

In-Vitro Comparison of Antifungal Activity of Herbs (Darehald and Pomegranate) with Azoles

Rabeea Rizwan¹, Shehla Shaheen¹, Zahida Memon¹, Faisal Afridi², Mubarak Zaib²

¹Department of Pharmacology, Ziauddin Medical College, Karachi, Pakistan

²Department of Microbiology, Ziauddin University Hospital, Karachi, Pakistan

Email: rabeea_rizwan@hotmail.com, drshel2011@gmail.com

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Abstract

Aim: Candida, an opportunistic organism is one of the commonest causes of hospital acquired infections among fungi. Currently available antifungal drugs have numerous adverse effects and drug-drug interactions (DDIs) along with increase in resistance over the time. Therefore, it is highly emergent to consider alternative treatments for candidal infections, having fewer adverse effects and is cost-effective. The current *in-vitro* study is undertaken to assess and compare the antifungal effects of the herbs, *Berberis aristata* (*B. aristata*, *Darehald*/*Darhald*) and *Punica granatum* (*P. granatum*, *Pomegranate*) with fluconazole and voriconazole, based on culture and sensitivity of candidal isolates. **Materials and Methods:** Ethanolic extracts of herbs (*Berberis aristata* and *Punica granatum*) and concentrations were formulated as per standard procedure. 130 samples were obtained for the study from in and out patients reported in clinical subsets of Ziauddin Hospital, Karachi from March to May, 2018. Samples were collected and grown according to the standard procedures like, wet mount test and gram's staining. Species were identified by CHROM agar candida and API 20 C AUX methods. Sensitivity tests were performed by Kirby Bauer's disc diffusion method according to CLSI guide lines M-44 A2, 2009. Data analysis was done by one-way ANOVA to compare the antifungal activities of drugs and herbs. **Results:** Mean inhibitory zones of herbs, *B. aristata* and *P. granatum* were highly significant against clinical candidal isolates with respective p-values of 0.00 and 0.02. Both of the herbs, *B. aristata* and *P. granatum* were found to be more sensitive, 98.5% and 97.7% respectively in comparison to fluconazole showing 42.3% and voriconazole showing 29.2% sensitivity against candidal isolates. The most resistant candidal specie was *C. tropicalis* that showed resistance against both fluconazole and voriconazole, contrary to that, this

specie was highly sensitive to both of the herbs, showing sensitivity of 100% respective for *Darehald* and *Pomegranate*. **Conclusion:** In comparison to azoles: Culture sensitivity of both herbs (*B. aristata* and *P. granatum*) displayed more sensitivity against candidal isolates of patients having non-invasive and invasive candidiasis. These herbs can be considered as substitute or alternative antifungal agents to the conventional antifungal therapy, particularly in cases of treating candidemia patients, which is a life threatening condition.

Keywords

Candida, Fungi, Azoles, Antifungal Sensitivity, Antifungals, *Berberis aristata*, *Punica granatum*, Pomegranate, Darhald, Darehald

1. Introduction

The use of herbs in folk medicine has been practiced by humans since ancient times. The traditional herbal therapy is considered as a “shotgun approach” because of multiple constituents of a herb’s extract, having many therapeutic mechanisms of actions which result in greater therapeutic activity against various diseases [1]. It is estimated by WHO that around 80% of world’s population rely on natural herbal treatments for their primary cure, mainly in developing world [1] [2]. In the field of ethnobotanic studies, unpurified herbal extracts considered to be safer and more potent as they contain multiple constituents having synergistic activity to achieve required therapeutic effects along with reduced toxicity in comparison to its isolated buffered ingredients [3].

Globally, the frequency of fungal infections is increasing just like bacterial infections [4] [5]. Among fungal infections, candidiasis is one of the common opportunistic organisms related to yeast family [6]. It is responsible for 70% of all hospital acquired mycological infections [7] [8]. In the field of medicine, till date synthetic drugs are considered as conventional empirical therapy for the treatment of fungal infections. About 18 antifungal drugs were synthesized and approved by authorities from 1980 to 2002 in which majority (83%) of the drugs were related to the azoles class [9]. For candidiasis, the preference of antifungal therapy depends up on type, nature and extent of the lesion along with patient’s generalized health and immune status [10]. Besides, numerous drug-drug interactions, the adverse effects of antifungal drugs are hepatotoxicity, nephrotoxicity, headache, altered taste sensation, anemia, GI upsets, hair loss etc. [11] [12]. Nowadays, the rising resistance to antifungals and cross resistance among its azole class of drugs is a big threat which shows variation topographically [13] [14]. These ground realities are sufficient for the motivation of researchers to consider and evaluate new antifungal drugs or alternative agents for the treatment of candidal infections, showing promising results with better

efficacy, lower resistance, fewer adverse effects and affordability of the patients [15].

