Anti-fungal Activity of Punica Granatum I.peels Powder and Extracts from Pathogenic Samples

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Abstract

Thirty five samples were collected from patients (1-30) years old, suffered from, infected skin , rushes, boils , oral thrush, anal & vaginal itches. *Candida albicans* 57.3% (20 isolates) and *Candida tropicalis* 22.٥% (8 isolates) *Aspergillus fumegatus* 11.5% (4 isolates) *Aspergillus nigar* 8.7%(3 isolates) , were isolated & identified from these samples. Alcoholic & water hot extracts of the *punica granatum (Pomegranate)* peels as well as the dried powder were prepared. The anti-fungal activity of the extracts was evaluated by means of the agar-well diffusion assay. The extract exhibited potent activity against yeast. The Minimum inhibitory concentrations were 128-1024 µg/ml against *Candida albicans* and *Candida tropicalis* .Their was little difference between the activities of alcoholic extract & aqueous extract. These results suggest the Pomegranate Peels extract which contains gallotanic acid as a promising anti-fungal agent.

Key wards : Antifungal agents, Plant extracts, Candida isolation

الخلاصة

تم جمع ٣٥ نموذج, من مرضى مصابين بإمراض جلدية مختلفة لأعمار من ١-٣٠ سنة, عزلت وشخصت الفطريات التالية: Candida albicans **(57.3%)** ,Candida tropicalis**(22.٥%)** , Aspergillus fumegatus **(11.5%) ,**Aspergillus nigar**(8.7%)** تم إيجاد فعالية المستخلصات الكحولية والمائية على الفطريات المعزولة باستخدام طريقة الانتشار في الوسط ألزرعي الصلب وطريقة التخفيف في أنابيب الاختبار ووجدت أعلى فعالية على عزلات Candida tropicalis , Candida albicans وكانت قياسات الجرع المثبطة الصغرى ١٢٨ - ١٠٢٤ مايكروكرام\مل وكانت فعالية المستخلصات الكحولية اعلى بقليل من المستخلصات المائية. أن فعالية المستخلصات التي تحتوي على حامض الكالوتانيك ضد الفطريات تجعلها مفيدة في علاج الالتهابات الجلدية , والتهابات الأغشية المخاطية وإصابات الفم.

Introduction

The common name of *Punica granatum* is *Pomegranate*, belong to Family *Punicaceae* , of the Order Myrtales, Subclass Rosidae,Class Magndiopsida Pomegranate has a long history as food Medicine and herbal use dating back more than 3,000 years[1]. Both the stem and the root barks contain unusual alkaloids, known as 'pelletierines', which paralyze tapeworms so that they are easily expelled from the body by using a laxative[2]. The plant is also rich in tannin, the dried peels of the fruit contains about 26% which makes it an effective astringent. It is used externally in the treatment of vaginal discharges, mouth sores and throat infections[3]. *Pomegranate*(*Punica granatum*) peel extracts have been shown to possess significant antioxidant activity in various in vitro models, it has already been established that antioxidant activity in *pomegranate* juices is higher when extracted from whole *pomegranate* [4 ,5, 6,7,8]. Australian researchers found that their scientific investigation of *pomegranate* flower extract improved hyperglycaemia in type II diabetes and obesity in which gallic acid is mostly responsible for its glycaemic activity[9,10, 11]. Concentrated pomegranate juice(CPJ) improves lipid profiles in diabetic patients with hyperlipidemia ,they concluded that (CPJ) consumption may modify heart disease risk factors in hyperlipidemic patients ,and its inclusion therefore in their diets may be beneficial[12,13]. Additionally,research findings on excess triglyceride accumulation and increased fatty acid oxidation in the diabetic heart, which contribute to cardiac dysfunction, suggested that pomegranate flower extract improves abnormal cardiac lipid metabolism[14]. In recent study, pomegranate juice was found to slow down cholesterol oxidation by almost half and reduce the retention of disproportionate LDL cholesterol [15]. Flavonoid –rich polyphenol fractions from pomegranate fruit have been shown to exert anti proliferative,anti-invasive

