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# Efficacy of Phytochemicals Present in Leaves of *Punica granatum* against *Malassezia* Species

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## ABSTRACT

*Punica granatum* is a valuable medicinal plant traditionally used to cure skin infections. This study was aimed to determine the antifungal activity of *P. granatum* leaves against *Malassezia* species which commonly causes superficial skin infections in humans.

Agar well diffusion method was performed using aqueous and methanol extracts of *P. granatum* leaves against three species of *Malassezia*. The chosen methanol crude extract was fractionated and the fractions were tested for antifungal activity. TLC was performed on the chosen ethyl acetate fraction followed by contact bioautography.

Methanol crude extract and the ethyl acetate fraction of methanol crude extract exhibited the highest antifungal activity against the tested *Malassezia* species. Phytochemical analysis using TLC revealed the presence many bio-active compounds in the ethyl acetate fraction. Contact bioautography of the detected spots of TLC indicated growth inhibitory activities in *Malassezia* species. Results reveal that many phytochemicals present in *P. granatum* are effective against *Malassezia* species.

**Keywords:** Leaves, *Punica granatum*, *Malassezia*, Medicinal plant.

## INTRODUCTION

*Malassezia* is a fungal genus which is commonly found in up to 90% of the human adult population and is present in the normal skin flora. It causes infection under warm and humid environments. Currently, several species belonging to *Malassezia* genus have been studied as the causal agent of pityriasis versicolor, which is the most common disease caused by them[1]. Additionally, they are known to cause pityriasis capitis (dandruff) which is the scaliness of the scalp skin without signs of inflammation[2,3].

Both pityriasis capitis and pityriasis versicolor are more or less the same, differing by the names due to the sites of occurrence; pityriasis capitis on the scalp and pityriasis vesicolor on other parts of the body such as neck and arms[4]. In tropical countries, fungal infections including pityriasis capitis are of common occurrence. Presently, a wide range of antifungal treatments are available but the complete control is not achieved[5]. Most of the available drugs have disadvantages such as being costly and having many side effects[6]. This situation makes the use of medicinal plants, which are highly important as anti fungal agents against common prevalent diseases. Studies should be done mainly to discover the potential phytochemicals that are active against the disease causing pathogens. In addition, the demand for Ayurvedic herbal medicines has increased rapidly due to relatively low side effects[7].

*Punica granatum* (Pomegranate) is a plant from family Punicaceae and it is considered as a valuable medicinal plant in traditional medicine. Further, *P. granatum* possesses antifungal, antiprotozoal and antioxidant activities and the arils, peel, leaves, rind and pericarp of this plant have exhibited antibacterial properties[8-11].

Since there is not much scientific evidence for bioactive compounds in widely used medicinal plants against *Malassezia* spp., systematic investigation is an effective approach in exploration of potential antifungal phytochemicals in the inestimable medicinal plant *P. granatum* against *Malassezia* spp.. Thus, the present study was aimed to assess the efficacy of phytochemicals of *P. granatum* leaves against three species of *Malassezia*.

## MATERIALS AND METHODS

### Collection of plant material

Fresh leaves of traditional medicinal plant *Punica granatum* were collected from home gardens in Colombo, Sri Lanka.

### Preparation of plant extracts

Leaves of *P. granatum* were used to prepare plant extracts as described in literature[12]. The washed leaves were dipped in 70% ethanol and dried in shade. They were then oven dried at 50 ± 2°C until a constant weight was obtained and were ground separately into fine powder using an electric grinder. The powder was suspended in both sterile distilled water and 100% methanol (10 g of plant material in 200 ml of the solvent) and extracted on a magnetic stirrer for 12 h. The rotary vacuum evaporator was used to evaporate the filtered sample to dryness at 40 rpm. The temperatures used were 40 ± 2°C for methanol extracts and 50 ± 2°C for aqueous extracts. The dried pellets were re-suspended in solvent (1 g of dried sample in 10 ml of solvent).