Therefore, this current study was undertaken to evaluate and compare the anticandidal effects of herbs *Punica granatum* (*Pomegranate*) peels and *Berberis aristata* (*Darehald*) root bark with commonly used azoles (fluconazole and voriconazole) antifungal drugs. The aforementioned herbs are commonly grown and available in regions of Asia [16] [17]. They are used in folk medicine for many therapeutic purposes, *Darehald* (*Darhald*) has been used as an antimicrobial, antipyretic, antioxidant, antihypertensive, anti-osteoporotic and anti-arrhythmic herb [18] [19]. Its root and stem barks extracts have potent antipyretic, antimalarial and antimicrobial effects [20] [21]. In the past, *pomegranate* was popularly called as a “fruit of dead” by Greek mythologists [22]. It is an extensively studied plant in the field of pharmacotherapeutics, having antimicrobial, neuroprotective and immune modulating properties, also being used in treating diabetes mellitus, infantile brain ischemia, weight gain, Alzheimer’s disease and arthritis [20] [23]. Regarding toxicity of herbs, *Berberis* was studied as safe drug with no mortality and hepatoprotective effects in therapeutic doses of even 2000 mg [24] [25] [26]. *P. granatum* (*Punica granatum*) was also reported to be safe with no noxious effects at therapeutic doses < 2000 mg daily along with hepatoprotective and nephroprotective effects [23] [27]. It is known as “superfood” by the mythologists because of its apparent non-toxic, curative and probable nutritious values [28].

2. Materials and Methods

Study settings:

Total 1020 samples were collected from in and out-patients of different wards, private rooms, operation theaters, emergency, coronary and intensive units of Dr. Ziauddin group of hospitals (Clifton, Kemari, Ziauddin Memorial and North Nazimabad Ziauddin Hospitals Karachi). All samples were transported within 24 hours to microbiology laboratory of North Nazimabad hospital where the bench work was performed.

Data Analysis:

The numerical data (mean zone of inhibitions and \pm standard deviations) of herbs (*B. aristata* and *P. granatum*) and azoles (fluconazole and voriconazole) were observed and compared by applying one-way analysis of variance (ANOVA) test through a statistical software program analysis, SPSS 21. 95% confidence interval was taken with p-value less than 0.05 was considered to be significant. While sensitivity and resistance pattern of these variables (herbs and azoles) was evaluated by percentage.

2.1. Preparation of Crude Extracts

All herbs were bought from a registered herb dealer, cleaned, dried and stored at room temperature. These herbs were authenticated and placed in herbarium of

Pharmacognosy department of Jinnah Sind Medical University (JSMU). They were grinded in electric grinder [29] [30]. The powder of each herb was placed in a glass container to mix and soak with the absolute (100%) ethanol at 3:10 ratio for 20 days. These extracts were filtered by using Whatmann No. 1 filter paper (**Figure 1(a)** and **Figure 1(b)**) [4] and further extracted by a rotary evaporator (BUCHI, Switzerland) (**Figure 1(c)**) [31]. These crude extracts were placed open in ventilated laboratory to let them dry for 7 to 8 days and stored in airtight bottles. [32] [33]. The minimum inhibitory concentrations (MICs) of *Berberis aristata* (25 mcg) and *Punica granatum* (20 mcg) extracts were made with per ml of 5% Tween 20 [31].

