Phytochemical screening, antifungal and antibacterial activity of *Psidium guajava* leaf extracts from dry zone and wet zone of Sri Lanka

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Abstract—Plants are capable of synthesizing various chemical compounds that support them to defend against attack from an immense diversity of predators such as fungi, bacteria, insects and herbivorous mammals. Although some of these chemical compounds are toxic to plant predators, they seem to be effective drugs for human diseases. *Psidium guajava* L. is used to treat some diseases such as diarrhea, diabetes, gastric cancers, osteoarthritis and skin diseases and this plant belongs to the family Myrtaceae. The aim of this study was to identify the phytochemical constituents and to determine the antifungal and antibacterial activities of *Psidium guajava* leaf extracts from dry zone and wet zone of Sri Lanka. The antifungal bioassay was performed by Agar well diffusion method with the concentration of 500 ppm of the leaf extracts against the fungi *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Rhizopus* sp. and *Trichoderma* sp. The antibacterial bioassay was performed against the bacteria *Acinetobacter* sp., *Bacillus* sp., *Escherichia coli*, *Pseudomonas* sp., and *Staphylococcus* sp. Methanol and acetone extracts of leaves of *P. guajava* displayed high degree of antifungal activity (mean diameter) for both dry and wet zones against *Aspergillus* sp. (20.8 mm, 18.5 mm, 23.0 mm and 20.3 mm) and *Trichoderma* sp. (16.3 mm, 20.3 mm, 20.3 mm and 22.5 mm) respectively. Methanol and acetone extracts of leaves of *P. guajava* revealed high degree of antibacterial activity for wet zone against *Bacillus* sp. (26.0 mm, 27.1 mm) and *Acinetobacter* sp. (20.0 mm, 23.0 mm) respectively. The phytochemical analysis revealed that the leaves of *P. guajava* from both zones contain secondary metabolites alkaloids, flavonoids, saponins, tannins, coumarins and cardiac glycosides which are the bioactive compounds and could play major role in antimicrobial activities.

Keywords- Antibacterial activity, Antifungal activity, Agar well diffusion method, Phytochemical analysis, *Psidium guajava* L.

I. INTRODUCTION

Plants are considered to be the important sources of medicines ever since the beginning of human civilization. Out of approximately 300,000 species of higher plants available, only a small proportion has been evaluated for medicinal uses, and a still smaller number to yield well-defined drugs. Therefore, the knowledge of plant constituents achieved so far is still insufficient, considering the immense number of species available in the world. Approximately, only 10% of the organic compounds are reported to be known and the remaining 90% are yet to be investigated (Balandrin *et al.*, 1985).

Psidium guajava L. is one of the medicinal plants used to treat various kinds of bacterial and fungal infections. *P. guajava* leaves and fruits are widely used in ayurvedic formulations because of the presence of significant amount of nutrients, thus called as a nutritional powerhouse. The plant *P. guajava* can adapt to a wide range of growing conditions from humid lowland tropics to the cooler elevations (Rymbai *et al*., 2010). The other uses of *P. guajava* include antidiabetic, anticancer, treatment of oral infections, gastrointestinal infections and respiratory infections etc. (Gutiérrez *et al.*, 2008).

In several studies, *Psidium guajava* showed significant antibacterial and antifungal activities. Methanol, hexane and ethyl acetate leaf extracts of *Psidium guajava* Linnaeus were tested against diarrhea-causing bacteria: *Staphylococcus aureus*, *Salmonella* spp. and *Escherichia coli*. Strains that were investigated included isolates from Seabob shrimp, *Xiphopenaeus kroyeri* (Heller) and laboratory-type strains. The test showed that the *Staphylococcus aureus* strains were mostly inhibited by the extracts (Gonçalves *et al*., 2008). Hexane, acetone and methanol extracts were sequentially extracted from *Psidium guajava* leaves and those were examined for their antifungal activities against *Trichophyton rubrum*, *Trichophyton tonsurans*, *Sporotrix schenckii*, *Microsporum canis*, *Cryptococcus neoformans*, *Candida parapsilosis* and *Candida albicans* by using the agar disk diffusion technique. Compared to control, hexane extract showed the highest antifungal activity, being active against all the tested dermatophytes (Beatriz *et al*., 2012). Antibacterial assay of aqueous and organic extracts of *Psidium guajava* leaves was tested against Multi Drug Resistant (MDR) clinical isolates of *Staphylococcus aureus* strains collected from hospitals in Northern (Malabar region) Kerala. The strains that showed resistance against all the antibiotics examined were selected for antibacterial assays. Minimum Inhibitory Concentration (MIC) for methanolic and aqueous extracts was investigated to be 625 µg/mL and 7.5 mg/mL respectively (Anas *et al*., 2008).

