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ANTIFUNGAL ACTIVITIES OF *PUNICA GRANATUM* RIND ALCOHOLIC EXTRACT AGAINST SOME NON-ALBICANS *CANDIDA* SPP.

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Punica granatum, *C. guilliermondii*, *C. tropicalis*, Zone of inhibition, MIC value

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ABSTRACT: *Punica granatum* (Pomegranate) external peel extract in 70% ethanol was challenged against two non-albicans *Candida* spp. to find out it's possible antifungal effects in disc diffusion technique as well as in microtiter dilution to find out MIC levels. Although both showed sensitivity zones in disc diffusion technique at 100 mg/mL concentration of the extract, at lower concentrations (<16.6 mg/mL), only *C. guilliermondii* showed MIC value at 0.52 mg/mL, while *C. tropicalis* did not show any inhibition of growth at these concentrations of the extract.

INTRODUCTION: *Punica granatum* (Pomegranate): *Punica granatum* (Pomegranate) is one of the first domesticated fruits that have been cultivated from past times. It is indigenous to Iran and neighboring countries that gradually developed in central Asia, the Himalayas, Eyalet of Anatolia, the Middle East, and the Mediterranean. It also thrives in Arizona and California and has been cultivated in the Mediterranean region, South Asia, and the Middle East countries; Kandahar in Afghanistan is famous for its high-quality pomegranate [1, 2]. Today, pomegranate is cultivated in most regions of the world, including Iran, Spain, Italy, Afghanistan, America, India, China, Russia, Uzbekistan, Morocco, and Greece. Iran is one of the biggest producers of pomegranate in World [2]. In Iran, Markazi, Yazd, Fars, Khorasan, and Kerman provinces have the highest production rates [3]. *P. granatum* L. belongs to the Punicaceae family **Fig. 1** and is the smallest plant family that includes one genus and two species, including *P. granatum* (edible pomegranate), which is indigenous to Iran and Mediterranean regions, and *P. protopunica* (inedible), which is endogenous to Socotra islands in the Pacific Ocean.

Pomegranate is a shrub that reaches 1.5 to 5m in height, with more or less irregular and thorny branches and glossy leaves that appear as a deciduous shrub in temperate regions and as evergreen in frigid regions [3]. Fruits are in light red colour to greenish-yellow and sometimes in dark purple. It is 5 to 20cm in diameter, and its weight ranges from less than 200g to more than 800g. Seeds are triangular, albumin free, and embedded in aril [3].

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This plant is mostly easy to grow in desert margins with hot dry summer and scorching heat of the sun and rather a cold winter having saline water and soil [4]. This wide compatibility range is considered a favorable eco physiological condition for *P. granatum* L, and so it is called Kavir ruby [5]. However, this plant is sensitive to soils that have low drainage, and its growth in this condition is low, and product quality is decreased [5]. The best soil condition to cultivate pomegranate is deep sandy clay soils, and the most growth, performance, and quality of the product is in regions with hot and long summers [5, 6]. The pomegranate's fruit, bark, stem, and peel have extensively been used as a traditional remedy against acidosis, dysentery, microbial infections, diarrhoea, helminthic infections, haemorrhage, and respiratory infections pathologies [7]. Pomegranate seeds have also been shown to contain the estrogenic compounds estrone and estradiol. Furthermore, the dried pericarp and the juice of the fruit are considered beneficial for the treatment of colic, colitis, menorrhagia, oxyuriasis, headache, kidney problems, acne, piles, allergic dermatitis, and treatment of oral diseases [7, 8].

