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Synergism effect of *Murraya koneigii* and *Piper betle* leaves extracts on antibacterial activity against Gram Positive bacteria

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ABSTRACT

*A*ntimicrobial resistance has become the major threat to the health worldwide. It has lead to the ineffective impact of the ability of the substance or antibiotics to kill the bacteria. Therefore,there is a need to find a new alternative to overcome the problem by using natural products. In this study *Piper betle (P. betle*) and *Murraya koenigii (M. koenigii*) leaf extracts were used to measure their synergism effect towards antimicrobial treatments. Previous study has shown that both plants has shown their ability in inhibiting the growth of bacteria separately. Three different concentration ratios of the synergised leaf extracts were used with 1:1(P.*betle*:*M. koenigii*) ratios, 2:1 *(P.betle:M. koenigii)* ratios and 1:2 (*P.betle:M.koenigii*) ratios. The synergised leaf extracts was at the highest inhibition zone with combination of 2:1 ratio of *P.betle* and *M. koenigii* with 22.6mm. However, it does not have the greater inhibition ability as compared to the *P.betle leaf* extract with 28.1mm. Therefore, from the study, antagonism effect was discovered when both plant extracts were combined.

Keywords:
Antagonism, Antibacterial activity, P.betle, M.koneigii

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1. Introduction

Antimicrobial resistance is the highest threat to the public health. The overuse and misuse of antibiotic has led to the development of bacteria in adapting the effect of the antibiotic, enabling them to survive from being killed by the antibiotics [14,15]. It is important to find new products that can inhibit the bacteria from developing into a Multi Drug resistance (MDR) bacteria, a bacteria that are resistance to many kinds of antibiotics [19,22].

Many research involving natural products has been done in finding potential antimicrobial substance. In this study, *Piper betle (P.betle)* and *Murraya Koneigii (M.koenigii)* plant were used as it has shown a greater effect on inhibiting the growth of bacteria [9,11]. Both plants are well known in the Asian region with their common names known as sireh for *P.Betle* and curry leaves for *M.koenigii* [13]. The leaves of *P. betle* contain several phenols in which the compound act as an antimicrobial

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agent (bacteriostatic) and killing microbe [10,17]. The use of *P. betle* that also known as sireh is a popular leaf that has been existed since ancient time in a variety of ways. In east Asia, where is the most population use to chewed the leaves for both nutritive and medicinal value [18]. Occasionally, was used for religious purposes in India [2]. While for *M. koenigii* or commonly called as curry leaves are widely used in Indian cuisine also in several Asian countries as condiments due to its calming scents and highly aromatic features. The part of the plant has been used in wound healing ,treating stomach-ache and dysentery [21].

In terms of chemistry, active substances such as phenolic compound presence in *P.betle* structure have a big impact in pharmacological potential [16]. While for *M. koenigii* leaves showed remarkable antibacterial activity against *Streptococcus mutans* and it is achievable due to the presence of gallic acid and phenolics in the extract. *M. koenigii* also found to have a therapeutic effectiveness against multidrugresistant pathogens when silver nanoparticles are extracted from the aqueous extract of the leaves [13,8,24]. Besides that, methanolic and ethanolic extracts of *M. koenigii* leaves both are reported to have significant inhibitory activity against *Escherichia coli (E. coli), Staphylococcus, Streptococcus*, and *Proteus* [1].

Due to the findings of the previous research for both plants, there is a need to investigate their synergised effect when they are combined as the *P.betle* and *M. koneigii* has shown a great potential as an antimicrobial agent.

2. Methodology

2.1 Collection of Plant Materials

Fresh *M. koenigii* and *P. betle* leaves were bought from market in Tapah, Perak and were washed clean with tap water to remove any dust or particulates present on the leaves' surfaces. The cleaned leaves were then dried by using oven at temperature of 70^0C for 3 days. The dried leaves of *M. koenigii* and *P. betle* were crushed separately by using a blender until fine powder is obtained for each leaf. The leaves powder was kept individually in an air- tight container and stored in a freezer at -20^0C.

2.2 Preparation of Ethanolic Extract

For the individual leaves extract, 3 g of the individual leaves powder was dissolved separately with 30 mL of 95% ethanol each for 12 hours. As for the combined extracts, 3 sets of concentrations were prepared that were *P. betle: M. koenigii,* 1:1, 2:1 and 1:2. For the *P. betle: M. koenigii* 1:1 combined leaf extract, 1.5 g of both leaves powder (*P. betle and M. koenigii*; giving a total of 3 g of leaves powder) was dissolved in 30 mL of 95% ethanol. While for the 2:1 concentration, 2 g of *P. betle* leaf powder was combined with 1 g of *M. koenigii* leaf powder and was dissolved in 30 mL of 95% ethanol. For the 1:2 concentration, 1 g of *P. betle* leaf powder was combined with 2 g of *M. koenigii* leaf powder and was dissolved in 30 mL of 95% ethanol. All three mixture were left to dissolved in the alcohol for 12 hours before the next procedure was implemented. Later, each of the mixture was filtered using 125 nm filter paper and the supernatant was evaporated by using rotary evaporator at 60^0 C until an oily texture is formed.