The aim of this study was to identify the phytochemical constituents and to determine the antifungal and antibacterial activities of *Psidium guajava* L. leaf extracts from dry zone and wet zone of Sri Lanka.

II. MATERIALS AND METHODS

A. Chemicals, solvents and instruments

Laboratory grade chemical reagents [$CHCl_3$ (99.5%), 18.4 $moldm^{-3}$ H_2SO_4 (95.0%), 11.65 $moldm^{-3}$HCl (35.0%), 18.1 $moldm^{-3}$ NH_3 (25.0-28.0%), 17.4 $moldm^{-3}$ Acetic acid (99.5%), 0.1% of $FeCl_3$ (98.0%), Ethyl acetate (99.5%), Acetic anhydride (99.0%)] were used during the phytochemical analysis.

Pure hexane (99.0%), acetone (99.5%) and methanol (100.0%) were used as solvents in order to extract non-polar, intermediate polar and polar leaf extracts of *Psidium guajava*. Soxhlet apparatus was used in order to perform hot extraction method. The solvents were evaporated under reduced pressure by using rotatory evaporator.

B. Source of plant materials

Plant material, soft leaves of *Psidium guajava* were collected. Leaves of wet zone were collected from fruit garden at Nawalakanda, Kalawana, Rathnapura and the leaves of dry zone were collected from Thirunelvely, Jaffna. These leaf samples have been authenticated with herbarium at the Department of Botany, University of Jaffna.

C. Preparation of leaf extract

Leaves from the plant of wet zone were taken and air dried for two weeks without exposing to the direct sun light. Then the plant material was ground into powder form and 80g of the powdered plant material was sequentially extracted by using Soxhlet apparatus with hexane, acetone and methanol (3 times, 24 hours each) as solvents. Plant material: solvent ratio was 1:5 (w/v). The solvent was evaporated under reduced pressure by using rotatory evaporator. The samples of hexane, acetone and methanol crude extracts were weighed out separately. This procedure was repeated for the plant material obtained from dry zone.

D. Preparation of test solution

Concentration of 500 ppm test solutions were prepared for each of the extracts obtained through Soxhlet extraction for both of the plants (dry zone and wet zone).

E. "In vitro" Antifungal assay

In vitro antifungal properties for the each of the extracts were tested against plant pathogenic fungi *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Rhizopus* sp. and *Trichoderma* sp. The antifungal assay was achieved by agar well diffusion method. Mancozeb (Dithane M-45, 0.3 mg/150 µL) and pure solvents (hexane; 99.0%, acetone; 99.5% and methanol; 100.0%) were used as standard and controls respectively.

E.1. Preparation of fungal spore suspension

A loopful of spores was taken by using an inoculation loop and suspended into sterile saline water aseptically. The spore concentration was determined by the Haemacytometer. After that the suspension was stirred well and serially diluted up to X $*10^5$ number of spores/mL.

E.2. Agar well diffusion method

0.1 mL of each fungal spore suspension was spread on the entire surface of the Potato Dextrose Agar (PDA) plate uniformly with the help of a sterile spreader. 9 mm diameter wells were made by using a sterile cork borer. Then 150 µL of test solutions, standard (mancozeb) and control (hexane, acetone, methanol) were added into each well separately with the help of a sterile microliter pipette. All the plates were incubated at room temperature for 4-5 days and the zone of inhibition around the well was measured after 72 hours. Each of the experiment was performed thrice and the mean value was obtained (Clark *et al.,* 1981). The above procedure was repeated for each of the fungus.

