Original Article

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Dentistry Section

"Rhetoric to Reality"- Efficacy of *Punica Granatum* Peel Extract on Oral Candidiasis: An In vitro Study

PREETHI MADUGULA[1], SUDHAKARA REDDY[2], JYOTHIRMAI KONERU[3], ATLA SRINIVASA RAO[4], RAYAPUREDDI SRUTHI[5], DIVYA TEJA DALLI[6]

ABSTRACT

Introduction: Global usage of synthetic drugs inadvertently has resulted in deleterious effects and antimicrobial resistance. Phytoextarcts with therapeutic properties appear to be appropriate substitutes for synthetic drugs. *Punica granatum* (Pomegranate) is a fruit rich in nutraceuticals and therapeutic properties that has lead to its widespread use as folk-medicine for treating innumerable diseases.

Aim: To determine the in vitro antifungal efficacy of *Punica granatum* peel extract against the oral *Candida* compared with clotrimazole.

Materials and Methods: An in vitro study was carried out on 60 saliva samples collected from patients confirmed by clinical and mycological examination as oral candidiasis and subjected to culture on Saborauds Dextrose Agar (SDA) medium and incubated at 37°C for 48 hours. The cultured *Candida* species were subjected to antifungal susceptibility test by agar well diffusion method. *Punica granatum* peel extract (Group-I), Ethanol (Group-II Negative control), Clotrimazole (Group-III-Positive control) were inoculated in wells and incubated. Zones of inhibitions were measured with a digital Vernier's callipers and subjected to statistical analysis. ANOVA (analysis of variance) was performed to compare inhibition zones and concentrations of all the three groups.

Results: Antifungal efficacy of *Punica granatum* group and Clotrimazole group were statistically significant with p-value <0.05. Additionally, with the increase in the concentration there was an increase in the inhibitory efficacy against *Candida* species. Minimum Inhibitory Concentration (MIC) of peel extract of *Punica granatum* approximated with that of the clotrimazole.

Conclusion: The present research was just a venture to usual clinical approach. The results of the study reveal that MIC of peel extract of *Punica granatum* approximated with that of the clotrimazole. Hence, peel extract of *Punica granatum* may be used as a substitute for antifungal agents in clinical trials with standardization so as to minimize the deleterious effects for patient compliance.

Keywords: Antifungal potential, Clotrimazole, *Candida* species, Ethanol, Minimum inhibition concentration

INTRODUCTION

Hippocrates, the Father of Medicine stated, "Let food be thy medicine and let medicine be thy food". Substantiating this statement, in the present scenario, an enormous engrossment has been extending worldwide in field of nutraceuticals and their role has advanced effectively in health management. Global uses of synthetic drugs have had deleterious effects, like recurrence of infections and antimicrobial resistance [1,2]. On the other hand, phytoextracts seem to be an appropriate substitute with their therapeutic properties and limited adverse effects.

Punica granatum is one of the phytoplant with admirable medicinal value, used often as a remedy in folk medicine for curing several diseases such as cardiovascular diseases, cancer, diabetes, gastritis, ulcers etc. Various parts of this plant have innumerable phytochemical compounds (flavanoids, polyphenols, tannins, organic acids) amid of which peel has surpassing phytochemical compounds [3]. Recent studies have stated *Punica granatum* as a potent phytoplant extract with innate therapeutic properties such as antioxidant, anti-inflammatory, anticarcinogenic, antiviral, antifungal etc., [4]. Owing to these inherent properties, *Punica granatum* has been widely used recently in the field of oral diseases and has known to alleviate many symptoms of oral mucosal diseases.

