Antifungal Activities of Alcoholic and Aqeuous Extracts of punica granatum against Some Non-Dermatophytic Fungi

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ABSTRACT:

A clinical and mycological study of superficial mycosis was conducted on 23 cases (7 males and 16 females) , and collected from patients (5-50) years old. Direct microscopy by KOH (potassium hydroxide) mount and culture was undertaken to isolate the fungal pathogen in each case. Non-dermatophyte molds were isolated from 18 cases (78.26%) and 21 isolates were identified from these cases ; 10 isolates *Candida albicans* (47.61%), 5 isolates *Rhizopus stolonifer* (23.8%), 2 isolates *Penicillium* sp. and 2 isolates *Aspergillus nidulans* (9.5%) respectively, 1 isolate *Alternaria alternata* and1 isolate *Fusarium* sp. (4.7%) respectively . Alcoholic & aqueous extracts of the *punica granatum (Pomegranate)* peels were prepared. The anti-fungal activity of the extracts was evaluated on isolated fungi by means of the agar-well diffusion assay. The Minimum inhibitory concentrations were 10-300 mg/ml against isolated fungi. Their was little difference between the activities of alcoholic extract & aqueous extract. These results suggest the Pomegranate Peels extract which contains active constituents as a promising anti-fungal agent.

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Introduction:

Phytotherapy is considered as a complementary approach for preventing and treating simple disease, although well grounded in medical tradition, it often lacks proper scientific validation (Cravatto *et al.,* 2010). The fact that some of plants have been used traditionally for centuries and modern scientific studies have shown the existence of good correlation between the traditional or folkloric application of some of these plants further pharmacologically active compounds from plants (Abba *et al*., 2009; Abalaka *et al*., 2009; Egharevba & Kunle 2010). One of such plants with wide ethnomedicinal use is *Punica granatum*, which belongs to the family of Punicaceae, is commonly known as pomegranate, grenade, granats and punica apple (Voravuthikunchai *et al.*, 2005). *Punica granatum* has been used extensively as a traditional medicine in many countries (Singh *et al.,*2002) for the treatment of dysentery, diarrhea, helminthiasis, acidosis, hemorrhage and respiratory pathologies(Ricci *et al.,* 2006; Sanchez-Lamar *et al.,* 2007). In addition, *P. granatum* is reported to have antioxidant (Related *et al.,* 2007; Parmar & Kar, 2008) anti-atherosclerotic (Aviram *et al*.,2004; Parmar & Kar,2007), antibacterial (Braga *et al.,* 2005; Naz *et al*.,2007) , antiviral (Zhang *et al.,*1995) and antifungal (Saad *et al.,*2010) properties. The constituents of *P. granatum* are very well known for their therapeutic properties (Lansky & Newman,2007) . Superficial mycosis refers to fungal infections of the outer layer of skin and its appendages like hair and nails (Chander,2009) . They are among the most prevalent of human infectious diseases(Collee *et al.,*1996). Over the last decades, an increasing number of non - dermatophytic filamentous fungi have been recognized as agents of skin and nail infections in humans, producing lesions clinically similar to those caused by dermatophytes. Though several reports on dermatophytosis are available from different parts of

the country, there are hardly any reports on non – dermatophytic fungi and yeast like fungi as causative agents of superficial mycoses along with dermatophytes (Aggarwal *et al.,*2002) The present study was undertaken with a view to find out the clinical pattern of non - dermatophytic fungi (superficial mycosis) and most common fungal pathogens are capable of causing superficial mycosis. Dermatologists are frequently faced with treatment failure, and microbiologists are frequently faced with failure to isolate dermatophytes in culture, this may be due to a possible infection by non-dermatophyte molds. Difficulties arising during chemotherapy of this fungi necessitate novel chemotherapeutic strategies. Therefore, the aims of this study are to investigate anti-fungal properties of water and ethanol extracts of *Punica granatum* L.Peels for treatment of several skin infections and inflammatory disorders using various *in vitro* models .

Materials and Methods:

Collection of plant materials

The *Punica granatum*. Peals were obtained from the local market. Washed, cleaned and dried at room temperature or under shade for nine days and then crushed into coarse powder using a grinder.

