Original Article

Punica Granatum v/s Lawsonia Inermis: An In Vitro Anti-Fungal study

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ABSTRACT

Background: Incidence of Fungal diseases is increasing with emerging immunodeficiency conditions. Discovery of antibiotics to combat these infections marked a resolution, but their inappropriate use blocked the action of antibiotics, change their target or ability to penetrate cells. Therefore there is a need for alternative potent antifungal products.

Aims & Objectives: To determine and compare the fungicidal action of pomegranate peel and henna leaves extract on candida.

Methods: Herbal extract was prepared from sundried, powdered Pomegranate peel and henna leaves and stored at 4˚c until use. Candida was grown in Sabouraud Agar media by the sample collected from throat swab. Sterile filter disc immersed in extract was loaded onto the prepared plates with the control as clotrimazole disc. The zone of inhibition was measured at time interval of 18-24 hours after incubation and readings were statistically analysed.

Results: The Henna lemon extract was superior to the Pomegranate ethanol extract. The minimum inhibition zone for Pomegranate ethanol extract was 14.3±0.58 mm which is lesser than that of lawsonia lemon extract and standard antibiotic with 18.6±0.58 mm of inhibition zone.

Conclusion: The results showed that punica granatum and lawsonia inermis has a potent antifungal activity and the potential use of these products as cheap, nontoxic with less side-effects and as a convenient adjuvants to pharmaceutical antifungal products and need for further investigations to be used for clinical implications in humans.

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Introduction

Incidence of fungal disease is increasing with emerging immunodeficiency conditions. Also the long term use of antibacterial antibiotics destroy harmful as well as beneficial flora in the body, eliminating the yeast's natural competitors for resources. A weakened or undeveloped immune system or metabolic illness such as diabetes, HIV/AIDS, mononucleosis, cancer treatments, steroids, stress and nutrient deficiency is significant predisposing factor for candida diseases.

Discovery of antifungal antibiotics to combat these infections marked a resolution, but their inappropriate use blocked the action of antibiotics leading to various drawbacks in terms of toxicity, side-effects, drug interactions, resistance, lack of fungicidal efficacy and cost. The nature of the resistance to a few drugs has been identified as related to altered transport, modification of an enzyme, and a change in membrane composition. [1] Therefore fungal infections have become an increasingly important cause of disease among both immunocompetent and immunocompromised individuals.[2]

The Henna plant, Lawsonia inermis Linnis known since with healing attributes and now is the subject of intense scientific study. The plant belongs to the family Lythraceae and is traditionally used to develop a red or black colouringto hands, feet and hair in occasions such as weddings and religious festivals. The plant is planted in home as hedges and as an ornament. The leaves of the plants are small, lanceolate, dark-green and glabrous, opposite, with very short petioles. The plant leaf contains a red orange color component, lawsone (2-hydroxy, 4-Napthoquinone). According to phytochemical analysis of henna, powdered leaves contain about 0.5-1.5% lawsone, the chief constituent responsible for the dyeing properties of the plant. Henna also contains mannite, tannic acid, mucilage, gallic acid and napthoquinone.[3]

In ancient Greek mythology, pomegranates are known as the "fruit of the dead", which is an ancient fruit that has not changed much throughout the history of man. It was found in the Indus Valley so early that there is a word in Sanskrit for pomegranate. Pomegranate belongs to punicaceae family. The major class of pomegranate phytochemicals is the polyphenols (phenolic rings bearing multiple hydroxyl groups) that predominate in the fruit. Pomegranate polyphenols include flavonoids (flavonols, flavanols and anthocyanins), condensed tannins (proanthocyanidins) and hydrolysable tannins (ellagitannins and gallotannins). Hydrolyzable tannins (HTs) are found in the peels (rind, husk, or pericarp), membranes and piths of the fruit. HTs are predominant polyphenols found in pomegranate juice and account for 92% of its antioxidant activity.[4]

Some researchers investigating the antifungal activities of pomegranate against *Candida* species reported that the fruit peel of *Punica granatum* L. was the most effective for inhibiting *C.albicans* growth.[5]

To substitute antibiotics therapeutic efficacy, many indigenous plants are used. Phytochemical analysis of punica granatum and lawsonia inermis had proved their antifungal efficacy which can offer viable alternative which can offer cheap, effective and less toxic module without any side-effects.

