and spot test assay against selected bacterial isolates. The results showed that the ethanolic crude extract of M. seguinii (highest in 100 mg/mL) displayed antibacterial activity against Staphylococcus aureus (9.67 + 1.15 mm), Pseudomonas aeruginosa (13.67 + 2.31 mm), and Methicillin resistant Staphylococcus aureus (13.67 + 2.31 mm) using spot test assay. However, the Extended Spectrum β-Lactamase (ESBL) Escherichia coli and Metallo-β-Lactamase (MBL) Pseudomonas aeruginosa were not inhibited in the same assay using the extract. Furthermore, all bacterial isolates used did not show sensitivity to the extract using paper disc diffusion method due to the actual absorbance of the paper discs and the diffusing property when the discs was placed in the agar. Thus, M. seguinii leaf extract can be a potential antibacterial agent for selected bacterial isolates.

Keywords: *Antibacterial activity; M. seguinii; regular and multi-drug resistant bacterial isolates; medicinal plants; leaf crude extract.*

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Introduction

Plants are very useful source of various bioactive compounds in which it contributes numerous necessities of life such as food, medicine, clothing and use in the treatments of various human conditions. Plants known to have biological properties, some of these properties are alkaloids, terpenoids, tannins and flavonoids. As a result of the discovery that herbs from various genera and species have antibacterial potential, new antimicrobials or medications have been created (Hammer et al., 1999; Obey et al., 2016; Sharifirar et al., 2009). It has been estimated that only about 14%-28% species has been used as medicaments and equivalent to 74% were observed for the use of ethnomedicinal plants (Mumbengegwi et al., 2015; Ncube et al., 2008).

Myrsine seguinii, locally known as "*kalumanay*," belongs to the family *Primulaceae*, while the genus is known as *Myrsine*. It is present mostly in tropical and subtropical regions that belongs to *Myrsinaceae* or *Myrsine* family, with 35 genera and 1,000 species (Abbhi et al., 2011). According to Ito et al. (2008), the Myrsinoic acid B inhibitory activities from *M. seguinii* was tested against predontal bacteria, which include *Porphyromonas gingivalis*, *Treponema denticola*, and *Fusobacterium nucleatum*. Moreover, their data suggested that the compound (myrsinoic acid) from *M. seguinii* possess anti-inflammatory activity. In addition, several studies of the genus *Myrsine* have biological activities such as antibacterial (de

Burger et al., 2015), anti-inflammatory (Ito et al., 2008) and anti-helmenthic (Abbhi et al., 2011; Gathuama et al., 2004).

Antibiotic resistance is characterized as sensitive, susceptible or resistant on the drug required to inhibit growth of the organism. The morbidity and mortality associated with multidrug resistant (MDR) bacterial infections has a convincing influence on the effectiveness of antibacterial drugs. Additionally, 700,000 persons were estimated to die yearly resulted by the infectious causing MDR pathogens, with millions of further grave complications. The development of the new antimicrobial agents is necessary in order to assure a possibility of various, sustainable and powerful antimicrobial drugs (Ambrose, et al., 2017).

The result of this study is beneficial to community health organizations for the discovery of new antibacterial agent to inhibit the growth of selected regular and multi-drug resistant bacterial isolates since *M. seguinii* is not yet fully known for its antibacterial use and eventually apply the antibacterial benefit as people are exposed in a community exposed to nosocomial infections.

The use of *M. seguinii* was already proven to have medicinal properties according to a number of studies published. Several studies of the genus *Myrsine*, possess to have anti-inflammatory, anthelminthic, anti-tumor, anti-fertility, hemagglutination, and antimicrobial activities (Abbhi et al., 2011; Cruz et al., 2013; Flores & Tercero, 2018). However, other studies about multi-drug resistant bacteria and regular strains have not yet been observed in the leaf. Therefore, this study aims to determine a new approach by testing the extract of *M. seguinii* against the selected multi-drug resistant bacteria and also regular strains which are known to have resistance against multiple drugs.

Objectives

The study aimed to determine if *M. seguinii* leaf crude extract possess antibacterial activity against selected bacterial isolates. Specifically, the study focused on the following objectives:

1. Determine the antibacterial activity of *M. seguinii* leaf crude extract against selected bacterial isolates which include *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, Extended Spectrum β-lactamases *Escherichia coli* (ESBL), Metallo-β-lactamase *Pseudomonas aeruginosa* (MBLPA), and Methicillin-resistant *Staphylococcus aureus* (MRSA); and
2. Ascertain the concentration of *M. seguinii* leaf crude extract (100 mg/mL, 50 mg/mL, 25 mg/mL, and 10 mg/mL) against selected bacterial isolates using paper disc diffusion method and spot test.

