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In Vitro Antimicrobial Inhibitory Effect of *Piper Aduncum* Leaves Extracts on Bacteria and Fungi.

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Abstract

Background : The usage of herbal medicines to cure variety illnesses caused by bacteria and fungi are increasing among Malaysians. Thus, the study of natural plants and their extracts as potential antibacterial and antifungal has been accelerated. The objective is to determine the *in vitro* antimicrobial effects of *P. aduncum* against pathogenic bacteria and fungi.
Methods: Extracts of *P. aduncum* at different concentrations were used to evaluate the effects on bacteria and fungi. Two different solvents were used in the extraction process and the differences between the effects of the products were also compared. The zones of inhibition were observed after 24-hour incubation. The evaluation of antimicrobial and antifungal activities were performed by introducing the concentrations of the extract onto petri dishes containing newly inoculated agar plates of pathogenic bacteria and fungi. Then, the agar plates were incubated at 37˚C for 24 hours. The zones of inhibition were measured to determine the antibacterial and antifungal activities.
Results: Both extracts of *P. aduncum* (500 mg/ml) show the largest zone of inhibition compared to other concentrations. However, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Candida tropicalis* showed insensitive towards n-hexane extract while *Bacillus subtilis* is resistant towards DCM extract.
Conclusion: In conclusion, the highest concentration from both solvents are the most effective antibacterial and antifungal where the most potent one is the DCM extracts of *P. aduncum*

Keywords: plant antimicrobial, natural source, n-hexane, dichloromethane, antimicrobial resistance

Introduction

The *Piper aduncum* exists in numerous regions in this world and it is commonly found throughout Brazil.[1] It is usually known as matico. It has been proven to produce essential oils that has potential antimicrobial effects against a culture of phytopathogens, such as bacteria, mollusks and fungi. In addition to that, *P. aduncum* also has shown an effective analgesic effect with lesser levels of toxicity. There are diverse types of Piperaceae species that exist in this world and *P. aduncum* is a high-quality 'essential oils' producer. High level of dillapiole can be obtained from essential oil of *P. aduncum*.

P. aduncum has antihelminthic, antioxidant and antiprotozoal activity.[1] Insecticidal activity of hexane, ethyl acetate and ethanol extracts of *P. aduncum* on Spodoptera and frugiperda has also been documented.[2] A study was done to elucidate the anti-bacterial and anti-inflammatory properties of 8 *Piper* species and revealed that most of them did not possess any antibacterial activity, except for *Piper betle* and *Piper chaba*, while *Piper sarmentosum, Piper argyrophyllum* and *Piper longum* showed potential acute anti – inflammatory activity. [3]

The antimicrobial efficacy of two Piper species, *Piper cubeba* and *Piper longum* was tested against bacterial and oral fungal pathogens. The piper species produced a larger zone of inhibition compared to the antifungal drugs used to treat *Candida albicans.* [4] Inspired by this findings, a study to investigate the antimicrobial property of *Piper aduncum* was carried out.

Methods

Plant Material and Plant Extraction

Piper aduncum plant which was collected in October 2019 from Pahang state was used in this study. 1kg leaves of *P. aduncum* was rinsed to remove any unwanted substances. They were dried in hot air oven at 40°C for 96 hours. The dried plant was then grinded to a powder form. and in a chiller at 4°C.

In the extraction process, the leaves were soaked in different solvents, which was n – hexane and dichloromethane at a ratio of 100 g to 1000 ml (1 g: 10 ml) for 7 days. (Sajna et al., 2019). The products were put in conical flasks, covered with aluminum foil and stored in air – dried oven at 38°C for 12 hours to evaporate the remaining solvent. The crude extracts were then kept at 4°C in sterile universal bottles.

Sterility Proofing of the Extracts

With reference to previous method performed on proof of sterility, we made some modifications to the sterility proofing of the extracts by introducing 2 ml of the extract into 10 ml of Muller Hinton broth, and incubated at 37°C for 24 hours. [5] The absence of turbidity or clearness of the broth after the period of incubation signifies the presence of a sterile extract. [5]

Fungi and Bacteria Strains Used

Bacteria and fungi strains used in this study were contributed from Department of Microbiology and Parasitology, Faculty of International Medical School, Management and Science University, and they included 3 yeasts - *Candida albicans* (ATCC 10231), *Candida tropicalis* (ATCC 13803) and *Candida glabrata* (ATCC 2001 and bacteria used were *Bacillus subtilis* (ATCC 6633), *Enterobacter cloacae* (ATCC 23355), *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 13883), *Proteus mirabilis* (ATCC 12453), *Pseudomonas aeruginosa, Salmonella typhimurium* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermis* (ATCC 12228), *Staphylococcus saprophyticus* (ATCC 15305) and *Streptococcus pyogenes* (ATCC 19615).