Material and method

The study was carried out in the department of Oral Pathology of Kothiwal Dental College, Moradabad, U.P., India. The ethical clearance was obtained from institutional ethical committee. Henna leaves and pomegranate peels used in this study were collected from the Moradabad city. Clotrimazole solution was used as a reference standard drug for comparison.

Preparation of Pomegranate Ethanol Extract: After washing, the peels were separated from the mesocarp and were sun dried for 3 days. The dried peels were ground in an electric grinder to produce a powder. 10 gm of powder was mixed with 100 ml of 100% ethanol and was continuously stirred at room temperature for 24 hours on a shaking device. The contents were then filtered with double filter paper and sterile filters to remove any impurities. The sample was stored at 4°C until use.

Preparation of Henna Lemon Extract: Fresh Henna leaves were dried in sun and were powdered in an electric grinder. 10 gm of powder was mixed with 10 ml of lemon juice to obtain a paste like consistency. Henna mix was kept for rest for 6-8 hours. The contents were centrifuged and supernatant was collected and used immediately.

Candida Strain: Throat swab of a diabetic individual was obtained and was swabbed over the surface of the Sabouraud Dextrose Agar (SDA) plate. The plate is then incubated in an incubator at 37°C for 48 hours.Creamy white, moist colonies were obtained (Fig. 1a) which were PAS (periodic acid Schiff) positive (Fig. 1b).

Method: The disc diffusion method was used to determine the anticandidal activity in vitro. The candida colonies were picked by an inoculation loop and were streaked over a new SDA plates. Sterile, Filter paper discs of 6 mm in diameterwere loaded with respective extracts and a control (Clotrimazole) and were placed with sterile tweezers onto the plates. The plates were then incubated at 37°C for 48 hours. The zone of inhibition around each disc was measured in mm. The descriptive results were presented as mean ± SD (standard deviation).

Comparison of antifungal activity of henna lemon extract and pomegranate peel extract with the control was done by one way ANOVA and significantly different pairs was identified by post-hocTurkey test. Statistical analysis was conducted using SPSS version 20.0 (SPSS, Inc., Chicago, IL, USA).

Results

A significant zone of inhibition was recorded (p=.000) from a total of 30 samples each (Table 1). Average Zone of inhibition (Figure 2) by Henna lemon extract (18.66 mm) is more than that of pomegranate ethanol extract (14.33 mm) but is comparable to Clotrimazole (18.6 mm).

Table 1: Comparison of antifungal activity of henna lemon extract and pomegranate peel extract with the control at 48 hours.

	Mean	SD	Variance	F-value	P-value
Control	18.6 (Fig 2a)	0.58	0.57	56.33	.000**
Pomegranate	14.33(Fig 2b)	0.577	0.94		
Henna	18.66(Fig 2c)	0.577	0.65		
Significant pairs (post hoc Turkey test)	Control & pomegranate; pomegranate & Heena				