2.2. Culture Assays Procedure for Yeast Sensitivity Testing

After approval from the institutional ethical committee, and written informed consent, the 130 (out of 1020) samples of the patients from suspected infected urine, pus, wound, vagina, tracheal aspirates and body fluids (ascitic, peritoneal and pleural fluids), nasogastric tube, incubate tips and blood was taken. Those suspected samples with positive sign and symptoms of infection (e.g. itchy subcutaneous regions of mouth etc., white velvety lesions and history of chronic fatigue, fever and recurrent infections) were identified candidal positive by the following procedure which was processed according to standard microbiological technique. The isolates of Candidal species from clinical samples of vagina, tracheal aspirate, pus, wound, body cavity fluids, sputum, urine, tips and blood identified by Wet mount (for vaginal swabs) (**Figure 2(a)**), Gram's stain (**Figure 2(b)**) and Germ tube test (for sputum specimens) (**Figure 2(c)**) and BACTEC 9240 system (Automated Blood Culture Systems, for candidemia samples) according to microbiological standard procedure. These isolates further identified on the species level by using CHRO Magar Candida (Oxoid Ltd., UK) (**Figure 2(d)**), and API 20 C AUX test [4] [34]. For screening purpose, simple discs soaked in 200 µL of each extract for 37 hours. Modified Kirby Bauer's disc diffusion method (**Figure 3**) at 0.5 McFarland turbidity was applied as per CLSI M-44 A2series guidelines, 2009 [35] [36] [37]. Muller Hilton Agar (MHA, Oxoid Ltd. UK) was prepared by adding 2% glucose and 0.5 µg/ml methylene blue in it. Fluconazole (SD 232, 25 mcg/disc) and voriconazole (SD 277, 1 mcg/disc, Hi-Media, India) discs were placed on MHA and incubated at 35°C for 37 hours [17] [38]. The inhibitory zones of samples > 14 mm for fluconazole (25 mcg) and >17 mm for voriconazole (1 mcg) were considered sensitive, as per standard by CLSI M 444-S3 guidelines, 2009 [39] [40] [41]. To the best of our knowledge the cut off values for herbs' (*B. aristata* and *P. granatum*) inhibitory zones (ZIH) were not mentioned in any of the previous studies and thus a pilot study was conducted on 20 candidal samples in order to calculate the cut offs for the inhibitory zones of *B. aristata* and *P. granatum* which were found to be greater than 12 and 8 mm respectively. All the tests were repeated and confirmed three times [4].

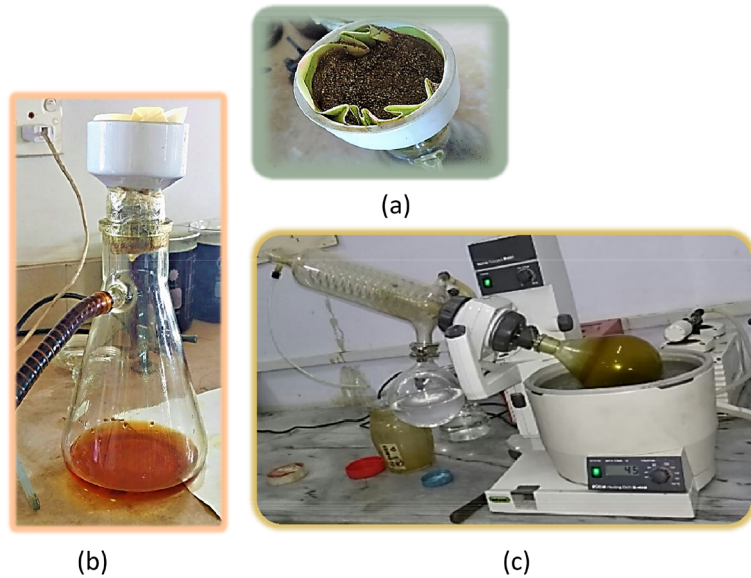


Figure 1. (a): Filtration of soaked pomegranate ethanolic extract by Whatmann number 1, filter paper. (b): Residual pomegranate coarse powder after filtration of its soaked ethanolic extract. (c): Final herb's (darehald/darhald) extraction by vacuum evaporator.