Scope and Limitations

The study evaluated the antibacterial activity of *M. seguinii* against selected bacterial isolates. The scope of the study includes a leaf crude extract of *M. seguinii* from crushed dried leaves. Ethanol (95%) was used for the leaf crude extract. The test organisms used for the antimicrobial assay include *E. coli*, *P. aeruginosa*, *S. aureus*, ESBL *E. coli*, MBLPA, and MRSA. Disc diffusion method and spot test were used in determining the antibacterial activity of *M. seguinii* crude extract against selected bacterial isolates. The concentrations of the ethanolic crude extracts used in disc diffusion method and spot test were 100 mg/mL, 50 mg/mL, 25 mg/mL, and 10 mg/mL. However, the study was limited to the phytochemical component of the extract was not quantified and correlated for its mode of action.

Methodology

Flow Chart

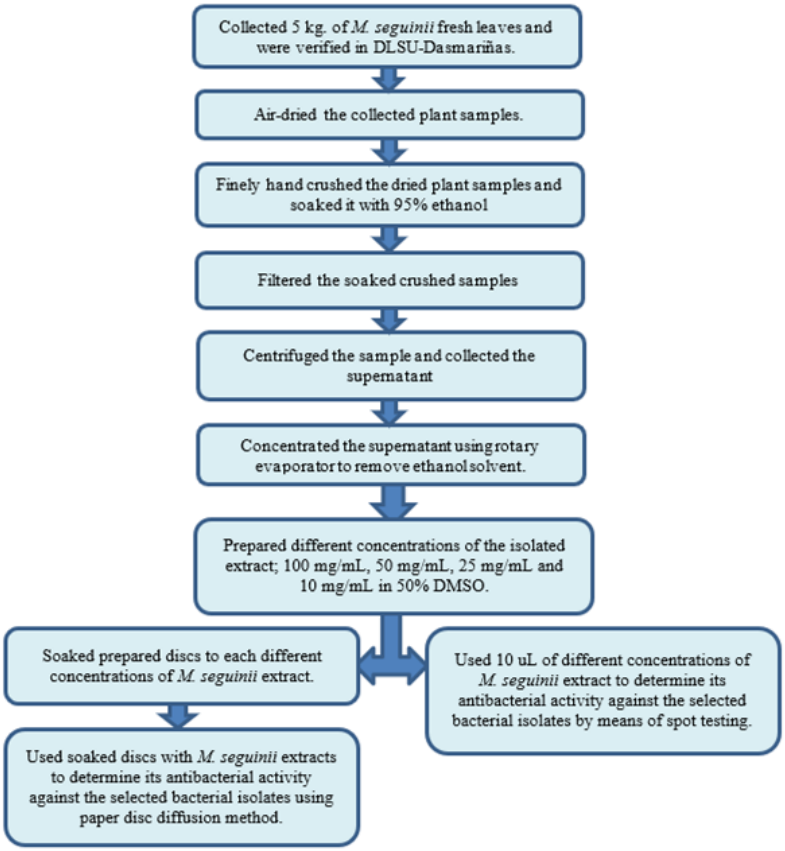


Figure 1. Flow chart

Collection of Plant Sample

Five (5) kilograms of fresh plant sample *M. seguinii* leaves were collected from Barangay Layong Mabilog, Maragondon, Cavite in November 29, 2018. As validated by the faculty and research director, Melanie M. Guiang, Ph.D., of De La Salle University – Dasmariñas, the plant collected was identified to be *M. seguinii*.

Processing and Extraction of Plant Sample

The collected 5 kg fresh leaves of M. seguinii were air dried for two weeks immediately after the collection to remove its water content. Then all dried leaves were crushed using bare hands and were later on weighed. This was later on soaked in 95% ethanol for 24 hours. Then the soaked leaves were filtered using a strainer to remove large plant materials. The filtrate were again filtered using Whatman filter paper #1. The filtered solution were then centrifuged for 5 minutes at 3,000 rpm. The supernatant were collected and were subjected to distillation using rotary evaporator (IKA©) at 50ºC (Valle et al., 2015).