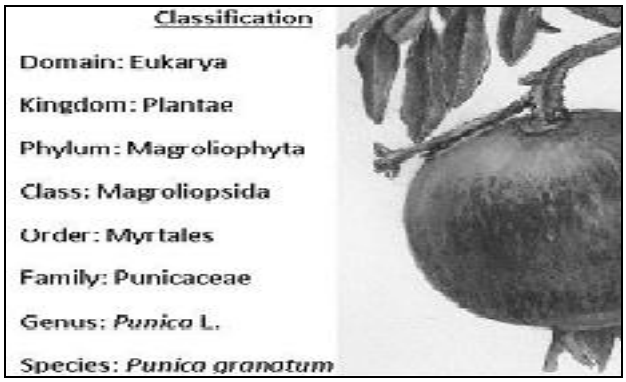

FIG. 1: TAXONOMY OF PUNICA GRANATUM

The pomegranate seed oil is also reported to contain phytoestrogenic compounds, and the fruit is rich in phenolic compounds with strong antioxidant activity [7, 8]. The juice and seeds are considered a tonic for the throat and heart [8]. It is used to stop nose and gum bleeding and treat haemorrhoids. *P. granatum* peel is used to treat infections found in human sexual organs as well as mastitis, folliculitis, piles, scalds, and tympanitis [9]. Today, *P. granatum* L. as a fruit not only attracts a lot of public interest, but research is also focused on its medicinal properties and food industry. Pomegranate plant has antitumor, antifungal, antibacterial, antidiarrheal, antioxidant, anti-inflammatory activities [2, 3]. They are also known to act as anti-ulcer agents [3]. They are used in osteoporotic cases [5, 9]. They also have anti-inhibitory effects in certain cancers of the breast and skin; that have been reported with various constituents of different parts of this plant [6, 9]. Many *Candida* isolates in West Bengal are now non-albicans variety [10]. Recent studies of Candida spp. in Kolkata indicated that about 12% and 10.67% of Candida isolates are *C. tropicalis* and *C. guilliermondii*, respectively [11]. Sometimes, they become drug-resistant and are drug-resistant related to biofilm production where antifungal agents cannot act properly. Thus we should look for newer antifungal agents to tackle these problems in the future.

MATERIALS AND METHODS:

Collection and Preparation of *P. granatum* Extract: The *P. granatum* fruit (Pomegranate), was collected from a local vendor. The fruit was washed and cleaned thoroughly with tap water, and the external peel was separated and kept in the incubator for drying purposes.

The drying of the peel was complete when its weight (serial weight) remained the same after repeated weighing using an electronic balance. This 1 g dried peel was crushed and dipped in 10mL 70% ethanol solution and was kept undisturbed for 48 h at room temperature. After 48 h, the obtained extract was separated and was preserved in the refrigerator for further experimental use.

Mueller Hinton (MH) Broth: 100mL MH broth was prepared by dissolving 2.1g of MH powder (HiMedia, India) in distilled water and sterilized by autoclaving.

Preparation of Muller Hinton (MH) Agar Plates: 100mL MH Agar was prepared by melting 3.8g of MHA powder (HiMedia, India) in distilled water, sterilized by autoclaving followed by pouring in petri-dishes to prepare the solid medium.

Collection and Isolation of the *Candida* spp. on MH Agar Plates: *Candida guilliermondii* was collected from the urine sample of a 79 years old female patient at Peerless Hospital, Kolkata on 14.08.2021. *Candida tropical* is was collected from

the urine sample of a 77-year-old male patient at Peerless Hospital, Kolkata, on 30.08.2021.

The subcultures of *C. guilliermondii* and *C. tropicalis* were maintained on MH agar petri plates in the laboratory for the experiment.

1.0 MacFarland Standard Preparation: *C. guilliermondii* and *C. tropicalis* suspensions in sterile normal saline were standardized at 1.0 MacFarland standard by the DensiCHEK Plus instrument (reading was 1.02McF).

Preparation of the 6 mm Whatman Filter Paper Discs: Standard 6 mm size discs were punched out, kept in petridishes and sterilized by autoclaving. After that, the discs were soaked in the extract and dried in the incubator. Control discs were also prepared with the vehicle 70% ethanol in a similar way.

Zone of Inhibition test (Similar to Kirby- Bauer Test) using the *P. granatum* Extract: Lawn cultures of the two Candida spp. were made on MH agar plates and then tested (in duplicates) and control discs were placed over the lawn cultures after 15 min and incubated at 37°C overnight. Sensitivity zones were measured with a standard scale (HiMedia, India) for measuring sensitivity zones.

Determination of MIC Values by Serial Dilution of the *P. granatum* Extract in a Microtiter Plate: A 96 well microtiter plate was used. In the first well of each row, 250 µL of the MH broth was added, and 50 µL of the extract was mixed (1:6 dilution; 16.66 mg/mL), followed by serial double dilution in 100 µL MH broth for successive 7 wells. The Candida suspensions were added in 10 µL amounts in each well, mixed gently, and a baseline reading of all the wells was taken at 620 nm in the Multiskan ELISA. The microtiter plate was then incubated 37 °C overnight, and a second reading was taken of each well. Subtractions of the first reading absorbance from the second reading were made for analysis.