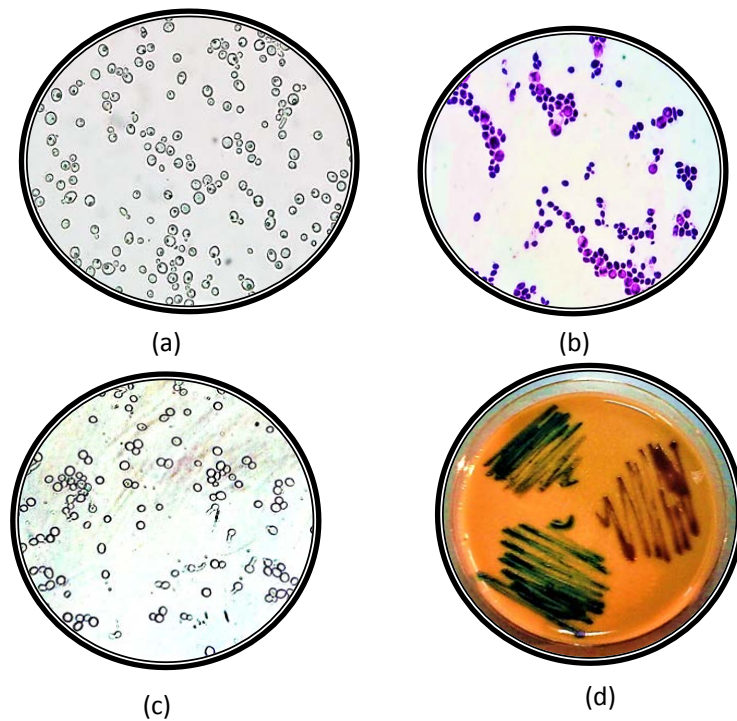


Figure 2. (a): Microscopic view of a candida positive wet mounted slide of a high vaginal swab specimen. (b): Microscopic view of a clinical specimen showing candidal positive on Gram stain. (c): Microscopic view of a clinical specimen showing candida positive germ tube test. (d): Candidal species identification of different candidal positive specimens on CHROMEagar media showing colonies of *C. albicans* (green color) and *C. glabrata* (pink/mergenda color).

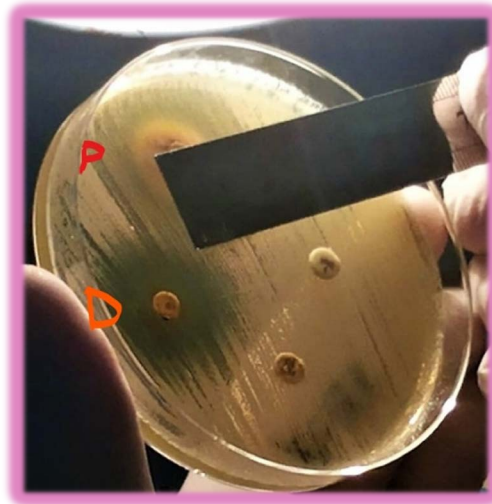


Figure 3. Measuring of inhibitory zones around both herbs (*B. aristata* and *P. granatum*) in azole (fluconazole and voriconazole) resistant strain.

3. Results

In this current study two herbs, *darhald* and *pomegranate* were evaluated for *in-vitro* antifungal activity against candidal isolates to assess their effectiveness in our clinical set ups. The inhibitory zones of azoles (fluconazole and voriconazole), *Berberis aristata* (*B. aristata*, *darhald/darehald*) and *Punica granatum* (*P. granatum*, *pomegranate*) peels' extracts against candidal strains measured in millimeters (mm). The highest mean zone of inhibition (ZIH) calculated on candidal samples was 22.27 ± 4.9 mm of *B. aristata* with statistically significant p-value < 0.05 while the least ZIH was 14.29 ± 12.86 of fluconazole with p-value > 0.05 , shown in **Table 1**. Though according to culture sensitivity pattern of clinical candidal isolates, *B. aristata* was the most sensitive antifungal agent 98.5% and the least sensitive agent was voriconazole 29.2%, displaying in **Table 2**.

The candidal species identified from clinical specimens were *Candida albicans* (*C. albicans*, 104), *Candida tropicalis* (13), *Candida glabrata* (12) and *Candida ciferrii* (1). While the sensitivity of antifungal agents identified for these various candidal species was; *B. aristata* - *C. albicans* 103 (99.03%), NCACS 25 (98.16%), *P. granatum* - *C. albicans* 101 (95.5%), NCACS 26 (100%), fluconazole - *C. albicans* 43 (37.85%), NCACS 16 (62.01%) and voriconazole - *C. albicans* 31 (30.15%), NCACS 13 (51%) as showing in **Figure 4**.

