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Antifungal activity of secondary metabolites present in *Psidium guajava* leaves against dermatophytes

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Abstract

Dermatophytes are a group of fungi that commonly cause skin diseases in animals and humans. *Psidium guajava* is a fruit tree with high medicinal value commonly used to cure various skin ailments. Methanol leaf extract of *Psidium guajava* was used to screen for the antifungal activity against five species of dermatophytes; *Epidermophyton floccosum, Trichophyton rubrum, T. mentagrophytes, Microsporum gypseum* and *M. canis*. The antifungal activity of the extract was tested using the agar well diffusion method from which the methanol crude extract revealed the highest antifungal activity exhibiting 22.5 mm against *E. floccosum*. Preliminary analysis of secondary metabolites was performed for the methanol crude extract which revealed the presence of many bio-active compounds including saponins, steroids and alkaloids. It was then subjected to fractionation and hexane, chloroform, ethyl acetate and aqueous fractions were obtained. Ethyl acetate fraction exhibited the highest antifungal activity against the test organisms recording 27.13 mm against *M. canis*. The secondary metabolites present in this chosen fraction were separated using thin layer chromatography (TLC) followed by the detection under UV light (365 nm and 254 nm). Contact bioautography was performed against the tested dermatophyte species using the detected spots from UV to determine the growth inhibitory activities. The secondary metabolites that were separated by TLC were identified using specific spray reagents which revealed the presence of alkaloids, steroids and flavonoids. Thus, *P. guajava*

possesses different types of secondary metabolites which are effective antifungal compounds against dermatophytes.

Key words: dermatophytes, *Psidium guajava*, secondary metabolites, antifungal activity

Introduction

Three genera; *Trichophyton, Microsporum* and *Epidermophyton* have been identified as dermatophytes which are a highly specialized group of fungi categorized on the basis of their habitat as anthropophilic, zoophilic, or geophilic (Weitzman and Summerbell, 1995). They are considered as the main causative agents of skin diseases of humans in the tropics and subtropics. Of the fungal infections of humans, superficial fungal infections such as dermatophytoses are very common in developing countries and people living in tropical areas.

Treating dermatophytoses is a challenge due to limitations in antimicrobial spectrum of most drugs and due to development of drug resistance (Dorman and Deans, 2008). Anthropophilic species are responsible for the majority of human infections. However, species from all three groups of dermatophytes have been associated with clinical diseases (Weitzman and Summerbell, 1995). Dermatophytes respond to various conventional antifungal drugs but there is a problem of recurrence of the disease at the same or different locations of the body (Natarajan *et al*., 2003).

Traditional medicinal systems including Ayurveda are preferred and commonly practiced due to high cost of modern medical treatments, side effects of many drugs and the development of resistance to many drugs. The demand for herbal medicines is increasing rapidly due to the lack of side effects (Bramono, 2012).

Psidium guajava is a traditional medicinal plant which is used to cure diarrhoea, treat wounds, rheumatism and problems in the lung. It exhibits various biological activities such as antimicrobial, antioxidant, antidiabetic and anti-inflammatory activities (Sanda *et al.,* 2011). This study was conducted with the objective of evaluating the efficacy of various secondary metabolites present in *Psidium guajava* leaves which possess antifungal activity against chosen dermatophyte species.

Materials and methods

Preparation of plant material

Fresh leaves of *P. guajava* were washed under running tap water, dipped in 70% ethanol and left to dry in shade. They were then oven dried at 50 ± 2°C until a constant weight was obtained and were ground into fine powder using an electric grinder (Mostafa *et al.*, 2011)**.**

Preparation of plant extract

The plant extract was prepared using the methods of Phongpaichit *et al.* (2004) and Mostafa *et al.* (2011). The powder was suspended in 100% methanol (10 g of plant material in 200 ml of the solvent) and was extracted on a magnetic stirrer for 12 h. Then the sample was filtered through Whatman No: 1 filter papers and the filtrate was evaporated to dryness using a rotary vacuum evaporator at 40 rpm. The temperature used was 40 ± 2°C. The dried pellet was re-suspended in 100% methanol (1 g of dried sample in 10 ml of the solvent).