Preparation of plant extract

The powder was used for the ethanol and water , 20 g of powder was added to a thimble and then placed in a Soxhlet extractor. Heat was applied to a round bottom flask which was placed at the base of the Soxhlet extractor. The process was continued for 18 hours. The extracts were then placed on rotary evaporators at 67 and 92 °C respectively to remove the ethanol and water. A sample of 500 mg of the dried extract was dissolved in 1 ml of water, and make serial dilutions, to give 5 extract concentrations (300, 100, 50, 30, and 10 mg/ml). These were used as the extracts in the microbial test (Barriada-Pereira, 2003).

Selection of Fungal Strains

Clinical specimens like skin scrapping, infected hair (by hair plucking)

and clipped nails were collected in small paper envelopes after cleaning the area with 70% alcohol. All specimens were subjected to direct microscopy for fungal elements in 10% KOH (20% for nail) and were cultured in Sabouraud's Dextrose Agar (SDA) with and without antibiotics . The culture studies and identification were done by standard methods (Koneman *et al.,*1997; Tony,2004 ; Padhye & Weitzman,2005).These organisms were chosen because they are commonly isolated pathogens from hospitalized patient with skin infections.

Media used

Twenty-three clinical isolates were obtained from AL-Hussain Teaching hospital. Sabouraud Dextrose Agar medium (SDA) was prepared from 10 g. of Neopepton with 10 g. Glucose and 20g. Agar, then added the distilled water to complete to 1 L.

Preparation of MacFrland Standard Solution

Solution A: 1.175gm of barium chloride BaCl2.2H2O in 100ml of distilled water.

Solution B: prepared by the addition of 1ml of concentrated H2SO4 to99ml distilled water.0.5ml of solution A was added to 99.5ml of solution B and the tube was compared with the fungal suspension to give number of cell approximatively 10^8x1.5 fungi/ml (Jawetz & Adelbergs,2001).

Preparation of Fungal Suspension

A sterile wire loop was used to place the test fungi into a test tube with distilled water over an open flame. The concentration of the inoculum was 0.5 McFarland's standards (ca. 10^8 CFU/ml) (Baker *et al,* 1983).

Well Diffusion Assay

Antifungal susceptibility testing was done using the well diffusion method to detect the presence of anti-fungal activities of the plant samples (Perez *et al.,* 1990). A sterile swab was used to evenly distribute fungal culture over the appropriate medium. The plates were allowed to dry for 15 minutes before use in the test. Wells were then created and a pipette was used to place 100 µl of the crude extract of *Punica granatum* into each well.

The same extract was used on each plate; with a total of three plates used for each extract including two wells for the positive and negative controls.. The plates were incubated at 26^{o}C for 24 hours after which they were examined for inhibition zones. A ruler was used to measure the inhibition zones. Three replicates were done for each concentration of the different extracts to ensure reliability.

Detection of *Punica granatum* Peels Constituants

Phytochemical Tests:

1- *Tannins Test:* A modified methods stated in (Trease & Evans,1996) was used to be presented of tannins on the extracts, A few drops of Ferric chloride reagent were added for 3 ml of extract. A blue-black color refereed to the present of tannins.

2- Alkaloids Test:A few drops of Marqus reagent (prepared from mixing 0.5 ml of Formaldehyde with 5 ml of concentration H_2SO_4), added to the 5 ml of extract. Turbidity refereed to the present of alkaloids (Harborne,1984).

3-Saponins Test: 3 ml of extract was added to the 2 ml of Ferric chloride, a white residue to be formed as evidence to the present of Saponins (Al-Khazaragi, 1991).

4- *Phenols Test*: many drops of (1%) Ferric chloride Reagent was added to 1 ml. plant extract,blue-green color refereed to the present of phenols(Ribereau – Gayon , 1972).

5-Flavonoids Test: Flavonoids test were implement in conformity with (Al-Khazaragi, 1991) 2 ml of extract mix with Alcoholic KOH (0.5 ml.), a yellow color as proofed to the present of Flavonoids.

6-Glygosides Test: 0.5g of dried extract was dissolved in 2 ml of glacial acetic acid containing one drop of Ferric chloride solution, then under laid with 1 ml of concentration H_2SO_4 .A brown ring indicated the present of Glycosides (Oloyede, 2005).