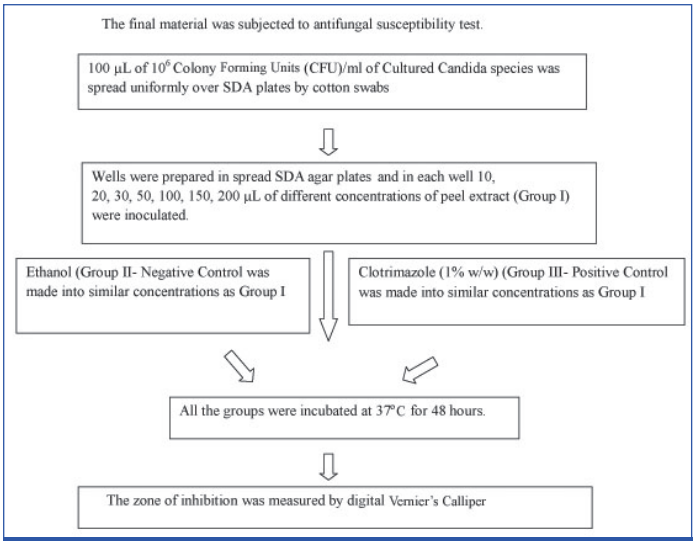
Oral cavity harbours diverse, copious and complex microbial community. *Candida albicans* is an innocuous commensal of the microbial communities which inhabits distinct surfaces of the mouth and is epitomized to be the most prevailing causative agent of oral candidiasis [5]. The most conventional treatment for oral candidiasis is the use of antifungal agents which pose disparate detrimental effects, uncommon infections and mainly augmenting resistance to them [6]. Hence, there is a need for a time-honoured approach to treat the disease by utilizing herbal drugs as an alternative to synthetic drugs, which have lesser detrimental effects and good patient compliance.

The present study was done to comprehensively report the antifungal potential of the *Punica granatum* peel extract against oral candidiasis. Hence, it was conceptualized to assess the in vitro antifungal efficacy of pomegranate peel extract on *Candida* species in comparison to synthetic drug.

MATERIALS AND METHODS

An in vitro study was conducted in the Department of Oral Medicine and Radiology, Vishnu Dental College and Hospital, Bhimavaram, Andhra Pradesh, India. A total of 60 patients who were diagnosed and confirmed by clinical and mycological examination as suffering from oral candidiasis were enrolled in the present study. The study protocol was approved by the Institutional Review Board which was held in the Mission Monitoring Cell (MMC), Vishnu Dental College, Bhimavaram (Ref no: IEC/VDC/MDS15 OMR 06) on November 24th, 2015. Signed informed consent was obtained from all participants before their inclusion in the present study for the collection of saliva. Patients who were under topical or systemic antifungal drugs were excluded.

Saliva was collected by spitting method in graduated container from patients diagnosed as having candidiasis. It was inoculated and cultured on 60 separate SDA plates and incubated at 37°C for 48 hours and then *Candida* species were collected.



[Table/Fig-1]: Step wise illustration of antifungal susceptibility test.

Preparation of the Extract

Peel extract of *Punica granatum* was prepared in Pharma Chemistry Lab, Shri Vishnu College of Pharmacy, Bhimavaram. Fresh samples of *Punica granatum* peel were air dried at room temperature in low light for seven days. Then dried peels were powdered finely and stored in air tight bottles.

About 100 grams of sample powder was soaked in 99.9% ethyl alcohol for four days and filtered by using Whatman filter paper. The obtained filtrate was subjected to rotary evaporator at a temperature of 70°C and 120 rpm and crude extract was obtained [4].

The weight of crude extract was calculated and measured by the difference between weights of beaker before and after the collection of extract. The final material (crude extract) was dissolved in ethanol and made into different concentrations of 10,20,30,50,100,150, 200 µl. Ethanol was considered as negative control group (Group-II) in the present study so as to investigate any antifungal property for ethanol as the crude extract was dissolved in ethanol. The final crude extract was subjected to antifungal susceptibility test (Table/Fig-1).

The MIC value is defined as the lowest concentration of plant extract in the medium that inhibited visible growth of the test fungal strains [4,7]. The Minimum Fungicidal Concentration (MFC) is defined as the concentration required to give 50% inhibition of hyphal growth [7].

STATISTICAL ANALYSIS

The data of disc diffusion values were entered into computer database for statistical analysis and responses were analyzed by SPSS software. One-way Analysis of Variance test (ANOVA) was applied to compare the inhibitory activity among the three groups and the p-value <0.05 was considered as statistically significant.