Afterwards, crude extracts were placed in sterile amber containers for storage. This was then diluted with 50% Dimethyl sulfoxide (DMSO) to prepare different concentrations that was used in the assay (100 mg/mL, 50 mg/mL, 25 mg/mL and 10 mg/mL) (Elumalai et al., 2011).

Antimicrobial Assay

Preparation of culture for assay

All test organisms (*E. coli*, *P. aeruginosa*, *S. aureus*, multi-drug resistant isolates: ESBL *E. coli*, MDRPA, and MRSA) used were acquired from the Department of Biology, De La Salle University – Manila. Fresh cultures were prepared for each isolates by inoculating it in a nutrient agar (NA) plate and were incubated at 37^{o}C for 24 hours before the conduction of the assay.

The bacterial cultures were standardized using 0.5 McFarland to get specific optimal density of 1.5 x 108 colony forming units (CFU/mL). This was obtained by inoculating one or two colonies from the culture plate into a test tube with 5 mL sterile distilled water (SDW). Inoculated test organisms were compared with the prepared 0.5 McFarland Standard using a Wickerham card. The turbidity produced by the standard was visually similar to the turbidity of the inoculated microbes. The standardized test organisms were inoculated on the entire surface of Mueller-Hinton agar (MHA) plate by streaking on a rotation of 60 degrees using sterile cotton swab applicator dipped on the inoculum.

Paper Disc Diffusion method

The method used was based on Kirby-Bauer test protocol or commonly known as the disc diffusion method. Whatman No. 1 filter papers were used for the paper disc diffusion assay. Using a puncher, cut filter papers were sterilized, dried and soaked in different concentration of *M. seguinii* leaf crude extracts for two hours (Ezeifeka et al., 2004). The soaked discs were then dried before inoculating into the MHA with standardized bacterial culture.

Antibiotic discs were used as positive control in all test organisms. This include the following: (1) meropenem for both *E. coli* and ESBL *E. coli*, (2) gentamicin for both *P. aeruginosa* and MBLPA, and (3) trimethoprim-sulfamethoxazole for both *S. aureus* and MRSA. On the other hand, disc infused with nutrient broth (NB) was used as negative control.

For every MHA plate, a maximum of three (3) paper discs were placed, thus a total of two (2) MHA was utilized for each bacterial isolates. One MHA plate contained paper discs infused with 100 mg/mL, 25 mg/mL and 10 mg/mL leaf crude extract while the other MHA plate contained 50 mg/mL leaf crude extract, positive control and negative control. Triplicates for each test organism were performed.

Spot test

This method of testing an antimicrobial agent was based on Syed et al. (2010) with some modifications. Instead of spotting 10 uL of tungsten-nanoparticles (W-NP), 10 uL of M. seguinii leaf crude extract was used. The MHA plate inoculated with the standardized test organisms was divided into four (4) quadrants allotted for each spot of the different concentrations of extract (100 mg/mL, 50 mg/mL, 25 mg/mL, and 10 mg/mL). From each concentrations, 10 uL was collected and spotted into the designated area of the cultured plate. Moreover, negative control of 10 uL nutrient broth was also spotted in the center of the quadrant. Positive control was not included in this assay, since the result will be based on the zone of inhibition exhibited in the agar plate used for the paper disc diffusion method. Triplicates for each test organism were performed. The spots that showed a clear zone indicated a positive activity against the test organisms and the diameter were measured using a ruler (mm).

Results and Discussion

Collection of plant sample

M. seguinii leaves were collected last November 28, 2018, in Barangay Layong Mabilog, Maragondon, Cavite. The identity of the plant was certified by the botanist of DLSU-Dasmariñas. The collected 5 kg of leaves were later on transported in the Biology Laboratory of St. Dominic College of Asia and were soaked in 95% ethanol then subjected to rotary evaporation. After the extraction procedure, only 600 g were obtained and were later on use for the antimicrobial assay. This were then diluted to different concentrations of 100mg/mL, 50 mg/mL, 25 mg/mL and 10 mg/mL using DMSO (50%).

Paper Disc Diffusion Method

In the paper disc diffusion method, all extract concentrations (100mg/mL, 50mg/mL, 25mg/mL, 10mg/mL) exhibited negative results to all bacterial isolates used (E. coli, P. aeruginosa, S. aureus, ESBL E. coli, MBLPA and MRSA) (Figures 2-7).