These results displayed *C. tropicalis* as the least sensitive among identified species, showing sensitivity of 30.55% (69.45% resistant) to fluconazole and 5.55% sensitive (94.45% resistant) to voriconazole (**Figure 4**).

4. Discussion

Nowadays herbs are gaining popularity across the globe and are a focus of interest in the field medicine and ethnobotany. Evidence based researches are playing

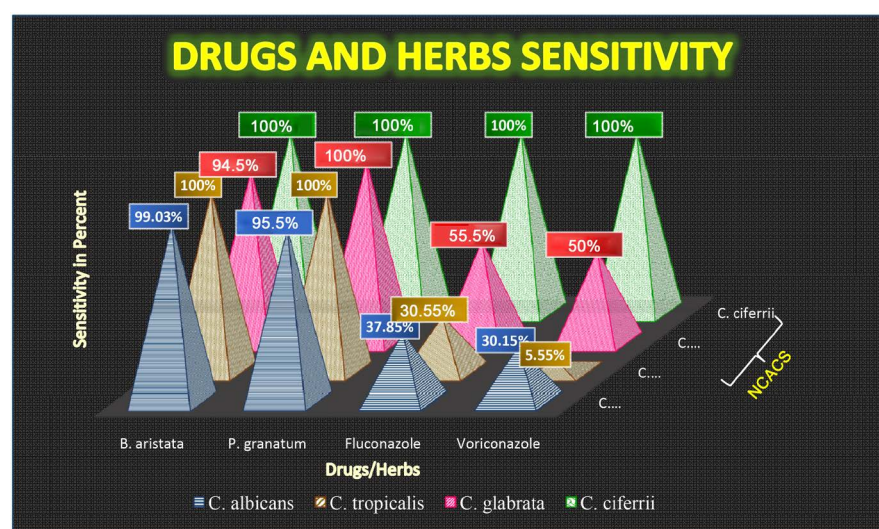
Table 1. Mean zone of inhibitions of herbs and azoles on candidal isolates.

S. No.	Drugs/Herbs (MIC in mcg)	Zone of inhibitions (mm)	P-value
		Mean \pm Std. Deviation	
1	Fluconazole (25)	14.29 \pm 12.86	0.434
2	Voriconazole (1)	16.90 \pm 13.92	0.648
3	<i>Berberis aristata</i> (25)	22.27 \pm 4.93	0.000*
4	<i>Punica granatum</i> (20)	17.48 \pm 4.03	0.020*

P-value calculated by one-way ANOVA followed by Post Hoc Tukey test. *P-value is significant (<0.05).

Table 2. Sensitivity pattern of azole antifungal drugs and herbs against clinical candidal isolates.

	Fluconazole N (%)	Voriconazole N (%)	<i>B. aristata</i> N (%)	<i>P. granatum</i> N (%)
Sensitive	55 (42.3)	38 (29.2)	125 (98.5)	124 (97.7)
Resistant	75 (57.7)	92 (70.8)	5 (3.8)	6 (4.6)

**Figure 4.** Antifungal sensitivity of azoles and herbs among candida albicans and Non-candida albicans (NCACS).

a crucial role in the exploration and development of new therapeutics [42] The limitations of recently available antifungal drugs, their rise in resistance along with recurrence in fungal (candidal) infections have emphasized the researchers to explore novel antifungal therapies with unique mode of actions against pathogens as well as have few adverse effects, inexpensive and easily available [9]. Since their discovery, azoles (fluconazole and voriconazole) are most widely prescribed antifungal drugs around the world and are fungicidal for aspergillus but are fungistatic for candida with subsequent rise in its resistance over the time [43]. Hence in the current study, two herbs were compared with two most commonly used antifungal drugs of class azoles, fluconazole and voriconazole on the basis of their zones of inhibition for various candidal strains isolated in

order to know their effectiveness in our community.