### Selection of fungal species

Three *Malassezia* species were selected for this study. *Malassezia furfur* CBS 1878, *Malassezia restricta* CBS 7877 (standard cultures) from the culture collection of the Medical Research Institute, Sri Lanka and a sample from a patient having pityriasis versicolor were obtained.

### Isolation and identification of the patient's sample

Skin scrapings from a patient having pityriasis versicolor were obtained and they were cultured on Sabouraud Dextrose Agar (SDA) supplemented with 2% (v/v) olive oil and incubated at 37 ± 1°C for 7 days as described in the literature[13].

Cell suspensions of the patient's sample were prepared in 5 ml of sterile

distilled water introducing a loopful of actively growing pathogen[14]. 16 ml of sterile SDA containing 0.05% chloramphenicol and 0.05% cyclohexamide was mixed well with 2 ml of spore suspension. The content was poured into a sterilized Petri-dish and four wells were made into which Tween 20, 40, 60 and 80 were filled (10 µl each). The same experimental design was performed for the standard cultures for comparison. The inoculated plates and controls were incubated at 37 ± 1°C for 1-2 days.

Preparation of inocula

Inocula were prepared as mentioned in literature[15]. The *Malassezia* cultures were grown on SDA slants supplemented with 2 % (v/v) olive oil at 37 ± 1 °C. Cell suspensions were prepared by adding 1 ml of sterile normal saline (0.85 %) to each slant and the cultures were gently probed with the tip of a sterile Pasteur pipette to obtain cells of *Malassezia* species. The suspensions were then transferred to sterile tubes and vortexed for homogenization. Finally, the volumes were adjusted to 0.5 McFarland standards.

Screening of methanol and aqueous leaf extracts of *P. granatum* for antifungal activity

The screening was carried out using a method described in the literature [3]. Well diffusion method was performed for the screening of antifungal activity of aqueous and methanol leaf extracts of *P. granatum*. The positive control used was Ketoconazole (10 µg) while the negative controls were 100% methanol and sterile distilled water. All samples were kept in an incubator at 37 ± 1°C for 2 days.

Phytochemical analysis for the chosen crude extract

The phytochemical analysis was done for the crude extract which showed potential antifungal activity by carrying out several chemical tests as described in the literature [16].

- **Alkaloids (Mayer's test)** - 1% HCl (1 ml) was added to the extracts (3 ml) and the mixture was heated for 20 minutes. It was filtered and Mayer reagent was added to the filtrate (2 drops to 1 ml of filtrate). Appearance of a creamy precipitate was an indication for the presence of alkaloids.
- **Tannins** - 5% $FeCl_3$ (2 drops) was added to the extracts (1 ml). Appearance of a greenish precipitate indicated the presence of tannins.
- **Flavonoids** - 3 ml of the extract was mixed with 1 ml of 10% NaOH. Yellow colour was an indication for the presence of flavonoids.
- **Saponins (Frothing test)** - 2 ml of the extract was shaken vigorously in a test tube for 2 minutes. The presence of saponins was shown by persistent frothing.
- **Steroids and terpenoids (Salkowski test)** – Conc. $H_2SO_4$ (5 drops) was added to the extracts (1 ml). Greenish blue colour is an indication for the presence of terpenoids and reddish colour was an indication for the presence of steroids.

Screening of fractions for antifungal activity

Fractions of the chosen crude extract were prepared as described previously with slight modifications[12,17]. Six grams of the evaporated crude sample from the chosen crude extract was re-suspended in 100 ml of sterile distilled water and shaken vigorously along with organic solvent in a sequential manner; hexane (100 ml x 3), chloroform (100 ml x 3) and ethyl acetate (100 ml x 3) respectively. The collected fractions were dried in a 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 for antifungal activity was carried out [3]. All fractions of the chosen crude extract were screened against the test organisms using well diffusion method by adding 20 µl fractions to each well. The positive control and negative controls were 10 µg Ketoconazole and 100% methanol respectively. The samples were kept in an incubator at 37 ± 1°C for 2 days.