Fungi and Bacteria Culture

Nutrient agar was used to culture *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Staphylococcus epidermis* (ATCC 12228) and *Staphylococcus saprophyticus* (ATCC 15305). Blood agar was used to culture

Streptococcus pyogenes (ATCC 19615), *Proteus mirabilis* (ATCC 12453) and *Pseudomonas aeruginosa*. *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 23355), *Klebsiella pneumonia* (ATCC 13883) and *Salmonella typhimurium* (ATCC 14028) were cultured using MacConkey agar. Candida species such as *Candida albicans* (ATCC 10231), *Candida tropicalis* (ATCC 13803) and *Candida glabrata* (ATCC 2001) were cultured by using Sabouraud Dextrose agar.

Three to five single colonies of each bacteria and fungi from the respective agar plates culture were suspended in 5 ml of specific nutrient, blood, MacConkey and Sabouraud Dextrose broth and incubated at 37 ºC until visibly turbid (0.5 McFarland standard). [6] The inoculum suspension was used within 15 minutes of standardization, which was very important factor in order to avoid any change of the size of inoculums or lose their viability.[7] A sterile cotton swab was dipped into the standardized bacterial inoculum suspension, and then streaked over the whole dried surface of 90 mm specific agar plates.

Disc Diffusion Assay

The antimicrobial properties of *Piper aduncum* was investigated by using the disc diffusion method. Dilution factor calculations was performed using the formula $df = V^1/V^2$. A stock solution of extract was prepared by dissolving 1000 mg of extract with 1 ml of their respective solvents Dimethyl Sulfoxide to produce a final volume of 2000 ml as a final volume. From this information, dilution factor of this investigation was 1/2.

Each micro test tubes were filled with 1 ml of Dimethyl Sulfoxide. About 1000 mg/ml was transferred from the previous micro test tube to another micro test tube. The maximum concentration was serially diluted in two-fold using micropipette in order to produce the following concentrations: 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml. 20 μL of each dilution was impregnated into sterile, blank sterile discs 6 mm in diameter. 5 μL of extract was spotted alternately on both sides of the discs and allowed to dry before the next 5 μL was spotted to ensure precise impregnation.

All discs were fully dried before the application on bacteria and fungi lawn cultures. The discs were pressed gently to ensure uniform contact with agar surface. All the plates were inverted and incubated in an incubator. Petri dish that contained fungi culture were incubated for 49 hours while the petri dish that contained bacteria culture were incubated for 24 hours at 37°C. Each of the tests were done in triplicates. The antimicrobial activity was determined by measuring the diameter of zone of inhibition.

Statistical Analysis

Measurements of each triplicated tests were taken and results were recorded as mean and standard deviation. One – way analysis of variance (ANOVA) was performed and it was considered as significant if the *P*-value <0.05.

Results

The present study showed antibacterial and antifungal effect on several Gram-negative bacteria, Gram-positive bacteria and fungi after an exposure towards different concentrations from n – hexane and dichloromethane extracts of *Piper aduncum* leaves.

Table 1 and 2 showed the zone of inhibition of Gram-positive bacteria, Gram-negative bacteria and fungi after being exposed to the test concentrations of *P. aduncum* at the concentration of 500 mg/ml, 250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml. Based on the table below, most of the Gram-negative, Gram-positive and fungi showed a positive result after being exposed to the test concentration of n-hexane extracts. Specifically, most of the test organisms showed a significant zone of inhibition at the test concentration of 500 mg/ml, except for *C. glabrata* which produced a non-significant reading. Similarly, the dichloromethane extract of

P. aduncum showed significant zone of inhibition at the test concentration of 500 mg/ml except for C. tropicalis. However, the Gram-positive and Gram-negative bacteria showed a significant zone inhibition results.

Discussion

Previous research has proved that there was presence of tannin and saponin compound in the extraction of *P. aduncum.* [8] Tanin has the ability to prevent the development of chitin in the cell wall of fungi. Presence of saponin in *P. aduncum* extract may bind with sterol component of cell wall causing damage to the cell membrane.[9] The destruction of cell membrane and cell wall of fungi will contribute to disruption of cell metabolism and inhibit the growth. Dichloromethane extract of *P. aduncum* showed significant zone of inhibition ($P<0.05$) except for *C. tropicalis*. n-hexane extract of P. *aduncum* did not show a significant zone of inhibition with *C. glabrata* . Both results might be due to a technical error during handling of this experiment. This error possibly might be caused by the prolonged storage of extracts that may lead to potential contamination. This potential contamination can be from the other species of fungi which may disrupt the efficacy of *P. aduncum* extracts on specific pathogenic fungi as antifungal agent.