F. "In vitro" Antibacterial assay

In vitro antibacterial properties were tested against the bacteria *Acinetobacter* sp., *Bacillus* sp., *Escherichia coli*, *Pseudomonas* sp. and *Staphylococcus* sp. Streptomycin (500 ppm) and pure solvents were used as standard and controls respectively.

F.1. Preparation of bacteria suspension

A loopful of bacterial culture was taken by a sterile inoculation loop and suspended into sterile saline water aseptically. Then the suspension was stirred well and the concentration of the bacterial suspension was maintained as X $*10^8$ cells/mL by comparing the turbidity with 0.5M McFarland standard using dilution techniques.

F.2. Determination of the antibacterial activity

Antibacterial activity of *P. guajava* leaf extracts was determined using agar well diffusion method. 0.1 mL of each bacterial suspension was spread on the entire surface of the Nutrient Agar (NA) plate uniformly with the help of a sterile spreader. 9 mm diameter wells were made by using a sterile cork borer. Then 150 µL of test solutions, standard (streptomycin) and control (hexane, acetone, methanol) were transferred into each well separately with the help of a sterile microliter pipette. All the plates were incubated at 37°C for 24 hours and the zone of inhibition around the well was measured. Each of the experiment was performed thrice and the mean value was obtained (Patience, OA., 2006). The above procedure was repeated for each of the bacterium.

G. Phytochemical screening

Different types of phytochemical constituents of the *P. guajava* leaf extracts were determined by using standard procedures (Harborne, J.B., 1984; Sofowora, A., 1993; Trease, G.E. and Evans, W.C., 1989). The colour intensities of each extracts and/or the appearance of solids in those extracts during the identification reactions revealed a semiquantitative evaluation of the presence of various kinds of secondary metabolites such as alkaloids, flavonoids, tannins, terpenoids, coumarins etc.

III. RESULTS AND DISCUSSION

In wet zone sample, acetone and methanol extracts of leaves inhibited the growth of almost all fungi tested except the *Rhizopus* sp. Hexane extract did not inhibit the growth of *Fusarium* sp. and *Rhizopus* sp. Acetone and methanol solvents (controls) also exhibited the inhibitory effect on the growth of *Fusarium* sp. (15.5mm, 19.0mm) and *Penicillium* sp. (16.0mm,16.0mm). But *Aspergillus* sp. growth was inhibited by all solvents as hexane, acetone and methanol (10.0mm, 10.0mm, 12.0mm). However, extracts had higher degree of inhibition than the solvents alone in terms of mean diameter of the inhibition zone. Growth of *Aspergillus* sp., *Penicillium* sp. and *Trichoderma* sp. was predominantly affected by the extracts of leaves from wet zone (Figure 1).

In dry zone sample, except *Rhizopus* sp. growth of all other fungi was inhibited by all three extracts. Growth of *Aspergillus* sp. was highly inhibited by hexane (17.0mm), acetone (23.0mm) and methanol (20.8mm) extracts when compared with other fungi tested. Hexane extract was less effective on the growth of *Penicillium* sp. (10.0mm). But acetone and methanol extracts were less effective on the growth inhibition of *Fusarium* sp. than compared to other fungi (Figure 2).

When considering the antibacterial activity of the wet zone sample, all bacterial growth was inhibited by the acetone and methanol leaf extracts. But acetone and methanol solvents alone exhibited antibacterial activity on all bacteria tested, except the growth of *Acinetobacter* sp. which was not inhibited by the methanol solvent. Degree of antibacterial activity was higher on *Bacillus* sp. (27.1mm), *Acinetobacter* sp. (23.0mm), *E.coli* (26.0mm) and *Staphylococcus* sp. (25.5mm) by the acetone extract compared with the standard streptomycin. Likewise, the degree of inhibition was higher on the growth of *Bacillus* sp. (26.0mm) by the methanol extract when compared to the standard. Hexane leaf extract did not exhibit any inhibitory effect on the growth of *E.coli* and *Staphylococcus* sp. and the hexane solvent alone did not show any inhibitory effect on the growth of all bacteria tested (Figure 3).