Selection of fungal species

Five dermatophyte species; *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Microsporum canis*, *Microsporum gypseum* and *Epidermophyton floccosum* were selected for this study. All species were obtained from the Medical Research Institute culture collection, Sri Lanka.

Preparation of inocula

The inocula were prepared as mentioned in CLSI M38- A2 method. All dermatophyte cultures were grown on Sabouraud Dextrose Agar (SDA) slants for 7 days at 26± 1°C. When preparing spore suspensions, 1 ml of sterile normal saline (0.85%) was added to each slant and the cultures were vortexed to dislodge conidia from the hyphal mat. The spore suspensions were then transferred to sterile tubes and vortexed to make them homogenized. Finally, the volumes were adjusted to 0.5 McFarland standard by adding normal saline.

Screening of methanol crude extract of *P. guajava* leaves for antifungal activity

The antifungal activity of plant extract was assessed using the well diffusion method as described by Ubulom *et al.* (2011). The positive control used was Econazole (10 µg) while

the negative controls were 100 % methanol and Sterile distilled water. All samples were incubated at 26 ± 1°C for 7 days.

Preliminary analysis of secondary metabolites

Preliminary analysis of secondary metabolites was carried out for the methanol crude extract of *P. guajava* leaves using methods described by Adebayo *et al.* (2010).

- **Alkaloids (Mayer's test)** – One millilitre of 1% HCl was added to 3 ml of the extract in a test tube. The mixture was heated for 20 minutes. It was cooled and filtered. Two drops of Mayer reagent was added to 1 ml of the filtrate. A creamy precipitate would indicate the presence of alkaloids.

- **Tannins** – Two drops of 5% $FeCl_3$ were added to 1 ml of the extract. Appearance of a greenish precipitate would indicate the presence of tannins.
- **Flavonoids -** One millilitre of 10% NaOH was added to 3 ml of the extract. Appearance of a yellow colouration indicated the presence of flavonoids.
- **Saponins (Frothing test) -** The extract (2 ml) in a test tube was vigorously shaken for 2 minutes. Persistent frothing indicated the presence of saponins.
- **Steroids and terpenoids (Salkowski test)** – Five drops of conc. H_2SO_4 were added to 1 ml of the extract. Appearance of a greenish blue colour indicated the presence of terpenoids and the appearance of a reddish colour indicated the presence of steroids.

Preparation of fractions from the methanol crude extract of *P. guajava* leaves.

The fractionation was done as described by Akinpelu *et al.* (2008) and Mostafa *et al.* (2011). Six grams of the evaporated crude sample from the methanol crude extract was re-suspended in 100 ml of sterile distilled water and was shaken vigorously along with hexane (100 ml x 3) and then with chloroform (100 ml x 3) and ethyl acetate (100 ml x 3), respectively. All samples were collected and evaporated to dryness using the rotary evaporator at 40 rpm. The temperatures used were 40± 2°C for hexane, chloroform and ethyl acetate samples and 55± 2°C for the aqueous sample. All dried samples were re-suspended in 100 % methanol (1 g of dried sample in 10 ml of methanol).

Screening of fractions for antifungal activity

All fractions of the methanol crude extract were screened against the five species of dermatophytes, according to the method described by Ubulom *et al.* (2011). The antifungal activity of fractions was assessed using well diffusion method. The positive control used was Econazole (10 µg) while the negative control was 100% methanol. All inoculated dermatophyte samples were incubated at 26± 1°C for 7 days.

Determination of Minimum Inhibitory Concentration (MIC) of the chosen fraction

The broth microdilution method was followed where micro-dilution plates were set up in accordance with the CLSI M27- A2 reference method with slight modifications. Columns 1 to 10 were filled with 100 µl of sterilized broth medium. From the chosen fraction, 50 µl was added into column 1 and a dilution series was prepared using the mixture in column 1. The spore suspensions of the test organisms were prepared using 0.5 McFarland standard and 50 µl of the relevant inocula were added into each column that contained the medium and the serially diluted fractions. Column number 11 was filled with 100 µl of sterilized medium (sterility control) and column 12 with 100 µl of the inoculum (growth control). All samples were incubated at 26± 1°C for 2 days.