In our study, the most sensitive agent on *in-vitro* culture sensitivity of candidal clinical specimens was *B. aristata* with mean ZIH of 22.27 ± 4.93 mm (**Table 1** and **Table 2**). Similarly, a study conducted on *B. aristata* displayed mean ZIH 20.1 ± 0.15 mm on candida samples [44]. It is well documented that antifungal activity of *B. aristata* is mainly due to its alkaloid constituent known as “*Berberine*” that inhibits fungal cell wall, ergosterol biosynthesis and mitochondrial functions. Interestingly it has been evaluated innocuous to human being cells [45]. According to our results shown in **Table 1**, the second highest mean inhibitory zone (17.48 ± 4.03 mm) against candidal strains was shown by *P. granatum*. This is in accordance to an Indian study in which *pomegranate* displayed 13 ± 1.225 mm of ZIH on candida samples [46]. The antifungal activity of *pomegranate*'s peels (*P. granatum*) is due to its alkaloid content, tannins containing punicalagin, granatins A and B, gallagylidilacton, casuarinin, pedunculagin, tellimagrandin I and corilagin. These constituents cause fungal cell wall alteration, inhibition of candidal cells' habitation, dwelling and growth on host [47] [48]. In our study the sensitivity of both herbs, *B. aristata* and *P. granatum* for candidal isolates were shown to be 98.5% and 97.7% receptively which was greater than both azoles (**Table 2**). To the best of our knowledge our study is innovative as we reported and compared for the first time the sensitivity pattern of the herbs for candidal samples as in the previous studies only mean zone of inhibitions were mentioned, which already have been discussed above. While the mean inhibitory zones of azoles noticed in this current study were 14.29 ± 12.86 mm for fluconazole and 16.90 ± 13.92 mm for voriconazole with 42.3% sensitivity to fluconazole (57.7% resistance), and sensitivity to voriconazole was 29.2% (70.8% resistance). These results are comparable with global surveillance data by Pfaller, 2010 reported sensitivity of 90.2% (6.2% resistant) to fluconazole and 95% (3% resistance) to voriconazole [48]. In the current research resistance pattern of fluconazole among species noted was, *C. tropicalis* 69.45%, *C. glabrata* 11.1% and *C. albicans* 62.15% while for voriconazole the resistance was shown for *C. tropicalis* 94.45%, *C. albicans* 69.85%, *C. glabrata* 50% (**Figure 1**). Our results clearly displayed that among all isolated candidal species the most resistant specie was *C. tropicalis* with respective 69.45% and 94.45% resistance against both fluconazole and voriconazole. Contrasting results were reported by Pfaller where the highest resistance to fluconazole was observed for *C. glabrata*, 15.7% followed by *C. tropicalis* 4.1% and *C. albicans* 1.4% while for voriconazole the resistance shown was *C. glabrata* 10%, *C. tropicalis* 5.4% and *C. albicans* 1.2% [49]. A study conducted in Iran also displayed contradictory results to our study; the most resistant specie to fluconazole was *C. glabrata* 79.1% though similar to our study the most resistant specie was *C. tropicalis* showing 41.6% resistance against voriconazole [50]. We hoped that the results of this current study may benefit clinicians to recommend appropriate antifungal drugs according to the culture and sensitivity patterns of the candidal isolates prevalent in our community. This current study also highlighted the role of commonly available herbs as

alternative treatment for candidal infection even for those showing high level of resistance to azoles.

5. Conclusions

Punica granatum and *berberis aristata* exhibited superior antifungal activity on all cultured samples of candida in comparison to two widely prescribed azoles, fluconazole and voriconazole in our population. To the best of our knowledge the current study is the first one to report sensitivity patterns of the *B. Aristata* and *P. granatum* on candidal isolates. *C. tropicalis* was greatly resistant to both of the azoles but in contrast, our studied herbs, *B. aristata* and *P. granatum* showed remarkable sensitivity for all candidal species including even those species showing high resistance to both of the commonly prescribed azoles, fluconazole and voriconazole.

Furthermore, both herbs are economical, easily available and documented to have better safety profiles with health boosting properties in general. While conventional antifungal drugs have lots of side effects including drug-drug interactions and are not cost-effective. Hence it is highly recommendable that both herbs, *B. aristata* and *P. granatum* can be considered as alternative drugs for the treatment of candidiasis in near future after subsequent clinical trials and further verification of our results.

6. Limitations of the Study

We conducted our study in few tertiary care health centers of only in one city (Karachi) of our country and could not include other cities and rural areas due to a limited budget.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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