Minimum Inhibitory Concentrations(MICs)

The Minimum inhibitory concentrations (MICs) were determined by agar well diffusion method. Inoculums of 10^8x1.5 fungi/ml were seeded on agar, Different concentrations of extracts (10mg/ml-300mg/ml) were added in each well in Sabouraud agar and incubated at 26C^0 . These results were compared with different

concentrations of Nystatin and Flucomin.The lowest concentration preventing growth (MIC) was estimated after 18 - 24 hours. The activity of different concentrations of *Punica granatum. L .* extracts were determined against *Candida albicans*, *Rhizopus stolonifer* , *Aspergillus nidulans* , *Penicillium* sp. , *Alternaria* sp. and *Fusarium* sp.

Statistical analysis:

Results were statistically analyzed using Duncan Multiple Rang Test, and least significance difference to compared between the means.

Results:

Clinical data (Table 1):

Table 1: Summary of clinical data of patients with skin infection

Patients	n=23
Age	5-50
Sex	7 males (30.43%) 16 females (69.56%)
Primary complaint	4 Thorax (17.39%) 2 Back (8.69%) 7 Finger (30.43%) 4 Nail (17.39%) 4 Hair (17.39%) 2 Face (8.69%)

Direct microscopy and culture on Sabouraud's dextrose agar :

Direct microscopic examination of the 23 specimens was done using 10- 20% KOH. Sixteen specimens (69.56 %) were KOH positive and 7 specimens (30.43 %)

were negative. Eighteen specimens (78.26%) were positive for growth and 5 specimens (21.73%) were negative, table (2)

Table 2: Results of direct microscopy and culture on Sabouraud's dextrose agar

Case no.	KOH	Culture on Sabouraud agar	Case no.	KOH	Culture on Sabouraud agar
1	+	*Candida albicans*	**13**	-	N.G
2	+	*Rhizopus stolonifer*	**14**	-	N.G
3	+	*Penicillium sp.*	**15**	+	*Fusarium sp.*
4	+	*Rhizopus stolonifer*	**16**	+	*Alternaria sp.*
5	+	*Aspergillus nidulans*	**17**	+	*Candida albicans*
6	+	*Rhizopus stolonifer*	**18**	-	N.G
7	+	*Aspergillus nidulans*	**19**	+	*Candida albicans*
8	+	*Candida albicans*	**20**	-	N.G
9	+	*Candida albicans*	**21**	-	Mix(*Rhizopus stolonifer* - *Candida albicans* .)
10	+	*Candida albicans*	**22**	-	Mix (*Rhizopus stolonifer* - *Candida albicans* - *Penicillium sp.*)
11	+	*Candida albicans*	**23**	-	N.G
12	+	*Candida albicans*			

Fungal culture on SDA:

Non-dermatophyte molds were isolated from 18 samples (78.26%) and 21 isolates were identified from these samples shown in table (3); 10 isolates *Candida albicans* in 10 patients (47.61%), 5 isolates *Rhizopus stolonifer* in 5 patients (23.8%), 2 isolates *Penicillium* sp. and 2 isolates *Aspergillus nidulans* in 2 patients (9.5%) respectively, 1 isolate *Alternaria alternata* and1 isolate *Fusarium* sp. in 1 patient (4.7%) respectively .

Table 3: Results of fungal culture on SDA

Fungal culture		No. Isolates	%
1	*Candida albicans*	10	47.61
2	*Rhizobus stolonifer*	5	23.8
3	*Aspergillus nidulans*	2	9.5
4	*Penicillium sp.*	2	9.5
5	*Fusarium* sp.	1	4.7
6	*Alternaria* sp.	1	4.7
Total		**21**	**100%**

Antifungal activities :

The antifungal screening of ethanol and aqueous extracts of *Punica granatum* showed good results **,** ethanol extract was most effective against all isolated fungi (31.18 mm). While, the aqueous extract was relatively found to be less effective (18.81mm). Negative control (well containing only solvent) showed no zone against any fungi . The positive controls (Flucomin and Nystatin) produce zone of inhibition against the tested fungi (table 4) .