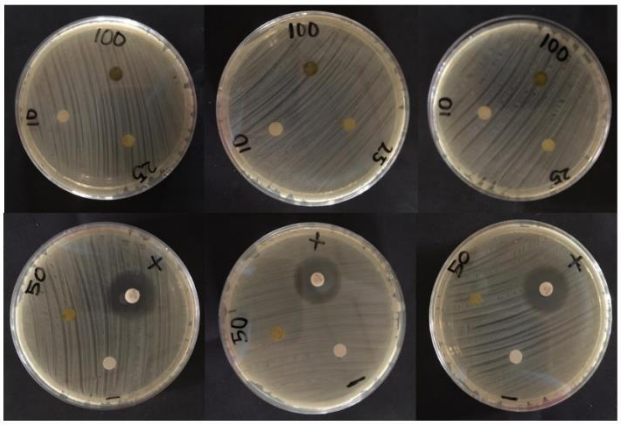


Figure 2. *E. coli* containing discs infused with 100 mg/mL, 25 mg/mL, and 10 mg/mL of *M. seguinii* crude extract in 50% DMSO in triplicates (from upper left to right); discs infused with 50 mg/mL, meropenem as positive control and nutrient broth as negative control in triplicates (from lower left to right).

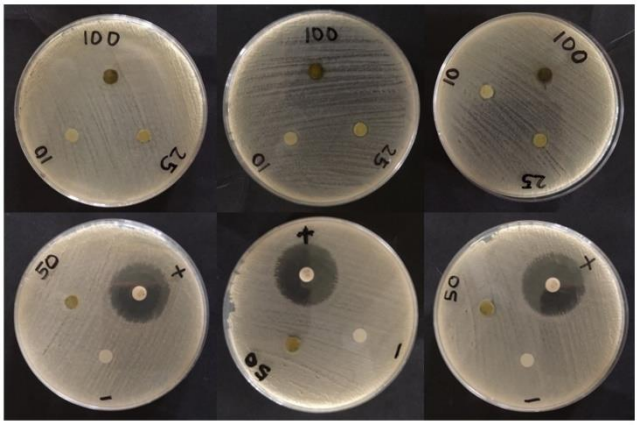


Figure 3. *P. aeruginosa* containing discs infused with 100 mg/mL, 25 mg/mL, and 10 mg/mL of *M. seguinii* crude extract in 50% DMSO in triplicates (from upper left to right); discs infused with 50 mg/mL, gentamicin as positive control and nutrient broth as negative control in triplicates (from lower left to right).

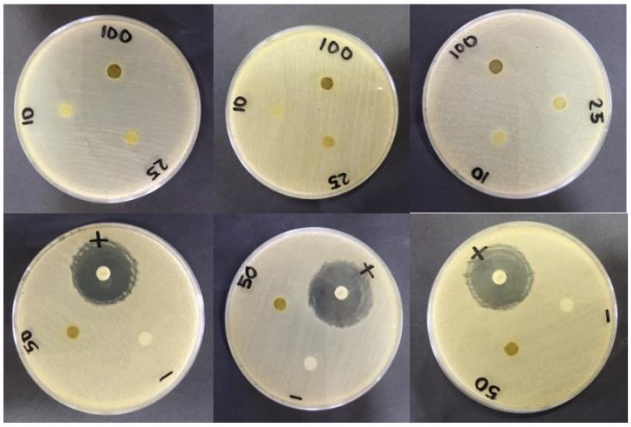


Figure 4. *S. aureus* containing discs infused with 100 mg/mL, 25 mg/mL, and 10 mg/mL of *M. seguinii* crude extract in 50% DMSO in triplicates (from upper left to right); discs infused with 50 mg/mL, trimethoprim-sulfamethoxazole as positive control and nutrient broth as negative control in triplicates (from lower left to right).