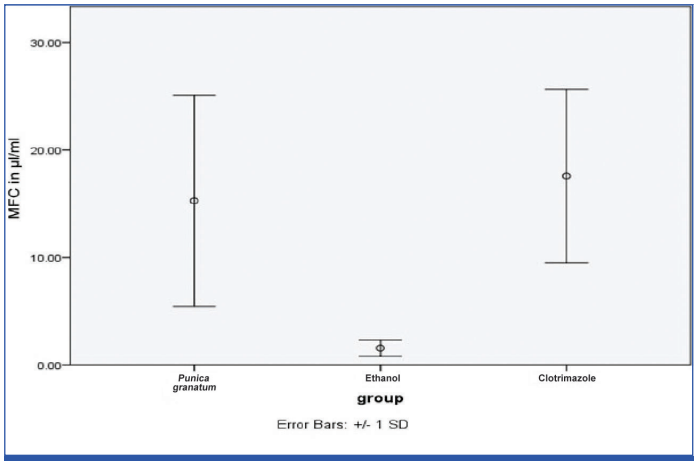
RESULTS

A total of 60 patients were enrolled in the present study. All the three groups were analyzed for antifungal susceptibility test and the mean MIC was calculated. The comparison of inhibition zones within the group and between the groups at different concentrations for all the three groups were tabulated [Table/Fig-2] and it depicts that the antifungal efficacy increased with increase in the concentrations and also Group-I (p=0.006) and Group-III (p=0.000) were statistically significant.

Inhibition zone of *Punica granatum* extract approximated with that of the clotrimazole inhibition zone which entails the antifungal property of *Punica granatum* peel extract was equivalent to clotrimazole as shown in [Table/Fig-2]. The upper and lower whiskers in *Punica granatum* group were approximated with those of Clotrimazole group as tabulated in [Table/Fig-3], and the so obtained variance in *Punica granatum* group also equates with that of the Clotrimazole group.

Inhibition Zones in mm			
Concentration	*Punica granatum*	Ethanol	Clotrimazole
10 µL	1.12±1.17	0	5.09±1.87
20 µL	5.3±1.89	0	11.4±1.24
30 µL	11.03±0.98	0	13.94±0.74
50µL	16.83±0.96	0	17.435±0.78
100 µL	21.14±0.73	0	22.24±0.74
150 µL	24.1±0.8	1.805±2.52	24.82±1.14
200 µL	27.32±0.77	3.105±2.90	28.055±0.71
p-value	0.006*	0.01*	0.000*

[Table/Fig-2]: Zones inhibition of *Punica granatum*, Ethanol, Clotrimazole group at different concentrations.
* p-value<0.05 is significant



[Table/Fig-3]: Comparison of means of inhibitory efficacy of three groups on *Candida* species.

	Minimum Inhibitory Concentration (MIC) in µl/ml	Minimum Fungicidal Concentration (MFC) in µl/ml
Punica granatum	20	10
Ethanol	0	0
Clotrimazole	10	5

[Table/Fig-4]: Determination of MIC and MFC for *Candida* species.

When the inhibitory zones of the three groups were compared, it was found that there is no antifungal activity for Ethanol group. The MFC of *Punica granatum*, ethanol and clotrimazole are 10 µl, 0 µl, 5 µl respectively and been tabulated in [Table/Fig-4] and shows half the inhibition efficacy on *Candida* species.

DISCUSSION

Plants and natural products from times immemorial have been used extensively for pharmacological pertinence. Indian subcontinent is a treasure trove of innumerable precious plants with therapeutic properties [8].

Punica granatum is an ancient, numinous, offbeat fruit and is predominantly used as a herbal drug for treating distinct ailments of several systems of the human body. Recently, there has been renewed awareness in use of *Punica granatum* as a herbal drug for oral and dental health as it possess potent antioxidants with anticarcinogenic, anti-inflammatory, antifungal, antimicrobial properties and the peel extract of this fruit has superior potent properties as documented in literature [9]. Usually, plants have high propensity to synthesize phenols and tannins and they accomplish defensive action against microorganisms [10]. It has been proven that *Punica granatum* peel extract has high reserves of tannins and phenols compared to different parts of *Punica granatum* plant in a study performed by the Pai MBH et al., [11]. So, as documented