Determination of minimum inhibitory concentration (MIC) of the plant extracts

The broth microdilution method was followed where microdilution plates were set up as described in literature[15]. Columns 1 to 10 were filled with 100 µl of sterilized broth medium and 50 µl of the chosen fraction was added into column 1 and a dilution series was prepared using the mixture in column 1. The cell suspensions of the test organisms were prepared in accordance to 0.5 McFarland standard and 50 µl of the relevant inoculum was 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 inocula (growth control). The samples were kept in an incubator at 37 ± 1°C for 2 days.

After the incubation period the samples from the columns were inoculated onto SDA supplemented with 2% (v/v) olive oil. Samples from column 1 to 10 were inoculated for each organism including the control samples. All samples were incubated at 37 ± 1°C for 2 days.

Identification of phytochemicals[16,20]

Identification of phytochemicals in the crude extract was done using different reagents.

- **Flavonoids** - 10% NaOH was sprayed on the TLC plates. Resulting yellow colour was an indication of the presence of flavonoids.
- **Steroids** - Anisaldehyde reagent was sprayed on the TLC plates and they were oven dried at 80°C for 2-3 min. Resulting red or purple shade was an indication of the presence of steroids.
- **Alkaloids** - Dragendorff's reagent was sprayed on the TLC plates. Red colour was an indication of 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** - 5% $FeCl_3$ solution was sprayed on the TLC plates. Resulting green colour was an indication of the presence of tannins.

Statistical analysis of data

Data were analysed using ANOVA Analysis of Variance. Significant differences between the values in each experiment were determined via Tukey's pair wise comparison at 5 % level of significance.

## RESULTS

Isolation and identification of the patient's sample

The patient's sample, which was freshly obtained for this study showed morphological features similar to the tested *M. furfur* CBS 1878 but with a faster growth.

Screening of methanol and aqueous leaf extracts of *P. granatum* for antifungal activity

An inhibition in the growth of all *Malassezia* species was observed around the wells filled with *P. granatum* aqueous crude extracts (Table 1).

A significant difference between the treatments and the positive control was observed. There were no inhibition zones for the negative control.

There was an inhibition in the growth of all *Malassezia* species around the wells filled with *P. granatum* methanol crude extracts (Table 2).

The highest inhibition zone by *P. granatum* methanol crude extract was obtained against *M. furfur* CBS 1878. Inhibition zone given by methanol crude extract against *M. restricta* CBS 7877 was significantly higher than that of positive control.

Phytochemical analysis for the chosen crude extract

The phytochemical analysis for the methanol crude extract indicated the presence of six groups of phytochemicals; alkaloids, tannins, saponins, steroids, terpenoids and flavonoids.

Screening of fractions for antifungal activity

There was an inhibition in the growth of both *M. furfur* CBS 1878 and *M. restricta* CBS 7877 around wells filled with all fractions of *P. granatum* separately. But the growth of *M. furfur* strain was inhibited only by the hexane and ethyl acetate fractions (Figure 1).

Ethyl acetate fraction exhibited the highest inhibition zone. All the zone values were significantly different from the respective positive control values except for the ethyl acetate fraction against *M. furfur* CBS 1878. Antifungal activity of the ethyl acetate fraction against both *M. furfur* CBS 1878 and *M. restricta* CBS 7877 was higher than that of the positive control. The inhibition by ethyl acetate fraction against *M. restricta* CBS 7877 was significantly higher than the positive control value.

Assessment of minimum inhibitory concentration (MIC) of the plant extracts

The MIC values of the ethyl acetate fraction of *P. granatum* methanol crude extract against the test organisms were assessed. *M. furfur* CBS 1878 exhibited a very low MIC value. Growth of *M. furfur* CBS 1878 was observed at 3.13 mg/ml but not at 6.25 mg/ml. The patient's sample (*M. furfur* strain) showed a visible growth at 12.50 mg/ml but not at 25.00 mg/ml (Table 3).

Testing for efficacy of bio-active compounds

The derived solvent system used for TLC was ethyl acetate- formic acid- acetic acid- water (8: 0.9: 0.9: 2.1). When the bio autographic technique was carried out an inhibition in the tested *Malassezia* spp. growth was observed around spots 01, 03 and 04.