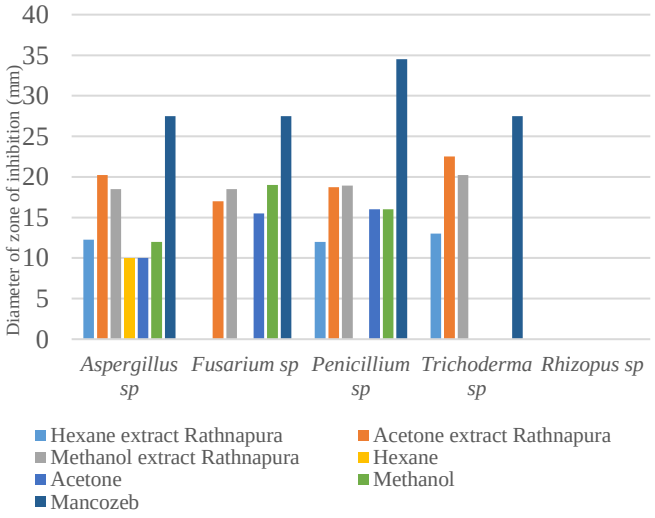

Figure 1: Antifungal activity of crude extracts of wet zone

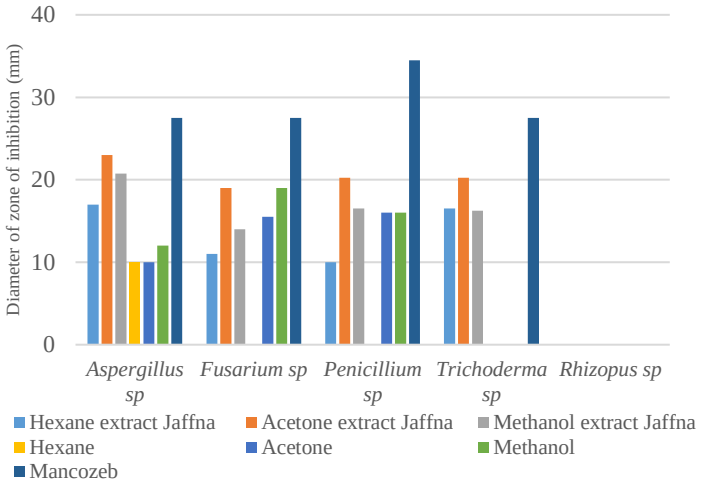

Figure 2: Antifungal activity of crude extracts of dry zone

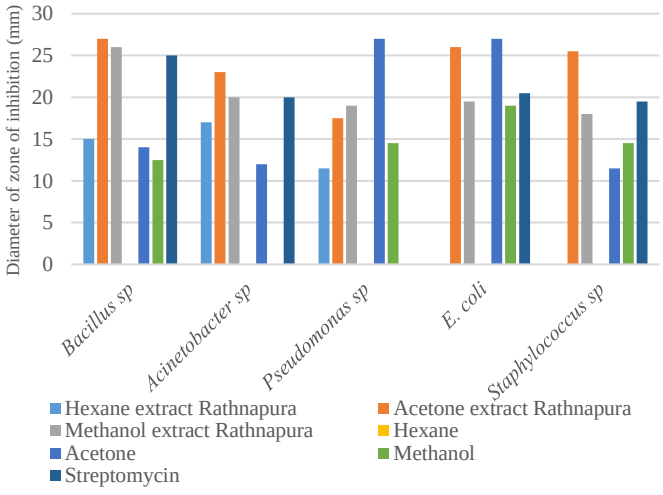

Figure 3: Antibacterial activity of crude extracts of wet zone

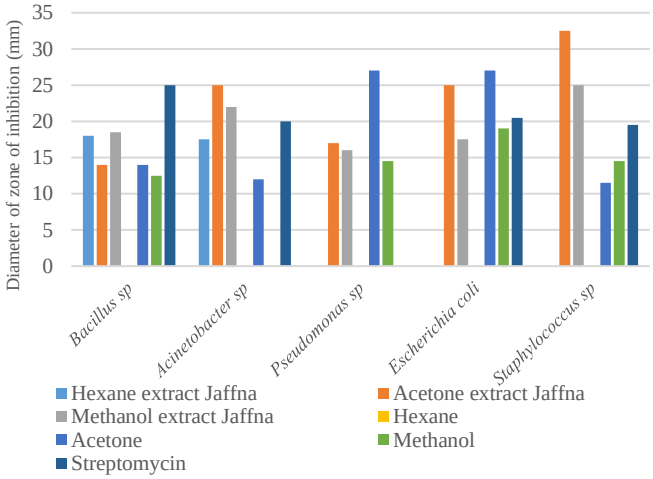

Figure 4: Antibacterial activity of crude extracts of dry zone

Organic extract	Alkaloids	Flavonoids	Saponins	Tannins	Terpenoids	Phlobatannins	steroids	Coumarins	Cardiac glycosides
Hexane	-	+	-	-	-	-	-	++	+
Acetone	+	+	+	++	+	-	-	+	++
Methanol	++	++	++	++	++	-	-	++	++

(++), abundant; (+), present; (-), absent.

Table 1: Phytochemical analysis of *P. guajava* wet zone leaf extracts

Organic extract	Alkaloids	Flavonoids	Saponins	Tannins	Terpenoids	Phlobatannins	steroids	Coumarins	Cardiac glycosides
Hexane	-	+	-	-	-	-	-	++	+
Acetone	+	+	+	++	+	-	-	+	++
Methanol	++	++	++	++	++	-	-	++	+

(++), abundant; (+), present; (-), absent.

Table 2: Phytochemical analysis of *P. guajava* dry zone leaf extracts

For the antibacterial activity of the dry zone sample, acetone and methanol crude leaf extracts inhibited the growth of all bacteria. But hexane crude extract only inhibited the growth of *Bacillus* sp. (18.0mm) and *Acinetobacter* sp. (17.5mm). Degree of growth inhibition was higher on *Acinetobacter* sp. (25.0mm), *Pseudomonas* sp. (17.0mm), *E.coli* (25.0mm) and *Staphylococcus* sp. (32.5mm) by the acetone crude leaf extract than the other two extracts, whereas methanol crude extract showed higher degree of inhibition on the growth of *Bacillus* sp. (18.5mm) (Figure 4).