2.3 Collection of Test Bacteria

A sterilized cotton swab was scrubbed over the surface of hand palms and was swabbed thoroughly on an agar plate. The agar plate was incubated for 24 hours at 37^0 C. The colonies

produced were isolated in order to obtain pure cultures. It was then was identified and classified under Gram positive bacteria by using a Gram stain method.

2.4 Antibacterial Activity

The antibacterial activity of was determined using the hole-inplate bioassay procedure. The pure cultures of the microorganisms were inoculated, and w swabbed on nutrient agar by using a sterilized cotton swab. Using a sterile corn borer of 5 mm diameter, three holes were made into the Petri dishes seeded with bacterial culture. The various concentrations of the synthesized compounds (two individual leaves extract and one combined extract), while the remaining well was let empty. were inoculated in the wells prepared on the agar plates. The wells are labelled on the lid of the agar plate with letters of "MK", "PB", "S" and "–" which corresponds to each of the extract filled in the respective well (*M. koenigii, P. betle,* Synergy, negative control). The plates were incubated at temperature of 37°C for 24 hours. The test was performed in triplicate and the inhibition zones formed were measured by using vernier calliper and recorded.

3. Results and Discussions

The extracts showed varying combination effects in inhibiting the gram-positive bacteria. Overall, the synergism extracts of ratio 2:1 (*P. betle*: *M. koenigii) concentration* exhibited the highest inhibition zone with 22.6mm compared to concentration of ratio 1:1 (*P. betle: M. koenigii)* with 20.3mm and 1:2 concentration ratio (*P. betle: M. koenigii*) extracts with 16.8mm. However, for each set of concentration, the inhibition zone for the respective combined extract is smaller compared to the *P. betle* extract which showed a much bigger inhibition zone with 28.1mm, reflecting better antibacterial activity. While for *M. koenigii* extract, it showed a moderate antibacterial activity in all plates since it exhibited the smallest zone of inhibition compared to the respective combined extracts and *P. betle* extract. The highest inhibition zone exhibited by *M. koenigii* is at 11.7 mm.

The combination of *P.betle* and *M.Koneigii* has shown ability in inhibiting the growth pf bacteria. Even though the individual leaves have been found to be effective against most of the gram-positive bacteria when these two leaves are combined, its antibacterial activity were not as potent compared to when these leaves are used individually. Instead of showing synergistic effects whereby the inhibition zone would be greater than that of individual leaves, it was found that these two leaves showed antagonistic effects, due to the notable reduction in inhibition zone when each of the combined extracts were used. There was a reduced of 19% of inhibition ability of the 2:1 ratio (*P.betle: M.koneigii*) synergism extract followed by a reduced of 27.8% of the 1:1 ratio (*P. betle: M.koneigii*) and a reduced of 40% inhibition ability for 1:2 ratio (*P.betle:M.koneigii*) synergism extract as compared to the *P.Betle* inhibition zone. From the study, it also shows that by *M. koneigii* has the antagonistic effect towards the synergism when combining with *P.betle.*This was proved when the concentration of *M.koneigii* is higher than *P. betle* ,it contribute to the reduction inhibition ability of the synergism effect. When the ratio of *M.koneigii* is lower than *P.betle*,the inhibition ability is higher. Figure 1 show the inhibition zone of the extracts on different ratio of concentration while Figure 2 and 3 show the Graph inhibition zone of individual extract of *P. betle* and *M. koneigii.* the graph of inhibition exhibits by the synergism extract based on different concentration.