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Received : 13/9/2005
Accepted : 4 /11/2007

and proapoptotic actions in breast and prostate cancer cells and other solid malignancies[16,17,18,19,20,21]. Topical application of *pomegranate* fruit and seed oil extract tested on mouse skin appears to posses chemopreventive activity in skin tumours[22]. It has been found that the methanolic extract of *pomegranate* peels posses wound healing activity against an excision wound on the skin of Wistar rats[23]. The whole plant, but in particular the bark, is antibacterial, antiviral Furthermore *pomegranate* juice provides an HIV-1 entry inhibitor by preventing the virus binding to the cellular receptor CD4[24]. The dried rind of the fruit is used in the treatment of amoebic dysentery and diarrhoea . It is a specific remedy for tapeworm infestation[25,26]. *Pomegranate* rind extract has been shown to have gastro-protective activity through its antioxidant mechanism , it posses strong antibacterial activity against different species of entropathogenes which cause diarrhoea and dysentery, *E.coli*, *Salmonella Shigella sonnei* and *Shigella flexner* [27,28,29,30,]. Pomegranate (outer rind) extract is also screened for their antimicrobial activity against Gram-positive bacteria and yeasts, results founded that *pomegranate* showed good activity against *Staphylococcus aureus* and *Candida* [31].Plants used in Argentin folk medicine screened for antimicrobial activity against *Staph. aureus* commonly present on skin and mucous membranes which causes boils and abscesses, showed that *pomegranate* rind extract produced one of the more active results. *Pomegranate* peels showed also bactericidal effect on Vibrio cholerae[32].

Aim of the Study

Candida and related yeasts are endogenous opportunists.Other opportunistic mycoses are caused by exogenous fungi that are globally present in soil, water and air. Several species of the yeast genus *Candida* are capable of causing candidiasis.They are members of the normal flora of the skin, mucous membranes and gastrointestinal tract. *Candida* species colonize the mucosal surfaces of all humans during or soon after birth and the risk of endogenous infection is ever present .Candidiasis is the most common systemic mycosis. Filamentous fungi such as *Aspergillus* are infected eye, ears, nose, and 5% of Natamycin drops used as treatment. Difficulties arising during chemotherapy of *Candida albicans* necessitate novel chemotherapeutic strategies. The aims of this study are to investigate anti-fungal properties of water and ethanol, extracts & powder of *Punica granatum* L.Peels for treatment of several skin infections and inflammatory disorders.

Materials and Methods

Materials :

Sabouraud agar, Potatos agar, Powder of Nystatin were obtained from (Russell, Beecham, and Special) Pomegranate peels powder, *Candida albicans* standard strain, Tannic acid.

Instruments **:**

Zone reader, Oven Memmert.Germany. Pasture pipett, Vortex mixer. Balances (Sartorius), Homogenizer, Mixer, Incubator, Ultrasonic (soniprep 150HSE) at 20KHZ. Centrifuge, Autoclave, Water bath, Rotary evaporator, Souxhlet apparatus, Magnetic stirrer, Shaker, Incubator.

3-Clinical isolates from different clinical samples collected from three hospitals

Methods **:**

Preparation of medium *(33)*

All media were prepared according to the manufacturers recommendations and were sterilized by autoclaving at 120C and 15 psi pressure for 15 minutes.

a-Sabouraud agar medium contain the following: Peptone 10gm, glucose 20gm, agar 15gm , distilled water(1000ml) ,pH 6-6.3 This medium recommended for the isolation of fungi from pathological samples.

b- Sabouraud conservation medium: Peptone 30gm, agar 20gm, distilled water (1000ml) pH= 6.5-6.7 this medium recommended for conservation of fungus.

c- Sabouraud agar medium with cycloheximide 0.5gm and Chloramphenicol pH 6-6.3 , &the same as(a).This medium was recommended for isolation of Dermatophytes and other pathological fungi. Cycloheximide inhibited the growth of saprophytic fungus and Chloramphenicol inhibits the growth of microbial contamination.

d- Sabouraud broth medium: meat pepton 5gm, tryptic casein 5gm, glucose, 20gm, distilled water(1000ml) ,pH 5.7

e- Sabouraud (Tetrazolium + Chloramphenicol) agar medium , contain the following: Pepton 10gm, glucose 20gm, agar 20gm 2,3,5, triphenyltetrazolium (H.C.L) 0.10gm, Chloramphenicol 0.5gm. For culture rapid differential media. The reduction of triphenyltetrazolium by the colonies of fungi appeared as different degree of red colour according to the type of fungusTable (1).

Preparation of MacFrland Standard Solution [33] **:**

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 bacterial suspension to give number of cell approximatively 10^8x1.5 fungi/ml.