Antibacterial properties

Piper species were proven to have various phytochemicals such as alkaloids, polyphenols, saponins, steroids and tannin, and hence, Piper species has the potential to possess a wide array of activities such as antioxidant, antibacterial and insecticidal activities. [10] The component known as saponin compound has numerous function and effects toward the fungi and bacteria. Most of the saponin can be classified as having an ability to carry out the antimicrobial activities. Saponin has produce a wide range of function including beneficial and harmful effects on human and also has allelopathic action in term of the growth of plant, pesticidal, insecticidal and it also may act as phyto-protectant that protect the plant against the microbes. Saponin effects cell membrane permeability due to its ability to create pores. However, saponin may not have any effect on certain Gram-negative bacteria.

Previous research done have tested the antimicrobial activities of tannins that had been extracted from a plant known as *Rhizophora apiculate* barks.[11] The antimicrobial activity was tested on each test organism using a mixed tannin, hydrolysable tannin and condensed tannin. The result showed that tannin has the ability to exhibit antibacterial and anti-yeast activity. After being exposed to the mixed tannin, *Acinetobacter calcoaceticus, S. epidermis, Yersinia enterocolitica, Micrococcus sp. P. mirabilis, S. aureus, P. aeruginosa, Erwinia sp., K. pneumoniae, B. cereus* and *Saccharomyces cerevisiae* showed a complete inhibition. However, *B. subtilis, C. albicans, E. coli* and *Cryptococcus neoformans* only showed a partial inhibition after being exposed to the mixed tannin. When the test organism is being exposed to the condensed tannin, only *S. saprophyticus, S. aureus, A. calcoaceticus, Erwinia sp., K. pneumonia, P. aeruginosa* and *S. cerevisiae* showed a complete inhibition and, *Serratia marcescens, S. paratyphi, S. epidermis, B. cereus, C. albicans, C. neofarms* and *Enterobacter aerogenes* showed a partial inhibition. Hydrolysed tannin showed complete inhibition with *P. mirabilis, A. calcoaceticus, S. saprophyticus, A. anitratus, B. licheniformis, Micrococcus sp., S. epidermidis, S. typhi, S. aureus, Erwinia sp., Klebsiella sp., C. albicans* and *S. cerevisiae*. *P. aeruginosa, Rhodotorula rubra, C. neoformans* and *B. cereus* showed a partial inhibition against the hydrolyzed tannin. [10]

This study showed *S. pyogenes* was best inhibited only at the concentration of 500 mg/ml with n-hexane extract of *P. aduncum* while, zone of inhibitions were seen in dichloromethane extract of *P. aduncum* at all concentration; 500 mg/ml,

250 mg/ml, 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml. Previous research showed that the crude extract of the aerial part of *P. aduncum* produced a zone of inhibition against *S. pyogenes* and with the MIC was 2 mg/ml, and MIC of positive control ciprofloxacin was 1 mg/ml. [12] There are obvious differences between previous and current studies where this study exhibited much higher MIC values. Previous research has used ethanol to extract the phytochemical components in *P. aduncum* and used only the aerial part. This may explain the differences in the results.

Conclusion

This research showed that *P. aduncum* n-hexane and dichloromethane leaves extracts have the ability to exhibit antibacterial properties against Gram-negative and Gram-positive bacteria and antifungal activity. Further research is required to improve the reproducibility of the antibacterial and antifungal activities of *P. aduncum*.

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Table 1. Zone of inhibition of Gram-positive, Gram-negative and fungi after being exposed to dichloromethane extraction of *P. aduncum* and incubated for 24 hours.