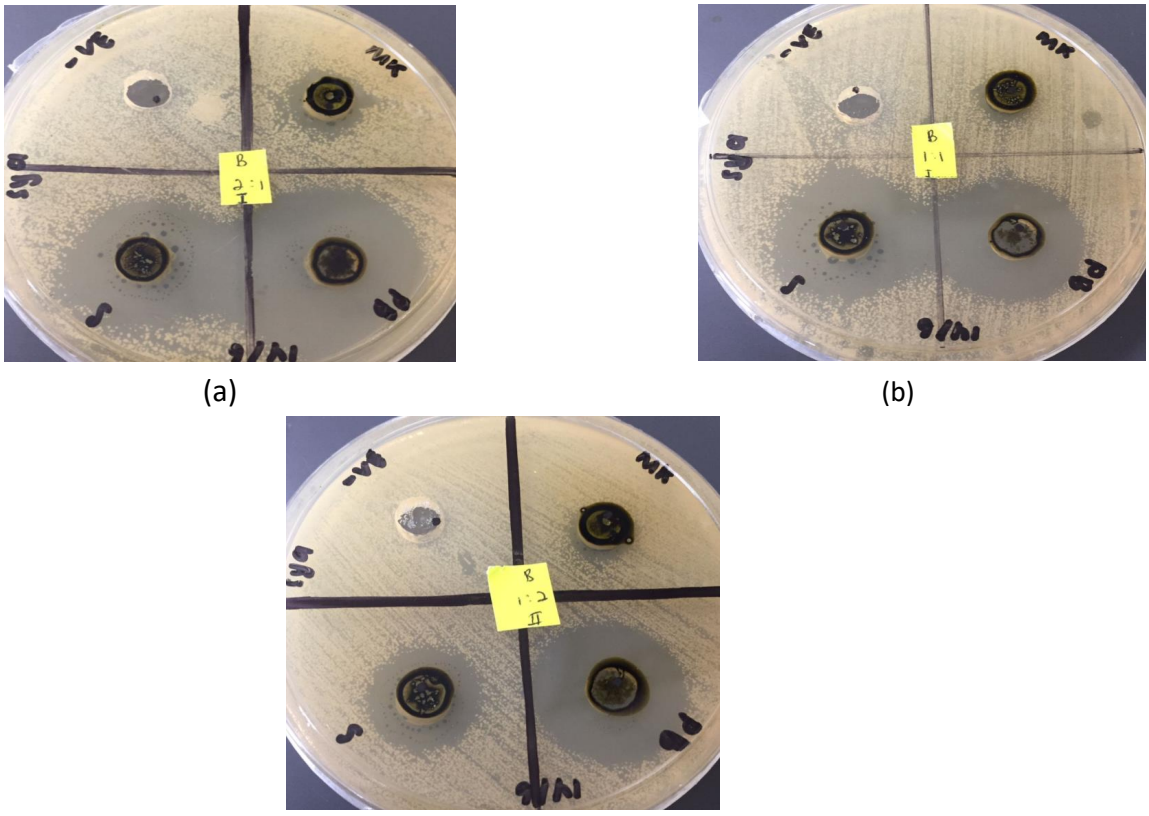

Fig. 1. Inhibition zone of the extracts on different ratio of concentration
a) Synergistic extract concentration ratio 1:1 (*P. betle*: *M. koenigii)* b)Synergistic extract concentration ratio 2:1 (*P.betle: M.koneigii*) c) Synergistic extract concentration ratio 1:2 (*P. betle*: *M. koenigii)*

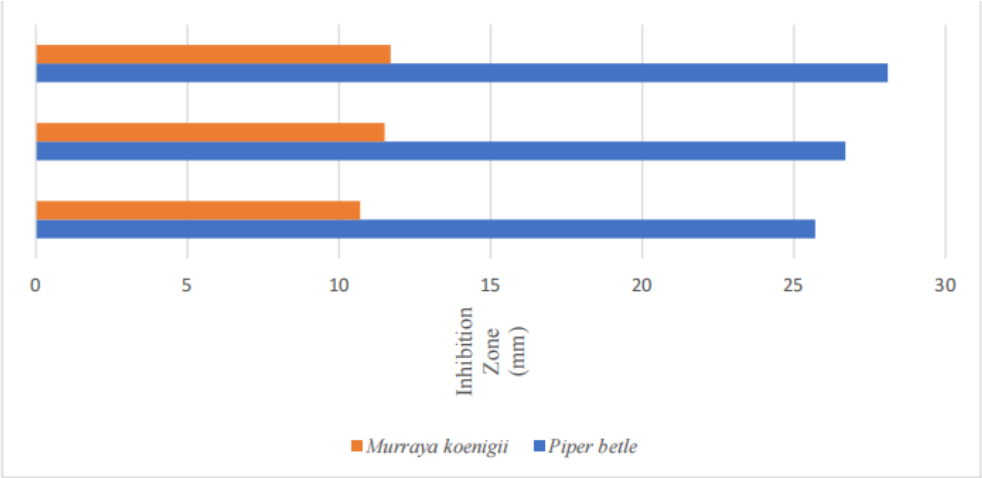

Fig. 2. Graph inhibition zone of individual extract of *P. betle* and *M. koneigii*