A particular crude extract showed different degree of antifungal and antibacterial activities among fungal and bacterial species. At the same time, different crude extracts exhibited different degree of activities on a particular species. The commercially available synthetic fungicide Mancozeb (Dithane M-45) was used as the standard and showed the highest antifungal activity than the crude extracts. This fungicide is commonly used to control the diseases like downy mildews, rust diseases, anthracnose and leaf blights in the field crops except rice plant (Gullino *et al.*, 2010). But the acetone and methanol crude leaf extracts exhibited higher degree of antibacterial activity than the standard streptomycin.

The qualitative phytochemical analysis revealed that alkaloids, flavonoids, saponins, tannins, terpenoids, coumarins and cardiac glycosides were present. Occurrence of the types of bioactive compounds was same in both wet and dry zones extracts. Seven different types of compounds were identified in both acetone and methanol extracts. But hexane extract had only three different types of bioactive compounds (Table 1 and Table 2).

The phytochemical screening and the qualitative estimation generally provide the way of identifying the presence of bioactive compounds which may indicate the medicinal value of *Psidium guajava* leaves. Acetone and methanol leaf extracts of *Psidium guajava* showed the presence of tannins and terpenoids that have a great potential to inhibit the growth of fungi, yeasts and bacteria (Chung *et al.*, 1998). Hexane leaf extract of *Psidium guajava* indicated the presence of coumarins. Various studies have been demonstrated that coumarin has a great inhibitory effect on microbial activities as well as a potential antioxidant (Lake, B.G., 1999). These effects are due to its ability to scavenge free radicals and foreign species and to chelate metal ions