Visual examination was performed after the incubation period of the cultures where the dermatophyte cultures were inoculated onto Sabouraud Dextrose Agar along with the control sample and incubated at 26 ± 1°C for 3-4 days.

Testing for the efficacy of secondary metabolites

The active secondary metabolites in the most effective fraction of the methanol crude extract were separated by using TLC as described by Cetkovic *et al.* (2003). The chosen fraction was spotted on 8 x 2 cm silica coated Aluminium plates. After drying, the plates were run using the

derived solvent system (ethyl acetate - formic acid - acetic acid - water 14: 0.9: 0.9: 0.9 v/v). All plates were visualized under UV light (254 nm and 365 nm) for the spot distribution.
Effect of different secondary metabolites separated on TLC was tested on fungal growth using contact bioautographic technique described by Das *et al.* (2010). The secondary metabolite groups that were separated as spots on the TLC plates were cut and placed on

Sabouraud Dextrose Agar inoculated with one dermatophyte species (*Microsporum gypseum*). The cultures were incubated at 26± 1°C for 72 h.

Identification of different secondary metabolites

Different spray reagents were used for the identification of different secondary metabolites in the chosen fraction as stated by Johann *et al*. (2007) and Adebayo *et al.* (2010).

- **Flavonoids**- Plates were sprinkled with 10% NaOH. Appearance of a yellow colour indicated the presence of flavonoids.
- **Steroids**- TLC plates were treated with Anisaldehyde reagent and oven dried at 80°C for 2-3 min. Appearance of a red or purple shade indicated the presence of steroids.
- **Alkaloids**- Plates were sprinkled with Dragendorff's reagent. Appearance of an orange colour indicated the presence of alkaloids.
- **Saponins**- Plates were sprinkled with Anisaldehyde reagent and oven dried at 80°C for 2-3 min. Appearance of a green colour indicated the presence of saponins.
- **Tannins**- Plates were treated with 5% ferric chloride solution. Appearance of a green colour indicated the presence of tannins.

Results

Methanol crude extract of *P. guajava* leaves showed inhibition zones against all five dermatophyte species tested (Figure 1). Different dermatophyte species showed different growth rates. Therefore, the observations were made starting from the 3rd day of incubation until the 7thday. There was a significant difference between the treatment and the positive control. The sizes of the inhibition zones obtained for the positive controls were much higher than that of test values. Inhibition zones were not observed in the negative control.