RESULTS: The disc diffusion study on MHA showed good sensitivity zones (Average 22 mm) of the extract (100 mg/ mL) when studied with *C. guilliermondii* and slightly less sensitivity zones (average 16 mm) when studied with *C. tropicalis* **Fig. 2.** In the study in microtiter plate with serial dilution of the extract starting from 16.6 mg/mL, *C. guilliermondii* showed inhibition of growth with MIC of 0.52 mg/Ml **Fig. 3**, which is highly significant as this is a crude extract of the plant. However, at these concentrations, *C. tropicalis* did not show any inhibition of the growth **Fig. 4.**

FIG. 2: ZONE OF INHIBITION OF THE EXTRACT AGAINST *CANDIDA GUILLIERMONDII* AND CANDIDA *TROPICALIS* ON MH AGAR PLATE. C IS THE CONTROL DISC

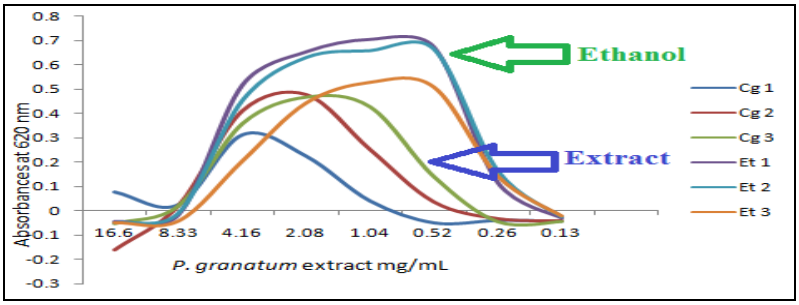

FIG. 3: GROWTH INHIBITION OF *C. GUILLIERMONDII* BY THE EXTRACT WITH MIC AT 0.52 MG/ML. THE BASELINE ABSORBANCE WAS MORE IN THE BEGINNING, AND AT THE END DUE TO COLOUR OF THE EXTRACT, THE DECLINE OF ABSORBANCE IN LOW CONCENTRATIONS WAS DUE TO PHASE OF DECLINE OF THE GROWTH AND LYSES OF THE FUNGI

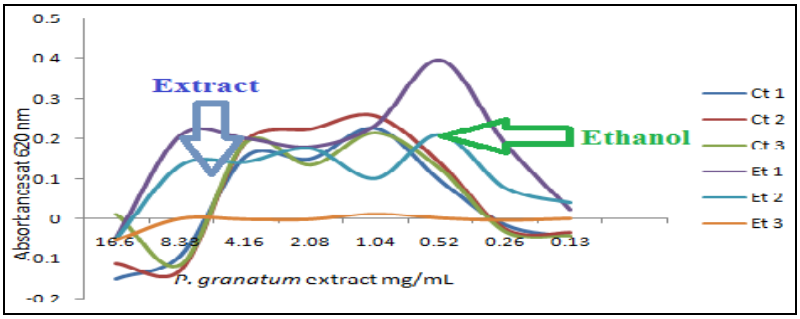

FIG. 4: NO GROWTH INHIBITION OF *C. TROPICALIS* BY THE EXTRACT. THE BASELINE ABSORBANCE WAS MORE IN THE BEGINNING, AND AT THE END DUE TO COLOUR OF THE EXTRACT, THE DECLINE OF ABSORBANCE IN LOW CONCENTRATIONS WAS DUE TO PHASE OF DECLINE OF THE GROWTH AND LYSES OF THE FUNGI

DISCUSSION: In this experiment, we observed good antifungal activities of fruit external peel alcoholic extract of *P. granatum* particularly against *C. guilliermondii*. Antibacterial activities of *P. Granatum* extract are well known against *Staphylococcus aureus* [12, 13], *Streptococcus mutans*, *E. coli* [13], *Pseudomonas aeruginosa* [13], *Propionibacterium acnes* [14], pathogenic Clostridium, Lactobacillus, Bifidobacteria, *Bacillus megaterium*, *Corynebacterium xerosis*, *Enterococcus faecalis*, *Micrococcus luteus* [15-18]. Duraipandiyan *et al.* experimented to determine the *in-vitro* antifungal efficacy of *P. granatum* peel extract against the oral *Candida* compared with clotrimazole. 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 Saboraud's Dextrose Agar (SDA) medium and incubated at 37°C for 48 h.