Identification of the bio-active compounds

Different bio-active compounds present in ethyl acetate fraction of *P. granatum* were identified using various spray reagents and the resulting appearance of different colours. Flavonoids were present as the major secondary metabolite showing in all spots given by ethyl acetate fraction of *P. granatum* while steroids and saponins were present in a few spots (Table 4).

## DISCUSSION

Antifungal activity of leaves of *Punica granatum* was evaluated using aqueous and methanol extracts so that differences in activity can be attributed to different phytochemicals present in the extracts. Methanol is a solvent that can extract a wide range of phytochemicals present in samples of plant origin from non polar to polar compounds[21]. Water is the most common solvent that can be used in preparing plant extracts and that is used in traditional

medicine to make pulps and extracts. Potential antifungal activity was demonstrated in the methanol crude extract of *P. granatum* against *Malassezia* species. There were quite similar high inhibition zones from *P. granatum* methanol crude extract and the positive control; Ketoconazole (35.5 mm and 36.4 mm) and these zones were not significantly different from each other. In a similar study, the fruit rind of *P. granatum* was used in preparing ethanol, chloroform, ethyl acetate and methanol crude extracts and these crude extracts were tested against *M. globosa*[22]. Their results revealed high antifungal activity of the methanol crude extract of *P. granatum* against the test organism.

A new *Malassezia* strain was also identified in this study. It was identified by using a standard method[14]. It demonstrated more or less similar results as the *M. furfur* CBS 1878 standard culture but exhibited a faster growth rate. Hence it was named as a new strain of *M. furfur*. This evidence based identification is supported by the results obtained for the antifungal activity of methanol extract against different test organisms and the MIC values. The inhibition zones obtained for *M. furfur* CBS 1878 standard culture, differed from that of the patient's sample. The MIC values are also remarkably different in the *M. furfur* strain (the patient's sample) and *M. furfur* CBS 1878.

Presence of many organic compounds; alkaloids, tannins, saponins, steroids, flavonoids and terpenoids in *P. granatum* methanol crude extract was observed when the preliminary phytochemical analysis was performed. These results are in broad agreement with those published previously. Presence of phytochemicals such as alkaloids, tannins, flavonoids, phytosterols, phenols, saponins, steroids, terpenes and volatile oils[23,24] has been reported. In addition carbohydrates, reducing sugars, sterols and glycosides have also been reported[8].

In this study the chosen crude extract was fractionated in order to purify the phytochemicals that showed antifungal activity against the relevant test organisms. The fractions possess high antifungal potential than the crude samples and the inhibition zones obtained for the fractions were much higher than that of the crude samples. Ethyl acetate fraction of *P. granatum* demonstrated a remarkable activity inhibiting the growth of *M. furfur* CBS 1878 giving a 36.6 mm inhibition zone, which is even larger than that of the positive control; Ketoconazole (35.9 mm). Most importantly, there was no significant difference between the inhibition zones given by the positive control and ethyl acetate fraction of the leaf extract. Thus the fractionation process was successful in further purifying the phytochemicals that were active against *Malassezia* species. These findings agree with previous results where higher inhibition of test organisms was obtained for fractionated *Garcinia kola* than the crude extracts[17].

The MIC value obtained for *P. granatum* was 3.13- 6.25 mg/ml against *M. furfur* CBS 1878. Similarly, in a previous study, the methanol crude extract of the fruit rind of *P. granatum* exhibited an MIC of 1.0 mg/ml against *M. globosa*[22]. The above result is also comparable with findings on *Curcuma longa* (turmeric) against *M. furfur*[3]. The antifungal activity of turmeric oil was tested and they found that the essential oil was very effective against *M. furfur* with an MIC value of 0.1 µl/ml. And the inhibition zones obtained were even larger than that of the positive control antibiotics (streptomycin and gentamycin).

In this study, further purification of the phytochemicals 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 exhibiting purified samples had activity against the tested *Malassezia* species. In the identification process of these separated phytochemicals, results revealed the presence of alkaloids, steroids, saponins and flavonoids in the separated spots on TLC plates which had antifungal activity.