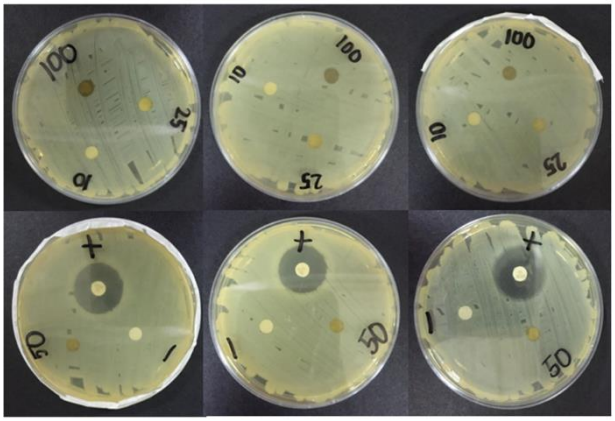


Figure 5. ESBL *E. coli* containing discs infused with 100 mg/mL, 25 mg/mL, and 10 mg/mL of *M. seguinii* crude extract in 50% DMSO in triplicates (from upper left to right); discs infused with 50 mg/mL, meropenem as positive control and nutrient broth as negative control in triplicates (from lower left to right).

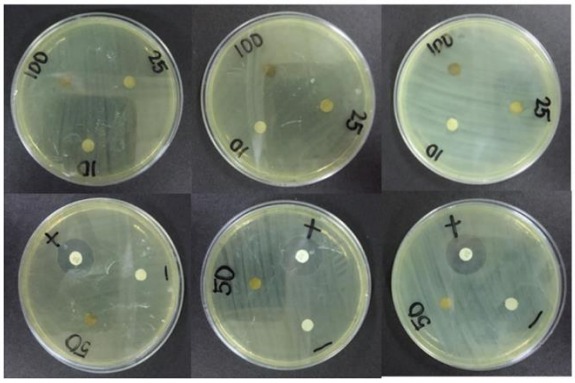


Figure 6. MBLPA containing discs infused with 100 mg/mL, 25 mg/mL, and 10 mg/mL of *M. seguinii* crude extract in 50% DMSO in triplicates (from upper left to right); discs infused with 50 mg/mL, gentamicin as positive control and nutrient broth as negative control in triplicates (from lower left to right).

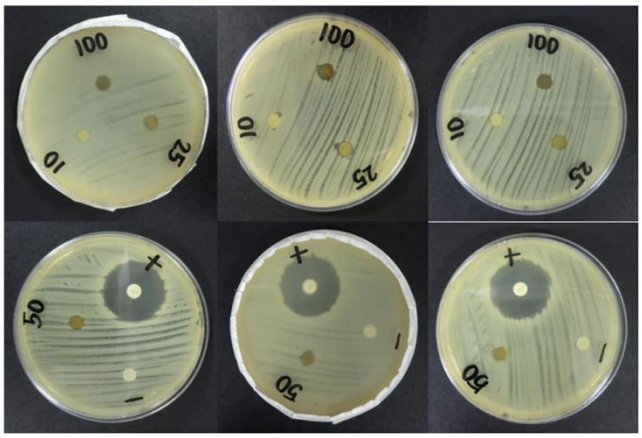


Figure 7. MRSA containing discs infused with 100 mg/mL, 25 mg/mL, and 10 mg/mL of *M. seguinii* crude extract in 50% DMSO in triplicates (from upper left to right); discs infused with 50 mg/mL, trimethoprim-sulfamethoxazole as positive control and nutrient broth as negative control in triplicates (from lower left to right).

Spot test

Using the spot test, regular isolates of P. aeruginosa, S. aureus and MRSA were inhibited by the leaf crude extract of *M. seguinii* in all concentrations (100 mg/mL, 50 mg/mL, 25 mg/mL, and 10 mg/mL). On the other hand, *E. coli*, ESBL *E. coli*, and MBLPA showed negative results to all concentrations of the leaf crude extract. Based on Table 1, the diameter of zone of inhibition of *P. aeruginosa*, *S. aureus*, and MRSA were observed.

On Table 1, *P. aeruginosa* exhibited the highest zone of inhibition in 100 mg/mL of the leaf extract (13.67 + 1.15) which still falls to the susceptible breakpoints for the control (gentamicin 10 ug) with reference to CLSI 2021. This is also the same for MRSA as shown on Table 2, in which the highest zone of inhibition was exhibited in 100 mg/mL (13.67 + 2.31) and is still accordance to the susceptible breakpoints for trimethoprim sulfamethoxazole (CLSI, 2021). However, for *S. aureus*, the highest zone of inhibition was observed in 100 mg/mL (9.67 + 1.15) in which it is lower than the susceptible value for CLSI (> 16) in comparison with the control antibiotic used (trimethoprim-sulfamethoxazole (1.25/23.75 ug = 26.67 + 0.58).