Isolation and Identification of Candida [33] **:** In culture or tissue, Candida species grow as oval, budding yeast cells(3-6 µm in size) .They also form pseudo hyphae when the buds continue to grow but fail to detach producing chains of elongated cells that are pinched or constricted at the septations between cells. *Candida albicans* is dimorphic, in addition to yeasts and pseudohyphae, it can also produce true hyphae . On agar media within 24 hours at 37°C or room temperature. Candida species produce soft cream colored colonies with a yeasty odor. Pseudo hyphae are apparent as submerged growth below the agar surface. Two simple morphology tests distinguish *Candida albicans* , the most common pathogen from the other species of *Candida.* After incubation in serum for about 90 minutes at 37°C yeast cells of *Candida albicans* will begin to form true hyphae or germ tubes on nutritionally deficient media. *Candida albicans* produce large spherical chlamydospores.. Sugar fermentation and assimilation test can be used to confirm the identification and speciate the more common Candida isolates Table (1).

Table(1) – Rapid Identification of *Candida albicans* [33]

Species	Respones in 4 hours	Respones in 24 Hours		
	Serum + Yeast	Media		
	37C Filamentation=+	P.C.B Chlamydospores = +	Sabouraud+Actidion Growth = + Inhibition =0	Sabouraud+Tetrazolium
Candida albicans	+	+	+	White
Candida stellatoidea	+	0	+	Rose
Candida tropicalis	0	0	0	Red-Violet
Candida pseudotropicalis	0	0	+	Rose
Candida guilliermondii	0	0	+	Red
Candida krusei	0	0	0	White
Candida .para krusei	0	0	0	Rose-Red
Candida zeylanoides	0	0	+	White
Candida pulcherrima	0	0	0	Rose

Isolation and Identification of Aspergillus *Aspergillus* species grow rapidly, producing aerial hyphae that bear characteristic conidial structures: long conidiophores with terminal vesicles on which chains of conidia present , the species are identified according to morphologic differences in these structures, including the size, shape, texture and color of the conidia. [33]

Collection of Samples Form Patients :

Candida albicans : 4 strains from skin infections, 2 strains from middle ear infections, four strains from rushes, 3 from infected boils , 2 from oral thrush, and 2 from anal and 3 from vaginal itches.

Microscopic Examination : On direct examination of above samples 10% 0f NaOH or 10% of KOH, the hyphae of *Aspergillus* species are hyaline, septate,uniform in width. Culture:*Aspergillus* species grow within a few days on most media at room temperature. Species are identified according to the morphology of their conidial structures.

Collection of Pomegrante Fruit Rinds： The Punica granatum. Peals were obtained from the local market. Washed, cleaned and dried at room temperature or under the sun.

Spesifictions of Pomegranate Fruit Rinds :

The rind of the fruit is usually is irregular concave fragments, 1/20-1/10in.thick, brownish red externally and dull yellow on the inner surface, with depressions left by the seeds. The toothed calyx is present on some pieces. Taste astringent.

Preparation of Punica granatum Peels. Water Extract .

A known quantity of *Punica granatum* peel was weighed and dissolved in 100ml distilled water boiled for 10-15minutes, soaked three hours, filtered twice, the filtrate was collected and evaporated by vacuum rotary evaporator at 55C until crud extract powder was obtained. The crud extract was weighed and dissolved in distilled water to calculate the concentrations needed for different experiments.

Reparation of Punica Granatum Peels. Alcholic Extract . *Alcoholic (Ethanol extract was prepared by soaking the peels in 75% ethyl alcohol using (Souxhlet apparatus) at 50C then filtered, evaporated by vacuum rotary evaporator at 45C and collected [34].*

Measuring PH :

Ten grams of peels extract were dissolved in 50ml of D.W, shacked well by magnetic stirrer for 12 minutes, filtered and measure the pH.

Detection of Punica granatum Peels Constituants[35]

Detection of Tannins

10gm of extract was dissolved in 50ml of distilled water, filtered and cooled 1% of lead acetate was added .The appearance of precipitation indicated positive reaction.

Detection of Glycosides

Equal amounts of Fehling reagent and extract were mixed and boiled 10 minutes in water bath, red precipitation indicated positive reaction [35]

Detection of Phenoles

10gm of Punica powder was dissolved in 50ml of d.w and boiled for 10minutes, filtered, cooled. 1% of iron chloride was added; greenish blue color appeared which indicated the presence of phenol.

Detection of Saponines :

Five ml of extract was added to1-3ml of $Hgcl_2$; white precipitate was indicated positive reaction.

Detection of Resin

Fifty ml of ethyl alcohol 96% was added to five gm of pomegranate powder and boiled in water bath for two minutes, filtered (Ederal N02) 10ml of acidified with HCl, was added to filtrate precipitation will occur in the case of positive reaction.