Author	Type of the study	Analysis of study and outcomes
Vasconcelos LC et al., [20]	In vitro study	Antimicrobial efficacy of *Punica granatum* extract gel was compared with miconazole gel. MIC of *Punica granatum* extract against different organisms was obtained as; Candidal strains (1:64), *S. mitis* (1:128), *S. mutans* (1:16) and it was proven that *Punica linn* extract has higher efficacy than miconazole.
Shafighi M et al., [12]	In vitro study	The antifungal efficacy of different parts of *Punica* phytoplant extracts was determined. All the extracts of peel, flower, leaf and stem were subjected to agar well diffusion method and found that MIC was higher for peel extract (10 µl) followed by flower, leaf and stem.
Solon J et al., (2014) [24]	In vitro study	Pomegranate (*Punica granatum*) peel decoction was used and tested for its antimicrobial activity. Positive results were obtained with antimicrobial effect against *Pseudomonas aeruginosa* and *Candida albicans*, suggesting that the *Punica* decoction can be used as a solution against oral diseases.
Singla S et al., [18]	In vitro study	In vitro analysis was carried out to evaluate the antifungal efficacy of *Punica granatum* peel, seed extracts (rind), and extract of Henna leaves (*Lawsonia inermis*) against *Candida albicans*. Henna leaves had proven maximum antifungal property with a higher inhibition zone (20 mm) followed by peel (13.3 mm) and seeds (3.75 mm)
Mehta V et al., [1]	In vitro study	Different extracts of pomegranate peel, lotus leaf, guava leaf and coffee against the *Streptococci* and *Candida* species were compared for antimicrobial property and in vitro analysis revealed that all the extracts were effective against *Streptococci* species but antifungal efficacy was maximum exhibited by *Punica granatum* peel extract and coffee.
Pai MBH et al., [11]	In vitro study	The in vitro antifungal efficacy of *Punica granatum*, *Acacia nilotica*, *Cuminum cyminum* and *Foeniculum vulgare* were determined. Analyzed data revealed that *Punica granatum* extract has higher inhibition zone of 22 mm against the *Candida albicans* as compared to other extracts.
Vasconcelos LC et al., [22]	In vivo study	A clinical trial was performed among 48 patients diagnosed as *Candida* associated denture stomatitis. Out of 48 subjects group A-(27) were treated with miconazole and group B-(21) with *Punica granatum* gel. *Punica granatum* has proved its antifungal efficacy against the *Candida* species. Moreover, authors suggested that this extract gel can be used as topical antifungal agent.

[Table/Fig-5]: Similar literature research and their inferences [1,11,12,18,20,22,24].

in the literature peel extract of *Punica granatum* was chosen in the present study to evaluate its antifungal efficacy against the *Candida* species [11].

Moreover, MIC can only be used for clinical trials to reduce side effects. Previously a study conducted by Shafighi M et al., had found MIC of peel extract at 10 µl which closely correlates with present study where, the MIC of *Punica granatum* peel extract was obtained at 20 µl as tabulated [Table/Fig-2] [12].

Clotrimazole (1% w/w) was used as positive control in present in vitro study which exhibited the inhibitory effect on *Candida* at MIC of 10 µl, which goes in accordance with the study conducted by Georgopapadakou NH et al., [13]. Moreover, extract prepared from the peel of *Punica granatum* exhibited antifungal efficacy against *Candida* with MIC at 20 µl. Interestingly, the antifungal efficacy of pomegranate peel extract against *Candida* was comparable to clotrimazole (positive control). The inhibition zones of both the groups increased with increase in concentration, which suggests that, a direct relationship exists between concentration and inhibition zone.

Ethanol acts as preservative and has no antifungal property which has been illustrated in the present study and was in accordance with the study conducted by Al-Hussaini JS [14].

Various in vitro studies have revealed that, punicalagin isolated from the peel extract can precipitate proteins on the cell surface, while polyphenols are known to interact with proteins and inhibited the growth of microbial co-aggregation and exhibited antifungal activity by deprivation of substrates and direct action on microbial metabolism [15-17]. Similar results were also observed in this present study. The ethanolic peel extract of *Punica granatum* inhibited *Candida* species which were responsible for oral candidiasis.