Table 1. Average diameter of the zone of inhibitions (mm) of *M. seguinii* leaf crude extract against the regular bacterial isolates using spot test

Bacterial isolates	100 mg/mL	50 mg/mL	25 mg/mL	10 mg/mL	+ Control
E. coli	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	20.33 ± 2.08
P. aeruginosa	13.67 ±1.15	12.67 ± 1.15	12.33 ± 1.53	6.33 ± 5.69	28.00 ± 2.00
S. aureus	9.67 ± 1.15	8.67 ± 1.54	8.33 ± 0.58	8.00 ± 1.00	26.67 ± 0.58

Control: Gentamicin (10 ug) for *P. aeruginosa*, meropenem (10ug) for *E. coli*, and trimethoprim-sulfamethoxazole (23.75ug/1.25 ug) for S. aureus.

Table 2. Average diameter of the zone of inhibitions (mm) of *M. seguinii* leaf crude extract against the regular bacterial isolates using spot test

Bacterial isolates	100 mg/mL	50 mg/mL	25 mg/mL	10 mg/mL	+ Control
ESBL *E. coli*	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	18.00 ± 6.08
MBLPA	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	20.00 ± 0.00
MRSA	13.67 ± 2.31	11.67 ± 2.08	11.00 ± 0.00	11.00 ± 1.00	29.67 ± 1.53

Control: Gentamicin (10 ug) for *P. aeruginosa*, meropenem (10ug) for *E. coli*, and trimethoprim-sulfamethoxazole (23.75ug/1.25 ug) for S. aureus.

Several human diseases can be prevented, treated, or cured with the help of herbs and herbal extracts. The development of a drug to act against germs in distinction to different sources such as plants, animals, and microorganisms is a result of the rapid growth of resistance in large quantities of production. Plants contain various bioactive compounds for traditional health care practices, both humans and animals (Habtamu et al., 2010; Nitta et al., 2002). In past decades, the benefits made from herbal medicines are sources of antimicrobial to cure various diseases. Herbal medicine established an adequate cause for both traditional and modern medicines (Erdemeier et al., 1996; Habtamu et al., 2010; Lino & Deogracious, 2006). It has been advertised to hold certain efficiency and the rural population that depends on it as their principal health care are about 80% (Akinyemi et al., 2005; Egamberdieva et al., 2021; Farnsworth et al., 1985). Plant materials have been estimated to have potential for developing antimicrobials that would lead to the development of a medicine to exploit against pathogenic microorganisms. A lot of commercially tested drugs utilized in modern medicine were originally applied in the form of crude in traditional or folk treatment processes that can be useful in biological activity (Egamberdieva et al., 2021).

In this study the antibacterial activity of *M. seguinii* extracts was determined against selected bacterial isolates by means of paper disc diffusion method and spot test. The paper disc method did not show any antibacterial activity for all bacterial isolates used in the study. This result can be assumed that the amount of extract absorbs on the discs and the diffusing ability through the agar inhibited the component of the extract to exhibit inhibition to the test organisms.

On the other hand, the result presented in Table 1 indicates that all concentrations of *M. seguinii* extract exhibited antibacterial activity based on the zones of inhibition using spot test method. With reference to CLSI (2021) which is the standard for testing antibiotics, *P. aeruginosa*, and MRSA still falls within the susceptible breakpoints of the control antibiotics used in the assay (gentamicin and trimethoprim-sulfamethoxazole). However, for *S. aureus*, although the extract exhibited zone of inhibition against the bacterium, the clear zone produced is lower than the accepted value of susceptibility as compared to the antibiotic used (trimethoprim-sulfamethoxazole) (CLSI, 2021). It indicates that the *M. seguinii* leaf crude extract has low antibacterial activity on the microorganism used.

Moreover, in spite of the clear zone exhibited by the extract against the bacterial isolates (*P. aeruginosa*, MRSA, and *S. aureus*), the diameter measured is lower than the control antibiotics used. Furthermore, based on the concentration of the extracts utilized in the assay which is 100 mg/mL, 50 mg/mL, 25 mg/mL, and 10 mg/mL and as compared to the dosage of gentamicin (10 ug) and trimethoprim-sulfamethoxazole (1.25 ug/23.75), it still produced a lower zone of inhibition. This indicates that *M. seguinii* has low antibacterial activity against the given bacterium, since what was utilized in the study was crude extract of the leaf and the actual chemical component of the leaf which may have antibacterial activity was not in full concentration or even determined.