** Highly significant

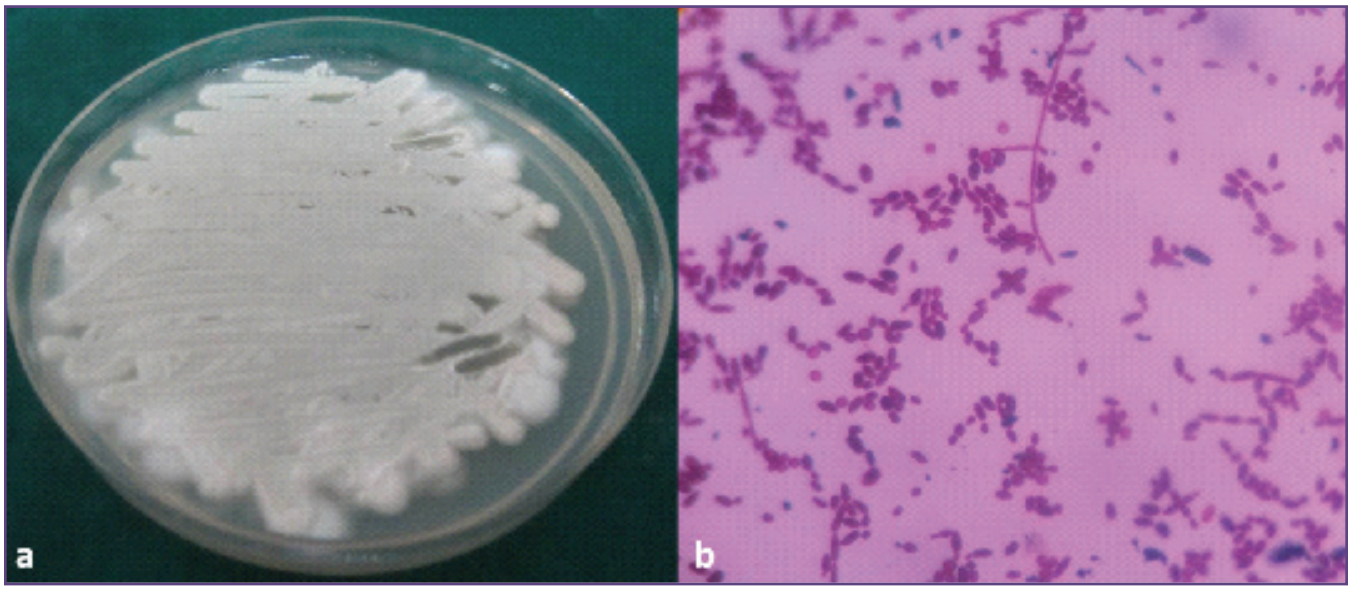

Fig. 1: a – Candida colonies, b – PAS test (Candida Hyphae, 10X)

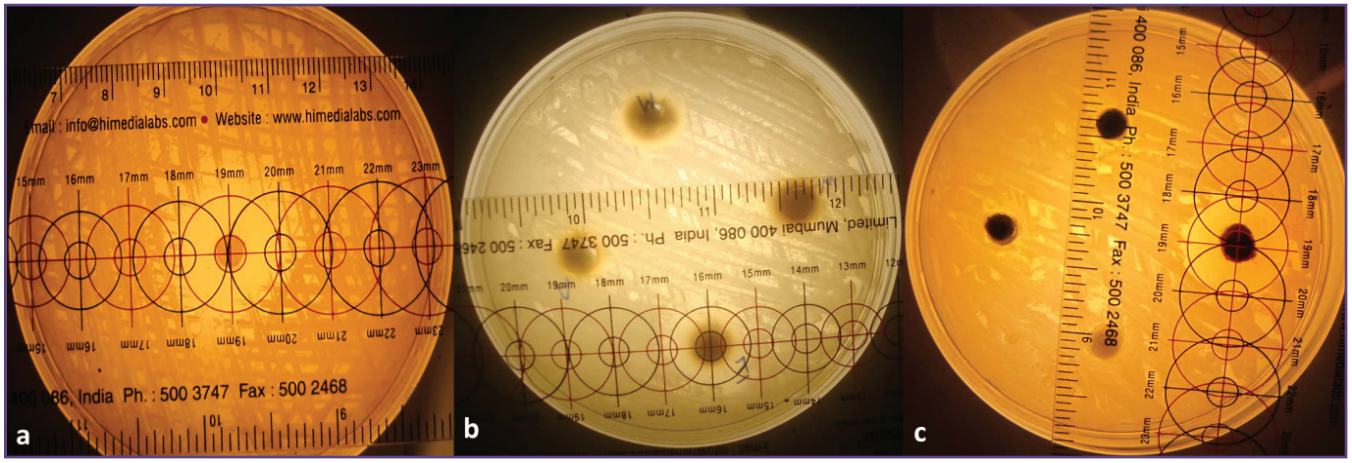

Fig. 2: Zone of inhibition; a- clotrimazole, b- Pomegranate, c-Henna

Discussion

Candidiasis one of the most common opportunistic infection secondary to immunodeficiency is treated by various synthetic antifungal agents topically. Azoles are becoming increasingly popular in the management of oral candidosis. They act by inhibition of cytochrome p-450 enzyme that is involved in cell membrane synthesis in fungi. The principle target is 14α-demethylase, which converts 14α-methylsterols to ergosterol in the fungal cell membrane, causing alteration of the fungal cell membrane by blocking the 14α-demethylation step in the synthesis of ergosterol, an important constituent of fungal cell membrane, which thus become permeable to intra-cellular constituents and leads to alteration in several membrane-associated functions.[6]But, these agents have limitations of having adverse effects and are costly.