The cultured Candida species were subjected to an antifungal susceptibility test by agar well diffusion method. Antifungal efficacy of *P. granatum* group and Clotrimazole group were statistically significant with a 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 *P. granatum* approximated with that of the clotrimazole. The Authors concluded that peel extract of *P. granatum* may be used as a substitute for antifungal agents in clinical trials with standardization to minimize the deleterious effects for patient compliance [15]. Anibal *et al.* conducted an experiment in which ethanolic crude extracts prepared from the arils and seeds, pericarp, peels, and the whole fruit of P. granatum had their antifungal activity tested against Candida spp. The ethanolic crude extracts were analyzed by Mass Spectrometry and yielded many compounds such as punicalagin and galladydilacton. The extracts from the pericarp and peel showed activity against *Candida* spp., with MICs of 125 µg/mL. The effect of pericarp and peel extracts upon the morphological and structure of *C. albicans* and *C. krusei* were examined by scanning and transmission electron microscopy, with the visualization of an irregular membrane and hyphae formation of vacuoles, and thickening of the cell wall. The data obtained revealed potential antimicrobial activity against the Candida genus yeast cells, and the bioactive compounds could be responsible for changes in cell morphology and structure. The data obtained open new perspectives for future research in continuation to this study, where information such as determination of the site of action of the compounds could contribute to an alternative therapy against these organisms [19].

Singh *et al.* conducted a research work in which; they took Arils from six pomegranate varieties grown in the Mediterranean region of Turkey that were tested for their antimicrobial properties by the agar diffusion and minimum inhibitory concentration (MIC) methods against three fungi (*Kluvyeromyces marxianus*, *Rhodotorula rubra*, *Candida albicans* ATCC 1023). It has been observed that the pomegranate aril extracts had antimicrobial effect on all microorganisms, giving inhibition zones ranging in size from 13 to 26 mm. The MIC values for active pomegranate extracts ranged between 30 and >90 µg/mL. The results obtained appeared to confirm the antimicrobial potential of the P. granatum varieties [17]. The chemical composition of the fruits differs

depending on the cultivar, growing region, maturity, cultivation practice, climate, and storage circumstances. About 50% of the total fruit weight corresponds to the peel, an important source of bioactive compounds such as phenolics, flavonoids, ellagitannins, proanthocyanidin, minerals and mainly minerals potassium, nitrogen, calcium, phosphorus, magnesium and sodium and complex polysaccharides. The edible part of the pomegranate fruit (50%) consists of 40% arils and 10% seeds. Arils contain 85% water, 10% total sugars, mainly fructose and glucose, and 1.5% pectin, organic acids, ascorbic acid, citric acid, malic acid, and bioactive compounds such as phenolics and flavonoids, principally anthocyanins [7]. The seed cover of the fruit contains delphinidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3, 5-diglucoside, cyaniding - 3, 5 – diglucoside, pelargonidin-3, 5-diglucoside, and pelargonidin-3-glucoside, with delphinidin-3,5-diglucoside being the main anthocyanin in pomegranate juice. 12–20% of the total seed weight of pomegranate comprises seed oil and is self-possessed with more than 70% of the conjugated linolenic acids [8].

The fatty acid component of pomegranate seed oil comprises over 95% of the oil, of which 99% are triacylglycerols. Minor oil components include sterols, steroids, and a key component of mammalian myelin sheaths, cerebroside [8]. Interestingly, punicic acid, a conjugated isomer unique to pomegranate oil, constitutes 70–76% of the seed oil [8]. Phenolic compounds, together with flavonoids, anthocyanins, and tannins, are the main group of antioxidant phytochemicals that are important due to their biological and free radical scavenging activities [7].

Phenolic acids, flavonoids, and tannins are present in different parts of pomegranate fruit, and this may be one of the reasons why many of the studies demonstrated that combinations of pomegranate extracts from different parts of the fruit were more effective than a single extract. In a comparative analysis, anthocyanins from pomegranate fruit possessed higher antioxidant activity than vitamin E (α-tocopherol), β-carotene, and ascorbic acid [9]. In this study, we did not observe the mechanism of action of the extract on the *Candida* spp. However, bioactive compounds such as phenolics and flavonoids, principally anthocyanins, may be responsible for the antifungal activities of this extract.

CONCLUSION: Ethanolic extract of pomegranate rind is effective on non-albicans *Candida* spp. particularly on *C. guilliermondii*.

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