As based on the resistance of *E. coli* and ESBL *E. coli* against *M. seguinii*, it may be assumed that the extract is sensitive against β-lactamase producers. However, some isolates which are not β-lactamase producers such as *S. aureus*, *P. aeruginosa*, and MRSA that are used in the study exhibited zone of inhibition. According to the study of de Burger et al., (2015), the fruit ethanolic extract from *M. coriacea* possess antibacterial activity. Myrsinoic acid A was used in acetylation and catalytic hydrogenation reaction to attain semi-synthetic derivatives. The Myrsinoic acid A from *M. coriacea* exhibited no reaction against selected microorganisms. However, semi-synthetic derivatives were effective against *Bacillus subtilis*, *E.*

coli, *S. aureus*, and *P. aeruginosa*. The aqueous extract of *M. africana* reported by Habtamu et al. (2010), has shown the highest zone of inhibition against *S. aureus* but no plant extract exhibited antimicrobial effect against *E. coli*. The findings of the present study in which *M. seguinii* was revealed to have antibacterial activity against gram positive and gram negative bacterial isolates was supported by a study performed by Obey et al. (2016) whereas Cruz et al. (2013) did not observe any antimicrobial activity of aqueous extract of *Rapanea ferruginae* (*Myrsine floculosa*) against E. coli and yeast. However, the *M. floculosa* presented selective antimicrobial activity against gram positive bacteria (*B. subitilis*, *S. aureus*, *Staphylococcus saprophyticus*, and *Streptococcus pyogenes*).

In this case, the ethanol extract of *M. seguinii* leaves were active in both gram positive and with gram negative bacteria, that indicates the presence of wide spectrum of antibiotic compounds in the plant. However, gram negative bacteria *P. aeruginosa* were more inhibited by the plant extracts compared with gram positive bacteria in *S. aureus*; these might be due to the different in cell wall constituents. Gram negative bacteria are more resistant to antibiotics, since they have a largely impermeable cell wall (Cruz et al., 2013; Tegos et al., 2002).

Peptidoglycan makes up the cell wall of gram-negative bacteria. The peptidoglycan, however, is only one thin layer that could inhibit the penetration of the bacterial cell to the plant extract whereas the cell walls of gram positive bacteria are rigid due to higher amount of peptidoglycan that is most susceptible to penetrate the bacterial cell. In contrast to gram positive bacteria, it seems that gram-negative organisms prevent some medications and antibiotics from entering the cell because they are ineffective (Slavin et al., 2017).

Conclusion

The main purpose of this study was to determine the antibacterial activity of *M. seguinii* leaf crude extract against selected bacterial isolates which was collected in Barangay Layong Mabilog, Maragondon, Cavite. This study investigated the antibacterial activity using different concentrations (100 mg/mL, 50 mg/mL, 25 mg/mL, and 10 mg/mL) of the *M. seguinii* extract using spot test and paper disc assay in which the extract exhibited negative inhibition against all bacteria using paper disc. However, *P. aeruginosa*, *S. aureus*, and MRSA were inhibited by the extract using spot test, although the zone of inhibition is lower compared to the positive control used (gentamicin and trimethoprim-sulfamethoxazole).

From these results, the leaf crude extract of *M. seguinii* was found to possess antibacterial activity especially to non-β-lactamase producer bacteria (i.e., *S. aureus*, *P. aeruginosa*, and MRSA). It is therefore suggested that *M. seguinii* possess compounds holding antibacterial properties that when extracted using a solvent such as ethanol, could effectively inhibit the growth of selected bacterial isolates.

Recommendations

Based on the results of the study, the following recommendations are suggested:

- Determine the phytochemical component of *M. seguinii* like alkanoids, tannins, flavonoids and quinones.
- Test the *M. seguinii* extract in a different set of concentrations.
- Use other solvents such as methanol, n-hexane, acetone, or chloroform for better extraction of the plant.
- Perform the antimicrobial activity of *M. seguinii* leaf crude extract against other microorganisms.

- Considering the presence of antibacterial activity of the *M. seguinii*, implement further testing if the extract can also exhibit to other test approximating anti-helminthic, hemagglutination activity, anti-fertility and anti-tumor.
- Check the level of toxicity of *M. seguinii* leaf crude extract if it can be used for human medication (Minimum Inhibitory Concentration).

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