Name of the Fungi	Average zone of inhibition in mm									
	Hx		Ac		Me		Hx	Ac	Me	Mz
	HLJ	HLR	ALJ	ALR	MLJ	MLR				
Aspergillus sp.	17.0	12.3	23.0	20.3	20.8	18.5	10.0	10.0	12.0	27.5
Fusarium sp.	11.0	-	19.0	17.0	14.0	18.5	-	15.5	19.0	27.5
Penicillium sp.	10.0	12.0	20.3	18.8	16.5	18.9	-	16.0	16.0	34.5
Trichoderma sp.	16.5	13.0	20.3	22.5	16.3	20.3	-	-	-	27.5
Rhizopus sp.	-	-	-	-	-	-	-	-	-	-

Mz, Mancozeb; Hx, Hexane; Ac, Acetone; Me, Methanol; HLJ, Hexane leaf extract dry zone; HLR, Hexane leaf extract wet zone; ALJ, Acetone leaf extract dry zone; ALR, Acetone leaf extract wet zone; MLJ, Methanol leaf extract dry zone; MLR, Methanol leaf extract wet zone; (-), no clear zone and the diameter of the well was 9 mm

Table 3: Antifungal activity for dry zone and wet zone samples

Name of the bacteria	Average zone of inhibition in mm									
	Hx		Ac		Me		Hx	Ac	Me	Str
	HLJ	HLR	ALJ	ALR	MLJ	MLR				
Bacillus sp.	18.0	15.0	14.0	27.1	18.5	26.0	-	14.0	12.5	25.0
Acinetobacter sp.	17.5	17.0	25.0	23.0	22.0	20.0	-	12.0	-	20.0
Pseudomonas sp.	-	11.5	17.0	17.5	16.0	19.0	-	27.0	14.5	-
Escherichia coli	-	-	25.0	26.0	17.5	19.5	-	27.0	19.0	20.5
Staphylococcus sp.	-	-	32.5	25.5	25.0	18.0	-	11.5	14.5	19.5

Str, Streptomycin; Hx, Hexane; Ac, Acetone; Me, Methanol; HLJ, Hexane leaf extract dry zone; HLR, Hexane leaf extract wet zone; ALJ, Acetone leaf extract dry zone; ALR, Acetone leaf extract wet zone; MLJ, Methanol leaf extract dry zone; MLR, Methanol leaf extract wet zone; (-), no clear zone and the diameter of the well was 9 mm

Table 4: Antibacterial activity for dry zone and wet zone samples

(Tseng, A., 1991). Therefore, antimicrobial properties of various extracts from many plants have recently been of great interest in both research and the food industry, because their possible use as natural additives and Ayurvedic formulations emerged from a growing tendency to replace synthetic antimicrobials and antioxidants with natural ones (Deba *et al.*, 2008).

There was a significant different in the degree of antifungal and antibacterial activities between dry and wet zone samples (Table 3 and Table 4). Previous study indicated that the antibacterial activities are influenced by the ecological conditions such as soil type, water quality, pH, climatic changes and the type and concentration of bioactive compounds (Clark *et al.*, 1981). Therefore, the existence of difference in the degree of antimicrobial activities was due to the ecological conditions of the two different habitats. Environmental factors also played a role to influence the degree of antimicrobial activities on the leaf of *Psidium guajava*.

IV. CONCLUSIoN

Leaf extracts of *Psidium guajava* exhibited antifungal as well as antibacterial activities. A particular crude extract showed different degree of antifungal and antibacterial activities among fungal and bacterial species. At the same time, different crude extracts exhibited different degree of activities on a particular species. Acetone and methanol crude leaf extracts exhibited higher degree of antibacterial and antifungal activities when compared to hexane extract. Significant difference was observed in the degree of antifungal and antibacterial activities between dry and wet zone samples. Environmental factors also played a role to influence the degree of antimicrobial activities on the leaf of *Psidium guajava*.

The active compounds of each of the extracts can be isolated by using flash chromatography technique. Further purification is possible by HPLC and characterization of those active compounds can be achieved by using spectroscopic methods (UV, IR, NMR and MS).

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