Detection of Alkaloides[36]

Ten gm of extract was boiled with 50ml of d.w acidified with 40% Hcl. The solution was filtered and cooled .0.5ml from filtrate was tested with the following solution:

Wagner solution- Grey precipitate positive reaction Mayer solution- white precipitate positive reaction

Detection of Comuurins [36]

A small quantity of extract was dissolved in alcohol in atest tube covered with filtered paper moisture with NaOH in water bath boiled 2-5minutes. The filter paper was exposed to U.V light (336 nm) the presence of yellow-green colour indicated the presence of comuurins.

Detection of Flavones[36]

Solution A –10gm of extract/ 5ml of ethyl alcohol 96%(Filtered) Solution B- 10ml of Ethyl alcohol 50%. Equal quantity was mixed,yellow precipitate indicated positive reaction, by exposing the spot of flavones to uv light, gave fluorescent spot, or by spraying with sulfomolybdic acid solution gave purple to rose color.

***Susceptibility Test*[37]**

Quantitative method, that require measurement of zone diameters give the most precise estimates of antibiotic susceptibility. 40-100 µl extracts from each concentrations (80%,70%, 60%, 50%, 25%) were poured in small holes applied at equal distances in Sabouraud agar seeded with 10^5-10^4/ fungi/ml , dried at room temperature , the inhibition zones were read ,after incubation at 28C for 18 hours. Inoculums of 10^5-10^4/ fungi /ml were prepared by dilutions with the same medium and spotted on Sabouraud agar.

***Minimum Inhibitory Concentrations(MICs)*[37]**

he Minimum inhibitory concentrations (MICs) were determined by agar dilution method. Different concentrations of extracts(2mcg/ml-8392mcg/ml) were diluted with Sabouraud agar in different Petri dishes. Inoculums of 10^8- 10^9 fungi /ml were diluted with the same medium to obtain 10^5-10^4/ fungi /ml spotted on agar, and incubated at $28C^0$. These results were compared with different concentrations of Nystatin and tannic acid diluted with dimethyl formamide and spotted in one cm distance in the same Petri dish .The lowest concentration preventing growth (MIC) was estimated after 18 - 24 hours of incubation by the disappearance of spots. As control, *Candida albicans*, strain was tested under the same conditions. The activity of different concentrations of *Punica granatum. L* .. extracts were determined *against Candida albicans, , Candida tropicalis , Aspergillus fumegatus& Aspergillus nigar* .

(16,17,18,23, 29 30.32.33)

Results and Discussion

Pomegranate has a long history as food Medicine and still continues in the evolution. It is act as antioxidant ,antibacterial anticancer, and anti fungal activities, a gel made from pomegranate peel has a high polyphenolic content demonstrated wound-healing capacity .*Candida albicans* 57.3% (20 isolates) and *Candida tropicalis* 22.٥% (8 isolates) *Aspergillus fumegatus* 11.5% (4 isolates) *Aspergillus nigar* 8.7%(3 isolates) , were isolated & identified from the following samples. *Candida albicans : 4* strains from skin infections, 2 strains from middle ear infections, 4 strains from rushes, 3 from infected boils , 2 from oral thrush, & 2 from anal &3 from vaginal itches.

Antibiotic Susceptibility test and Minimum inhibitory concentrations (MICs)

Table (2) and Table (3) - Shows the results of activity of alcoholic & water extract by disk diffusion technique of thirty-five strains comparing with control organisms(*Candida albicans*).The results were the following:

57.3% (20 isolates) *Candida albicans* 19.5-22 mm zone of inhibition with different concentrations of extracts and *Candida tropicalis* 22.٥% (8 isolates) 21-23.5 , also good activity was noted with water extract with the same microorganism, these results indicated ,excellent activity of alcoholic and water extrat on *Candida tropicalis* and *Candida albicans* at different concentration comparing with standards. On the other hand no activity was observed against *Aspergillus fumegatus* 11.5% (4 isolates) *and Aspergillus nigar* 8.7 %(3 isolates) These results were in agreement with the studies of Holetz FB. Et al.,Fundacao-O-C.. *pomegranate* activity on *candida albicans* [31, 32].The comparative study of minimum inhibitory concentrations of extracts under test against all strains were studied.The results were as follow: MICs for alcoholic extract and water extract against *Candida albicans* and *Candida tropicalis* were 128-1024µg/ml, and for The MICs of for alcoholic extract and water extract against strains of *Aspergillus fumegatus and Aspergillus nigar* were very high as demonstrated in Table (4) and (5). Fig (1) demonstrated the diameters zone of inhibition of different dilutions of alcoholic extract against *Candida albicans* . The results were compared with the activity of Nystatin and Tannic acid. Table (6),Table (7) demonstrated the active ingredients of *Pomegranate* peels.