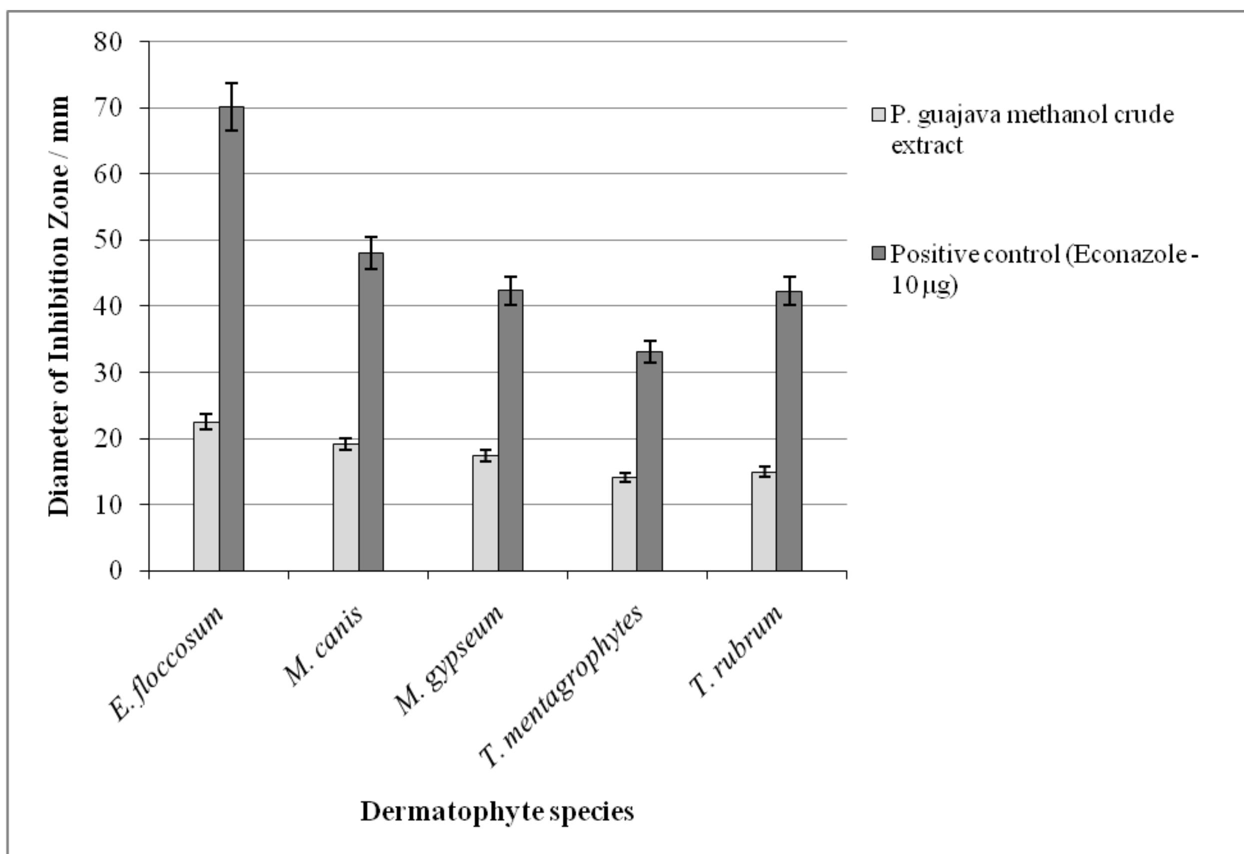

Figure 1. Antifungal activity of methanol crude extract of *P. guajava* leaves against dermatophytes. Values are means of four replicates ± SE

Preliminary analysis of secondary metabolites of the methanol crude extract revealed the presence of six groups of bioactive compounds; alkaloids, tannins, saponins, steroids and flavonoids in the *P. guajava* methanol crude extract.

Both ethyl acetate and aqueous fractions of *P. guajava* methanol crude extract inhibited the growth of all dermatophytes. The highest inhibition was demonstrated by ethyl acetate fraction of *P. guajava* against all dermatophytes while aqueous fraction also exhibited relatively higher inhibition. These values were higher than that of *P. guajava* methanol crude extract. *M. canis* is the most susceptible species to the ethyl acetate fraction (Figure 2). Hexane and chloroform fractions did not show any inhibitory effect on growth of all test fungi and there were no zones present in the negative control (100% methanol).