In the present study throat swab of diabetic individual was used to isolate candida, which was then cultured on SDA. Analysis of the Candida species isolated from differentanatomical sites showed a remarkable diversity of Candida species distribution. It was noted that C. albicans were isolated mainly from the oral cavity, respiratory tract and high vaginal swabs.[7]Fungal infections are more common in Diabetes mellitus, particularly those caused by *Candida*.[8]The most frequently used primary isolation medium for *Candida* is SDA which, although permitting growth of *Candida*, suppresses the growth of many species of oralbacteria due to its low Ph.[9]

During an attempt to stain the capsules of certain fungi, brilliant staining with the periodic-acid-Schiff-reagent technique (Hotchkiss-McManus stain) was observed. [10] Cawson & Lehner demonstrated that microscopical examination of PAS-stained smears was the most helpful single investigation in candida infection.[11]

Disc diffusion method used to determine the anti-fungal efficacy of pomegranate and henna leaves compared with Clotrimazole was statistically significant with maximum inhibition zone of 14.33±0.58, 18.66±0.58, and 18.66±0.58mm respectively in which the findings of henna extract andClotrimazole are similar.Disc diffusion method is easy to perform and gives accurate and precise results. [12] The findings of our study are comparable with Saadabi MAA(2007) and Pai MBH et al (2010).[13]

Therapy for Candida infections has become a challenge. Treatment is difficult due to the eukaryotic nature of fungal cells, which are similar to host cells. Few antifungal agents are in clinical use, and therefore therapy is limited by drug-safety considerations and their narrow spectrum of activity, efficacy and resistance.[14]

The active antifungal compounds in the peel extract of pomegranate are Punicalagin, Castgalagin, Granatin, catechin, Gallocatechin, kaempferol, and querectin. The synergistic interaction of these compounds increases the antifungal activity of pomegranate peel extract. Tannins are known to precipitate proteins which might be responsible for inhibitory action of extract.[13]The effect of tannins on microbial metabolismcan be measured by their action on membranes.They can cross the cell wall, composed ofseveral polysaccharides and proteins, and bind to itssurface. This adhesion can also help determining minimuminhibitory concentrations for yeasts and bacteria. [15]By transmission electron microscopy, treated cells with punicalagin showed a thickened cell wall,changes in the space between cell wall and the plasmamembrane, vacuoles, and a reduction in cytoplasmic content.[16]

Application of lemon on hand after applying henna paste has been practiced since ancient times to darken its colour. The acid in the lemon juice releases the dye (Lawsone) out in the henna.Phytochemical analysis of henna leaves showed tannic acid, napthaquinone (hennotannic acid), crysophanic acid, anthraquinones, mucilage, mannite, gallic acid, cyanogenic glycosides, sterols and triterpenes. [3]Lawsonia leaves contain 0.5%-1.5% lawsone (2 hydroxy-1,4-napthaquinone) and exhibit strong fungitoxicity where napthaquinones were found to be active factor which acts on the cell membrane, alter the enzyme secretion of the fungi and inhibit metabolic activity of fungi and catalase production.Catalase is essential enzyme in fungi for the conversion H_2O_2 in to oxygen and water. If this enzyme is not produce then H_2O_2 will accumulate in large quantity and toxic to the cell.[17]

Naphthoquinones interact with biological targetsby forming covalent bonds or via their ability to undergo reversible oxidation-reduction reactions. The mechanism of action usually involves the generation of reactive oxygen species (ROS) by the redox cycle under aerobic conditions, by the inhibition of electron transport, by DNA intercalating and/or alkylating agents of biomolecules, and/or as topoisomerase inhibitors.[18]Lawsone has been shown to be effective against candida albicans isolated from patients with HIV/AIDS.[19]

It is not possible to make a direct correlation between the observed activity of the plant extracts in vitro and the actual effects when used in vivo for the diseases observed by the indigenous people and traditional healers. Therefore it is important that these extracts should also be further investigated to evaluate their role clinically.

Conclusion

Candida spp. Is the most common fungal pathogen responsible for invasive infections. Apart from *C. albicans*,

other species such as *C.glabrata*, *Candida tropicalis*, *C. parapsilosis*, *Candida krusei*and *Candida dubliniensis*have also been isolated from saliva of infected subjects.The present study is just a venture from the usual clinical approach. The use of medicinal plants against candida can be a viable alternative to other antifungal agents as these offers cheap and effective module. It is to be concluded that punica granatum and lawsonia inermis extract can serve as an effective means to control candidal infections and can be used as a replacement to topical antifungal products. Therefore these herbs needs further investigations so as to isolate and characterize their active components for pharmacological testing and studies on toxicity in humans, formulations, optimal concentration for clinical applications and comparative studies with antifungal drugs currently in use.

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Competing Interests
None declared

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