Table(2) –Diameters Zone of Inhibition /mm of Fungi Under test (Ethanol Extracts)

Average diameters zone of inhibition/mm for different concentrations of *Punica granatum*						
Type of microorganisms		**80%**	**70%**	**60%**	**50%**	**25%**
1-	***Candida albicans* 10**	22	21.5	21	20	19.5
2-	***Candida albicans* 10**	22	22	21	21	20
3-	***Candida tropicalis* 5**	23.5	23	22.5	22	21
4-	***Candida tropicalis* 3**	23	22.5	22	21	20
5-	***Aspergillus fumegatus* 4**	5	0	0	8	0
6-	***Aspergillus nigar* 3**	0	4	2	4	0
7-	***Candida albicans* Standard**	21	21	21	20	19.5

Table (3) - Diameters Zone of Inhibition /mm of Fungi Under test (Water Extracts)

Average diameters zone of inhibition/mm for different concentrations of *Punica granatum* water extracts						
Type of microorganisms		**80%**	**70%**	**60%**	**50%**	**25%**
1-	***Candida albicans* 13**	21	20	19.5	19	18
2-	***Candida albicans* 7**	21.5	21	20	19.5	19
3-	***Candida 4 tropicalis***	22	21	20.5	19	18.5
4-	***Candida 4 tropicalis***	23	22	21.5	21	20
5-	***Aspergillus fumegatus* 4**	4	4	0	0	0
6-	***Aspergillus nigar 3***	0	0	0	0	0
7-	***Candida albicans* Standard**	22	21.5	21	20	19.5

Table (4)- Minimum Inhibitory Concentrations µg/ml of *Punica granatum* Peels Alcoholic Extract of Different Concentrations

Type of microorganism	**Minimum inhibitory concentrations/ml**				
	80%	**70%**	**60%**	**50%**	**25%**
Candida albicans	128*	256	1024	1024	1024
Candida tropicalis	64	128	512	1024	1024
Aspergillus fumegatus	≤4196	4196	4196	≤8192	≤8192
Aspergillus nigar	2048	2048	2048	4196	4196
***Candida albicans* Standard**	128	256	1024	1024	2048

*N=6

Table (5) - Minimum Inhibitory Concentrations µg/ml of *Punica granatum (Pomegranate)* Peels Water Extract of Different Concentrations

Type of microorganism	Minimum inhibitory concentrationsµg/ml				
	80%	70%	60%	50%	25%
Candida albicans	256	512	512	1024	1024
Candida tropicalis	128	256	512	1024	1024
Aspergillus fumegatus	2048	2048	2048	4196	4196
Aspergillus nigar	4196	4196	4196	≤8192	≤8192
***Candida albicans* Standard**	128	256	1024	1024	2048

*N=6

Table (6) - Minimum Inhibitory Concentrations µg/ml of *Punica granatum (Pomegranate)* Peels and Peels Powder,

Fungus	Minimum Inhibitaory Concentration / mcg/ml			
	Powder/pomegranate peels	Solution/ water extract-80%	Standard Tannic acid 80%	Nystatin*/ In DMF
Candida albicans	512	256	128	4
Candida tropicalis	128	128	64	4
Aspergillus fumegatus	4196	4196	1024	16
Aspergillus nigar	2048	2048	1024	16
***Candida albicans* Standard**	128	128	64	2-4

- Nystatin powder activity 4976 I.U= 93.8% .DMF- Dimethyl formamide.

Table (7) - Active Ingredients of *pomegranate* Fruit Rinds

Constituents	Peels powder	Ethyl alcohol extract	Water extract
Tannins/ as Gallotanic acid	28%	29%	30%
Glycosides	+	+	+
Total Ash	5.14%	5%	%5.3%
Non soluble materials	30%	NT	NT
Alkaloides	_	_	−
Phenoles	_	_	−
Saponines	_	_	−
Couumarins	_	_	−
Flavones	_	_	−
Non soluble ash in acid	0.3%	0.2%	0.3%
Colour	+	+	+
Resinss	+	+	+

Conclusions

From above study one can concluded that the extract of *Pomegranate* peels which contains Gallotanic acid is useful for the treatment of several infections and inflammatory disorders due to *Candida albicans* & *Candida tropicalis* , these results suggested the possibility of using this raw material in pharmaceutical as cream, ointment, skin solution, lotion ,powder, mouth wash, gargles and even ear drops. Further studies and investigations were needed .

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