Table 4: Effect of Aqueous and ethanol Pomegranate Extracts and antifungal drugs on the Fungal Cultures

Fungi	Aqueous extract	Ethanol extract	Control	
			Antifungal Flucomin (7 mg/ml)	Antifungal Nystatin (3mg/ml)
Candida albicans	25	27	6*	30
Rhizobus stolonifer	25	35	12	30.6
Aspergillus nidulans	29.6	30	19	24.6
Penicillium sp.	6	23.6	6	35
Fusarium sp.	6	34.5	16	22
Alternaria sp.	33.3	37	6	14.3
Average	**20.81**	**31.18**	**10.83**	**26.08**

*6:Diameter of well

Phytochemical Compounds:

Pomegranate peels extract was screened for the presence of biologically active compounds like as Tannins, Alkaloids, Phenols, Flavones, Glycosides and Saponins ,table (5) .

Table 5:Phytochemical Compoundsin ethanol extract of *Punica granatum*

Constituents	Result
Tannins	+
Alkaloides	+
Phenoles	+
Flavones	+
Glycosides	+
Saponines	+

Minimum Inhibitory Concentrations (MICs)

Phytochemical Compounds in ethanol extract of *Punica granatum* revealed a high activity for different concentrations *in vitro* against the species of non-dermatophytic fungi included in this study, Statistical analysis showed high significant different between the concentrations of *Punica granatum* expect 50 mg. concentration showed no significant different, table (6) .

Table 6 : Diameters of Inhibition Zone (mm) of Fungi Under test (Ethanol extract of *Punica granatum*)

Type of fungi		Average diameters of inhibition zone (mm.) for different concentrations of *Punica granatum* (mg.)				
		300	**100**	**50**	**30**	**10**
1	*Candida albicans*	24	21	17	14	12
2	*Rhizobus stolonifer*	22	18	17	17	15
3	*Aspergillus nidulans*	27	25	20	20	18
4	*Penicillium sp.*	22	21	19	17	11
5	*Fusarium* sp.	21.5	20	19	17.5	14
6	*Alternaria* sp.	25.6	23.6	15.6	11.3	9.3

P≤0.001

Discussion:

Non-dermatophyte molds(NDMs) are not easily identified with routine fungal cultures, and if discovered, they need a much longer duration of therapy with systemic antifungal agents than dermatophytes, which patients do not always receive. Thus, we carried out this study in order to detect the possible prevalence of NDM in patients with abnormal skin . In this study NDM were isholated from 18

treating acace of skin disorders and the common practice of discardin them as contaminant should be avoided and, at the same time, unequivocal evidence of existence has been obtained (Ghannoum et al.,2000; Summerbell et al.,2005;Baran et al., 2006). Candida albicans 10 isolates (47.61%),Rhizopus stolonifer 5 isolates(23.8%),Penicillium sp. And Aspergillus nidulans 2 isolates (9.5%) for each genus, Alternaria sp. and Fusarium sp. 1 isolate(4.7%) for each genus were isolated & identified from the following regions: Thorax (17.39%),2 Back (8.69%).

NDMs should be considered in evealuating and treating abnormal skin. In the recent years, the use of plants with preventive and therapeutic effects contributes to health care needs (Holetz et al.,2002). There are three main reasons to be interested in the treating and healing power of plant extract. First, pharmacological studies have demonstrated that many of plants are known to possess antimicrobial agents; second, people are becoming aware of the side effects associated with the over prescription of traditional antibiotics; third, time to time resistant microorganisms against antibiotics are increasing (Holetz *et al.*,2002; Meléndez *et al*., 2006; Naz *et al.,*2007) . Among these plants, *punica granate* has an important role in folk medicine. Pomegranate is known as a rich source of pharmacological properties which have been evaluated due to antiparasitic, antibacterial, antifungal, antiproliferative, apoptotic and anti-cancer effects as well as protection against herpes virus and decrease in atheromatous plaque formation and reduction of systolic blood pressure (Kim *et al*.,2002; Naz *et al.,*2007) . peel extracts were previously reported to be able to inhibit the growth of some pathogenic fungi, as well as yeasts(Jayaprakasha *et al.,*2006 ; Tayel *et al*.,2009 ; Osorioa *et al.,* 2010 ; Endo *et al.,*2010; Tayel *et al.,*2011). The present study showed that ethanol and aqueous extract was samples (78.26%), table (2). Hence, yeasts and NDM should always be kept in mind while investigating and