## CONCLUSION

The present study reveals that phytochemicals are rich in *P. granatum* leaves, and they possess antifungal properties which act against *Malassezia* species. However, efficacy of the treatment is determined by the potential of secondary metabolite which is in a form of active ingredient in the herbal product. Evaluation of the efficacy of medicinal plants which possess antifungal activity against *Malassezia* species under clinical conditions is recommended.

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**Table 1.** Antifungal activity of aqueous crude extract of the leaves of *P. granatum* against *Malassezia* species. Values are means of four replicates ± SE

| *Malassezia* spp. | Inhibition zone (diameter/ mm) ± SE | | |
|---|---|---|---|
| | Aqueous crude extract *P. granatum* | Positive control - Ketoconazole (10 µg) | Negative control - Sterile distilled water |
| *M. furfur* CBS 1878 | 9.63 ± 0.32 * | 35.75 ± 0.65 | - |
| *M. restricta* CBS 7877 | 8.88 ± 0.30 * | 29.63 ± 0.57 | - |
| *M. furfur* strain (the patient's sample) | 8.63 ± 0.46 * | 30.00 ± 0.57 | - |

*Significantly different from positive control (P< 0.05).

**Table 2.** Antifungal activity of methanol crude extract of *P. granatum* against *Malassezia* species. Values are means of four replicates ± SE

| *Malassezia* spp. | Inhibition zone (diameter/ mm) ± SE | | |
|---|---|---|---|
| | Methanol crude extract of *P. granatum* | Positive control - Ketoconazole (10 µg) | Negative control – 100% Methanol |
| *M. furfur* CBS 1878 | 35.50 ± 0.38* | 36.38 ± 0.38 | - |
| *M. restricta* CBS 7877 | 31.50 ± 0.46 * | 29.63 ± 0.57 | - |
| *M. furfur* strain (the patient's sample) | 21.38 ± 0.75 * | 30.00 ± 0.57 | - |

* Significantly different from positive control (P< 0.05).

**Table 3.** MIC values of *P. granatum* ethyl acetate fraction against tested *Malassezia* spp.

| Organism | MIC value (mg/ ml) |
|---|---|
| *M. restricta* CBS 7877 | 6.25 - 12.50 |
| *M. furfur* CBS 1878 | 3.13 - 6.25 |
| *M. furfur* strain (the patient's sample) | 12.50 - 25.00 |

**Table 4.** Different phytochemicals present in ethyl acetate fraction of *P. granatum*

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

+ = present, - = absent

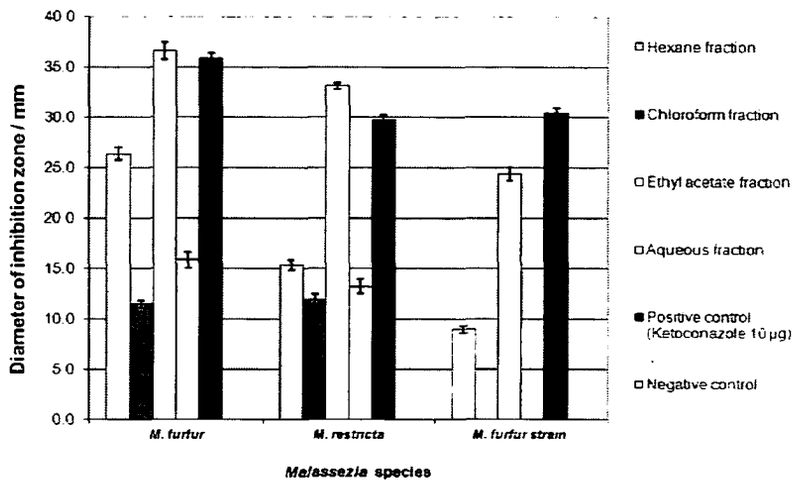


**Figure 1.** Antifungal activity of *P. granatum* fractions against *Malassezia* species. Values are means of four replicates ± SE