Species	Zone of Inhibition (mm) Mean					*P*-Value
	500 mg/mL	250 mg/mL	125 mg/mL	62.5 mg/mL	31.25 mg/mL	
Candida albicans	14.33 ± 1.15	11.00 ± 1.00	9.00 ± 0.00	8.33 ± 0.57	8.33 ± 1.15	*P* < 0.05 (0.000)
Candida glabrata	11.66 ± 2.08	10.00 ± 1.00	8.66 ± 0.57	8.00 ± 0.0	7.00 ± 0.0	*P* < 0.05 (0.003)
Candida tropicalis	21.00 ± 4.35*	17.33 ± 3.21*	16.33 ± 3.21*	14.66 ± 2.88*	14.66 ± 2.51*	*P* > 0.05 (0.191)
Bacillus subtilis	16.00 ± 3.46	12.33 ± 0.57	9.00 ± 0.00	8.00 ± 1.00	7.00 ± 1.00	*P* < 0.05 (0.000)
Staphylococcus aureus	23.33 ± 2.08	20.66 ± 2.08	18.66 ± 0.57	15.33 ± 0.57	14.00 ± 0.00	*P* < 0.05 (0.000)
Staphylococcus epidermis	14.00 ± 1.00	11.33 ± 0.57	9.33 ± 0.57	9.00 ± 0.00	7.66 ± 0.57	*P* < 0.05 (0.000)
Staphylococcus saprophyticus	16.00 ± 2.64	14.33 ± 1.52	10.66 ± 1.52	10.00 ± 0.00	9.00 ± 0.00	*P* < 0.05 (0.001)
Streptococcus pyogenes	28.33 ± 2.08	22.00 ± 1.73	18.66 ± 1.52	15.33 ± 2.08	9.66 ± 2.30	*P* < 0.05 (0.000)
Enterobacter cloacae	19.33 ± 0.57	18.33 ± 0.57	17.66 ± 0.57	13.33 ± 0.57	11.00 ± 1.00	*P* < 0.05 (0.000)
Escherichia coli	23.00 ± 2.00	18.00 ± 1.73	14.66 ± 0.57	13.00 ± 1.00	9.00 ± 1.00	*P* < 0.05 (0.000)
Klebsiella pneumonia	17.33 ± 2.08	16.66 ± 0.57	15.33 ± 0.57	11.66 ± 1.15	10.00 ± 0.00	*P* < 0.05 (0.000)
Proteus mirabilis	23.66 ± 1.15	16.00 ± 0.00	13.33 ± 1.52	10.66 ± 1.52	10.00 ± 1.00	*P* < 0.05 (0.000)
Pseudomonas aeruginosa	16.33 ± 1.15	13.00 ± 1.00	10.33 ± 0.57	9.66 ± 0.57	9.66 ± 0.57	*P* < 0.05 (0.000)
Salmonella typhimurium	17.00 ± 1.00	14.66 ± 0.57	11.33 ± 2.30	9.66 ± 2.08	8.00 ± 1.73	*P* < 0.05 (0.000)

(*) *P*-value showed not-significant reading *P*<0.05

Table 2. Zone of Inhibition of Gram-positive, Gram-negative bacteria and fungi after being treated with n-hexane extraction of *P. aduncum* and incubated for 24 hours.

Species	Zone of Inhibition (Mean) mm					*P*-Value
	500 mg/mL	250 mg/mL	125 mg/mL	62.5 mg/mL	31.25 mg/mL	
Candida albicans	17.00 ± 1.00	12.33 ± 3.51	11.00 ± 2.00	9.66 ± 2.08	8.00 ± 2.00	*P* < 0.05 (0.006)
Candida glabrata	18.00 ± 6.08*	13.66 ± 2.08*	11.00 ± 2.64*	11.00 ± 264*	11.66 ± 2.88*	*P* > 0.05 (0.159)
Candida tropicalis	10.33 ± 0.57	8.66 ± 1.52	9.33 ± 1.15	5.66 ± 0.57	6.66 ± 0.57	*P* < 0.05 (0.001)
Bacillus subtilis	12.00 ± 1.00	10.33 ± 1.15	9.33 ± 0.57	6.00 ± 0.00	6.33 ± 0.57	*P* < 0.05 (0.000)
Staphylococcus aureus	16.33 ± 3.21	8.00 ± 1.00	8.33 ± 2.08	7.66 ± 2.08	8.00 ± 0.00	*P* < 0.05 (0.001)
Staphylococcus epidermis	25.66 ± 1.15	12.66 ± 2.08	9.66 ± 0.57	9.33 ± 0.57	9.33 ± 0.57	*P* < 0.05 (0.000)
Staphylococcus saprophyticus	17.33 ± 1.52	11.00 ± 0.00	9.33 ± 0.57	8.66 ± 1.15	7.66 ± 1.15	*P* < 0.05 (0.000)
Streptococcus pyogenes	10.33 ± 0.57	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	*P* < 0.05 (0.000)
Enterobacter cloacae	16.66 ± 1.52	13.00 ± 1.00	10.66 ± 1.52	10.66 ± 1.15	9.33 ± 1.15	*P* < 0.05 (0.000)
Escherichia coli	22.66 ± 3.51	17.66 ± 5.03	11.33 ± 1.52	9.66 ± 0.57	7.66 ± 0.57	*P* < 0.05 (0.000)
Klebsiella pneumonia	17.66 ± 1.52	15.00 ± 1.00	14.66 ± 1.52	11.66 ± 0.57	10.66 ± 0.57	*P* < 0.05 (0.000)
Proteus mirabilis	11.33 ± 2.30	10.33 ± 1.52	9.33 ± 1.52	8.00 ± 1.00	5.66 ± 0.57	*P* < 0.05 (0.008)
Pseudomonas aeruginosa	21.66 ± 2.88	15.66 ± 3.05	13.00 ± 2.64	11.33 ± 2.08	9.33 ± 0.57	*P* < 0.05 (0.001)
Salmonella typhimurium	19.66 ± 2.51	16.00 ± 3.60	12.66 ± 3.05	10.00 ± 0.00	9.00 ± 1.00	*P* < 0.05 (0.002)

(*) *P*-value showed not-significant reading *P*<0.05

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