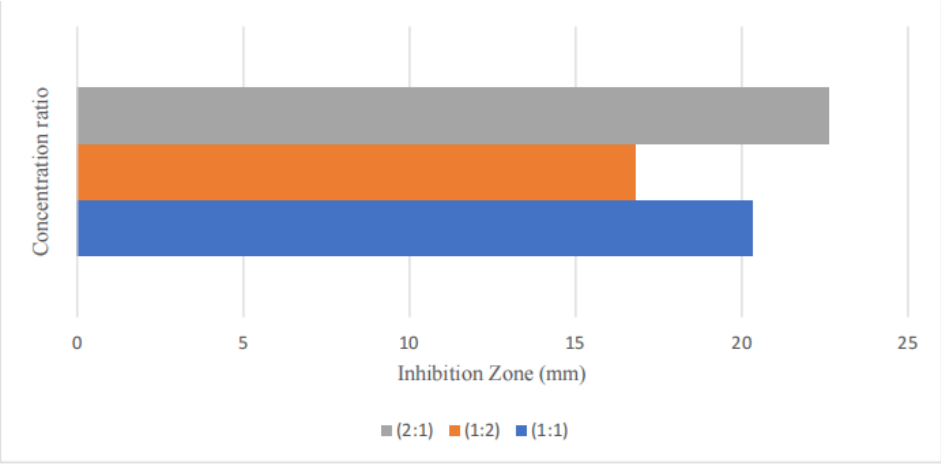

Fig. 3. Graph inhibition zone of synergism extract at different concentration ratios

This also was found in the research conducted by Raj and Yogini [4] where the synergistic effect between *M.koneigii* and *Azadirachta indica leaves does not show antibacterial effect against Streptococous mutans.* Hartini *et al*., [8] also stated antagonistic effect was recorded when *P. betle* and red betel (*Piper crocatum)* were combined for antibacterial activity against *Staphylococcus aureus, Staphylococcus epidermidis* or *Eschericia coli*. There was no significant difference between betel and red betel antibacterial activity against the three bacteria.

According to Caesar [5], the reaction between several agents can be classified as synergistic, or additive, or antagonistic. Additive effect is purely an effect of summation of the agents involved, whilst antagonistic effects are indicated by a result that is much lesser than the additive effect. As for synergistic effects or positive interaction it is indicated by the greater combination effects of the combined sample compared to the additive effect. By that means, since in plates, the combined sample (regardless its concentration) exhibited results that are much lesser than the potential additive effects of the individual leaves, making it rule out the synergistic effects. Hence, in this experiment, we found that the combined extract of *M. koenigii* and *P. betle* leaves showed antagonistic effects. The antagonist is a phenomenon when the combination of the compound produces a lower overall effect than the additive effect of the agent alone [4]. There should be investigation made on the constituents present and the plant extracts as the activity can be lost upon fractionation [8]. This multiple constituent is important in performing the biological effects.

This finding is parallel to what Bhuva [3] found where it is better for the individual leaves of *M. koenigii* and *Azadirachta indica* (neem leaves) to be used separately for an enhanced antibacterial activity against the bacteria strain such *Streptococcus mutans, Streptococcus gordonii,* and *Pseudomonas aeruginosa*. Likewise, it is found that *M. koenigii* is being slightly selective with respect to the use of leaves it is combined with, against gram- positive bacteria, like in the work conducted by Subramaniam [19]. They found that when *M. koenigii* leaves were combined with leaves of *A. indica,* the zone of inhibition against *Staphylococcus sp* greater compared to the use of individual leaves. However, when the two combined leaves extract is combined with *Plectranthus amboinicus*, a significant reduction in inhibition zone was observed. Same antibacterial affect was found by Hartini [7] when the occurrence of antagonistic effects was recorded when betel and red betel were combined.

Based on this study it is important to find out the the presence of phytochemicals in the synersitic extracts in order to help in detecting mechanisms of action for the respective combination effects. This is since when the correlation between the individual leaves' compound properties and

their respective combination effects are made, the leaves' modes of action can be recognised.The results obtain can be help in contributing to the knowledge on findings the best synergistic effect of an antimicrobial substance. The promotion of advancements in antimicrobial discovery would benefit humanity as it is less toxic compared to synthetic chemicals used in modern medicine. This would potentially lower risks of side effects commonly found in conventional treatment. By knowing the synergism effect of *P.Betle* and *M.Koneigii e*nable to find out other natural product that will have synergistic effect to antibacterial.

4. Conclusions

The experimental data of the inhibition zone of the synergistic extract shows an antagonistic effect toward the antibacterial activity. The antibacterial activity was lower compared to the individual agent inhibitory. Different concentration and different solvent extraction can be used for future research in order to go in depth for the synergism effect. Moreover, identification of phytochemical can help in detecting the mechanism for the synergism.

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