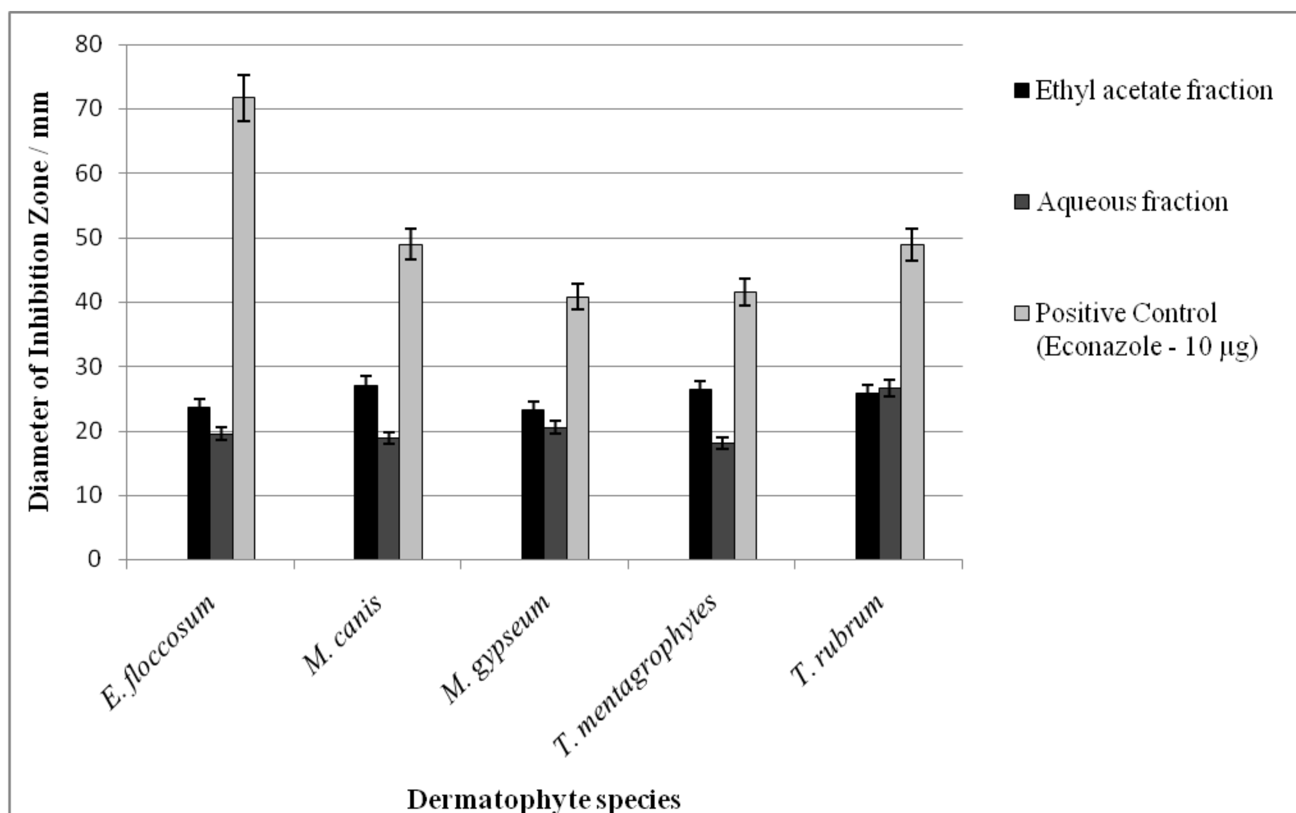

Figure 2. Antifungal activity of *P. guajava* fractions against dermatophytes. Values are means of four replicates ± SE

The MIC values were determined using ethyl acetate fraction of *P. guajava* methanol crude extract against the dermatophyte species. *M. canis* showed visual growth at the concentration of 3.13 mg/ ml but not at 6.25 mg/ml while in *T. mentagrophytes* plates there was a visual growth at 6.25 mg/ ml but not at 12.50 mg/ ml. In other three species a slight growth was observed at comparatively higher concentrations with more or less similar values (Table 1).

Table 1. MIC values of *P. guajava* ethyl acetate fraction against dermatophytes

Dermatophyte species	MIC value (mg / ml)
M. canis	3.13 - 6.25
T. mentagrophytes	6.25 - 12.50

M. gypseum	12.50 - 25.00
T. rubrum	12.50 – 25.00
E. floccosum	12.50 – 25.00

The derived solvent system used for TLC was ethyl acetate- formic acid- acetic acid- water (14 : 0.9 : 0.9 : 0.9 v/v). Bioautographic technique revealed an inhibition in the dermatophyte growth around spot number 06 that was separated by TLC.

Different secondary metabolites which exhibited antifungal activity present in ethyl acetate fraction of *P. guajava* methanol crude extract were identified using various spray reagents. According to the appearance of different colours the secondary metabolites were identified. Steroids and flavonoids were the major secondary metabolites found in *P. guajava* ethyl acetate fraction. Tannins and alkaloids were also observed in spot number 01 and number 06 respectively (Table II).

Table II. Different secondary metabolites present in the ethyl acetate fraction of *P. guajava*

Fraction	Spot number	Alkaloids	Steroids	Saponins	Flavonoids	Tannins
***P. guajava* ethyl acetate fraction**	**01**	-	-	-	-	+
	02	-	+	-	-	-
	03	-	+	-	-	-
	04	-	+	-	+	-
	05	-	+	-	+	-
	06	+	+	-	+	-

+ = present, - = absent

Discussion

Psidium guajava was chosen for this study to screen and experiment for its antifungal properties against dermatophytes as it is very commonly used in the traditional medicine systems to cure many illnesses including skin diseases.