effective against some common non-dermatophytic fungi such as*, Candida albicans , Rhizopus stolonifer, Aspergillus nidulans* , *penicillium* sp. ,*Fusarium* sp. and *Alternaria* sp. The inhibition ability was suggested to be attributed to the high levels of polypolyphenols (Gil *et al.,*2000 ; Tzulker *et al.,*2007; Samy & Gopalakrishnakone,2008). These results were in agreement with the most reports worldwide which detected activity of *Punica granatum* against fungi such as Vasconcelos *et al* (2006) showed that *Punica granatum* may be used as a topical antifungal drug against *C. albicans* and Siham *et al* (2007) suggest the Pomegranate Peels extract which contains gallotanic acid as a promising anti-fungal agent. The real mechanism of the antifungal effect of tannins (the major components of *Punica granatum* extract) may be related to their toxicity, astringent, molecular structure or other ways (Vasconcelos *et al*.,2006) .Table (4): Shows the results of activity of alcoholic & water extract by well diffusion technique of twenty-one strains comparing with control antifungal drug (Flucomin and Nystatin) , good activity was noted with extracts , Antifungal activeties of ethanolic extract had a very good activity against the species most commonly isolated in clinical samples Effectiveness can be returned to the active compounds owned by the pomegranate (Table 5). These results were in agreement with the studies of Siham *et al.,*2007. Table (6) shows the following: 10 isolates (47.61%)*Candida albicans* 12-24mm zone of inhibition with different concentrations of extracts and 5 isolates (23.8%) *Rhizopus stolonifer* 15-22 mm zone , 2 isolates (9.5%) *Penicillium* sp. 11-22mm and 2 isolates(9.5%) *Aspergillus nidulans* 18-27mm , 1 isolate (4.7%) *Fusarium* sp. 14-21.5mm and 1 isolate(4.7%) *Alternaria* sp. 9.3-25.6mm, these results indicated , excellent activity of alcoholic and water extract on all isolated fungi at different concentration comparing with antifungal drug , this antifungal activity may be related to the presence of hydrolysable tannins and polyphenolics in the pomegranate extract specifically punicalagin and gallagic acid (Vasconcelos *et al.,*2006 ;

Reddy *et al.,*2007). It means that the antimicrobial effect of tannins is related to its toxicity and molecular structure. Tannins may act on the cell wall and across the cell membrane because they can precipitate proteins (Naz *et al*(2007) demonstrated that gallic acid (a tannic acid) has the highest antibacterial effect against tested sensitive strains even at low concentrations. Hence, the antifungal activity of *Punica granatum* may be related to polyphenol structures because polyphenols may affect the cell wall, inhibit enzymes by oxidized agents, interact with proteins and disturb co-aggregation of microorganisms (Vasconcelos *et al.*,2003 ; Naz *et al*.,2007).

Conclusion:

Extracts of *Punica granatum* L. bark in this study demonstrated a therapeutic potentials against non-dermatophytic fungi with different diameter zone of inhibition. The antifungal activities of the plant extract, possibly due to the secondary metabolites such as tannins, phenolic compounds or saponins that were abundant in this plant.

This study paves the way for further attention and research to identify the active compounds responsible for the plant biological activity. Further studies should be undertaken to elucidate the exact mechanism of action by which extracts exert their antifungal effect.

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الفاعلية المضادة للفطريات للمستخلص الكحولي والمائي لقشور نبات الرمان ضد بعض مسببات الاصابات الفطرية الغير جلدية

فاتن نعيم عباس

قسم الاحياء المجهرية-كلية الطب-جامعة ذي قار

الخلاصة:

أجريت دراسة سريرية وفطرية على الاصابات الفطرية السطحية للمسببات الغير جلدية على 23 حالة مرضية (7 ذكور والإناث 16)، والتي تم جمعها من مرضى تتراوح أعمارهم (5-50) سنة. أجري الفحص المجهري باستخدام هيدروكسيد البوتاسيوم ، وتم عزل وتشخيص 21 عزلة من الفطريات الغير جلدية

تم أيجاد الفعالية المضادة للفطريات للمستخلص الكحولي والمائي لقشور نبات الرمان باستخدام طريقة الانتشار من الحفر .وتحديد أقل تركيز مثبط (10- 300 ملغم/مل) ضد الفطريات المعزولة. وكانت فعالية المستخلص الكحولي أعلى بقليل من فعالية المستخلص المائي . تشير النتائج امكانية أرجاع الفاعلية الى وجود المركبات الفعالة في المستخلصات لقشور الرمان كعامل مضاد للفطريات .