Anibal C et al., has evaluated the morphological alterations on the yeasts using scanning electron microscopy. In addition to cell aggregation and growth inhibition it was found that phytochemical compounds could be responsible for changes in cell morphology, producing viscous material and rupturing the cells which have been acknowledged for their antifungal activity [4].

Furthermore, *Punica granatum* peel extract was efficacious against *Candida* owing to inherent antifungal property has been proven in the current study, was in accordance with the study conducted by Endo EH who exemplified that the peel extract of *Punica granatum* was efficient for inhibition of *Candida* growth [18,19]. The results were also supported by the studies conducted by Vasconcelos LC et al., Duraipandiyan V et al., [20,21]. In a clinical trial, Vasconcelos LC et al., showed that, *Punica granatum* peel extract may be used as a topical antifungal drug against *Candida albicans* in two cases of denture- stomatitis patients [22].

An in vitro study conducted by Abdollahzadeh SH et al., is in contrast to the present study and it has been suggested that methanolic extract of *Punica granatum* might be used as an antibacterial agent but not as an antifungal agent in preventing oral infections [5]. This is explained by the fact that the percentage of polyphenols obtained from peel of *Punica granatum* fruit is evaluated by the solubility of phenol compounds in the solvent used for the extraction process. In the present study, ethanol and water mixtures have been used for the extraction of plant materials instead of methanol as it can dissolve a wide range of phenols. Furthermore, ethanolic mixtures were appropriate for human consumption [23]. Details of similar literature research are tabulated [Table/Fig-5] [1,11,12,18,20,22,24].

Various studies conducted in past have reported the antifungal activity of *Punica granatum* alone or in comparison with other natural products. While in the present study, apart from demonstrating the antifungal efficacy of the plant extract, we have analyzed and compared it with standard drug clotrimazole, indicating that *Punica granatum* peel extract has antifungal efficacy which was almost similar to clotrimazole.

The extract of pomegranate peel can be used as an alternative to regular antifungal agents in clinical trials with standardization and as it has been proven to have antifungal property inherent in peel extract, it open new perspectives for future research related to other fungal diseases. The methodology was a laborious procedure which required proper temperature maintenance and need to undertake appropriate sterilization control as there were chances for contamination. On other hand, it required accurate mixture of solvent to crude extract ratio and it's a technique sensitive procedure.

The results of the present study are limited because of its in vitro nature. Further in vivo longitudinal studies can be conducted to confirm the results of the present study.

CONCLUSION

An explosion of interest in the copious therapeutic properties of *Punica granatum* over the past decade has led to immense interest in conducting numerous in vitro, animal, and clinical trials. The results of the present study, suggests that antifungal efficacy of *Punica granatum* peel extract was analogous to clotrimazole against *Candida* species. Indeed, owing to antifungal potential of *Punica granatum* peel extract it might be possible to use it as a therapy for oral candidiasis. Thus, in view of present scenario, judicious dealing of such natural products might not only help to minimize the deleterious effects of synthetic drugs but also prove to be cost effective, especially in developing economies.

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PARTICULARS OF CONTRIBUTORS:

1. Postgraduate Student, Department of Oral Medicine and Radiology, Vishnu Dental College, Bhimavaram, Andhra Pradesh, India.
2. Professor and Head, Department of Oral Medicine and Radiology, Vishnu Dental College, Bhimavaram, Andhra Pradesh, India.
3. Reader, Department of Oral Medicine and Radiology, Vishnu Dental College, Bhimavaram, Andhra Pradesh, India.
4. Professor and Head, Department of Pharmaceutical Analysis and Quality Assurance, Shri Vishnu College of Pharmacy, Bhimavaram, Andhra Pradesh, India.
5. Postgraduate Student, Department of Oral Medicine and Radiology, Vishnu Dental College, Bhimavaram, Andhra Pradesh, India.
6. Postgraduate Student, Department of Oral Medicine and Radiology, Vishnu Dental College, Bhimavaram, Andhra Pradesh, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:

Dr. Preethi Madugula,
Postgraduate Student, Department of Oral Medicine and Radiology, Vishnu Dental College, Bhimavaram-534202,
Andhra Pradesh, India.
E-mail: dr.preethimadugula@gmail.com

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