Antifungal activity of *P. guajava* leaves was evaluated using methanol extract since alcohol can extract a wide range of secondary metabolites present in plants (Rojas *et al.,* 2006). Both polar and nonpolar compounds of plant origin can be extracted using alcohol. Thus, in this study 100% methanol was used for extraction purposes.

In the initial screening with methanol crude extract of *P. guajava*, growth of all dermatophytes was inhibited by it. The highest inhibition zone obtained was 22.5 mm against *E. floccosum* while the smallest inhibition zone obtained was 14.13 mm against *T. mentagrophytes*. The active methanol crude extract of *P. guajava* gave inhibition values that were significantly different ($P < 0.05$) from the positive control values in each dermatophyte tested. These results are in broad agreement with previous work done by Pandey and Shweta (2011) where the antifungal activities of ethanol, methanol, ethyl acetate and hot water extracts from leaves and fruits of *P. guajava* were tested against two dermatophyte species; *M. canis* and *T. rubrum.* The results revealed that the methanol extract of *P. guajava* leaves inhibited growth of *M. canis* and *T. rubrum* giving mean inhibition zones (diameter) of 15.5 mm and 16.5 mm respectively. Further, the methanol extract of fruits of *P. guajava* had shown inhibitory effect against *T. rubrum.*

Analysis of the presence of secondary metabolites revealed that methanol crude extract of *P. guajava* leaves contained alkaloids, tannins, saponins, steroids and flavonoids. Previous studies conducted by Adeyemi *et al.* (2009), Uboh *et al.* (2010) and Arya *et al.* (2012) also mentioned the presence of alkaloids, flavonoids, glycosides, polyphenols, saponins, tannins, sterols and carbohydrates in *P. guajava* leaf extracts. Further, they also mentioned that alkaloids, flavonoids, polyphenols and saponins were present in high quantities in *P. guajava* leaves. Results suggest that these secondary metabolites are potential antifungal compounds which remarkably inhibited growth of dermatophytes.

In this study methanol crude extract of *P. guajava* leaves was fractionated in order to purify the secondary metabolites that showed antifungal activity against the tested dermatophyte species. The fractionation process followed was successful and the inhibition zones obtained for the fractions were much higher than that of the crude sample. Thus, it contributed to further purifying the secondary metabolites that were active against the dermatophytes. These findings agree with the results obtained by Akinpelu *et al.* (2008) where higher inhibition of test organisms was obtained for fractionated *Garcinia kola* than the crude extracts.

In the present study the results demonstrated an MIC value of 3.13- 6.25 mg/ ml for *M. canis* and 6.25- 12.5 mg/ ml for *T. mentagrophytes*. While relatively higher MIC value (12.5- 25.0 mg/ ml) was recorded for *E. floccosum, M. gypseum* and *T. rubrum*. These results agree with those of de Souza *et al*. (2010) for *Hymenaea martiana* crude extracts and fractions. They found an inhibitory activity against *T. rubrum, T. mentagrophytes* and *M. canis*, and the best results with MIC values between 8 and 256 µg/ml for methanol and butanol fractions.

Further, purification of the secondary metabolites of the chosen fractions was carried out by using TLC and the separated samples were tested by performing contact bioautography. This method gave a qualitative result as to which purified samples had activity against the dermatophytes. This method was successful since only spot number 06 of the separated spots in *P. guajava* ethyl acetate fraction gave a positive result. Thus, the compounds had successfully separated according to their polarity and only the secondary metabolites present in spot number 06 had antifungal activity against the tested dermatophytes. The identification process revealed the presence of alkaloids, steroids, saponins and flavonoids in the separated spot number 06 on TLC plates which exhibited antifungal activity. As described by Ponnusamy *et al.* (2010), many other plants such as *Wrightia tinctoria* contain secondary metabolites which act as antifungal compounds against *E. floccosum, T. rubrum, T. tonsurans, T. mentagrophytes* and *T. simi*.

The results of this study reveal that secondary metabolites are rich in *P. guajava* leaves and they can act as antifungal compounds against the growth of species of all three genera of dermatophytes. Further, these secondary metabolites can be used in treatments as active ingredients for many herbal medicines. Thus, the efficacy of secondary metabolites which exhibit antifungal activity against dermatophytes in plants such as *P. guajava* can be further studied under clinical conditions.

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