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Antifungal resistance and herbal sensitivity of oral *Candida* isolates from HIV-infected patients in a rural community in Western Uttar Pradesh, India

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Drug-resistant *Candida* species in HIV-infected patients are the result of the selective pressure of currently used azoles. In the course of screening for active plant products against drug-resistant *Candida* species, we paid special attention on the effects of commonly available herbal plants. A total of 123 oral *Candida* isolates which were previously isolated from 172 HIV-infected patients were included in this study. *In vitro* antifungal susceptibility to fluconazole (FCZ), itraconazole (ITZ) and amphotericin B (AMB) was evaluated using Clinical and Laboratory Standard Institute (CLSI) guidelines. Antifungal potency of garlic, neem, *Aloe*, *Calendula*, *Citrus*, mint, tea and ginger extracts were tested against drug-resistant isolates. Out of 123 isolates, 26.8% were resistant to FCZ, 21.9% to ITZ and 8.1% to AMB. All drug-resistant isolates tested, were completely inhibited by garlic and neem leaves extracts with minimum fungicidal concentration (MFC) of 0.781 and 1.562 mg/ml, respectively. *Aloe* and *Calendula* extracts were also found to be effective with MFC of 3.125 mg/ml each. The observed growth inhibition zones and minimum inhibitory concentrations (MICs) showed that the isolates exhibited susceptibility. Our results provided scientific justification for the use of garlic, neem, *Aloe* and *Calendula* extracts in health products and herbal remedies against multidrug-resistant candidiasis.

Key words: Oral lesions, antifungal susceptibility, drug resistant, herbal, *Candida albicans*.

INTRODUCTION

Oral candidiasis (OC) is the most frequent human immunodeficiency virus (HIV) infection-associated oral disease and can also act as a marker for immuno-suppression (Moura et al., 2006; Chunchanur et al., 2009; Merçon et al., 2009; Moura et al., 2010; Thompson et al., 2010). Fluconazole (FCZ) is frequently used for the treatment of mucocutaneous candidiasis in patients with AIDS (Vazquez et al., 2001). In the 1990s, there was a significant increase in the prevalence of drug-resistant fungal infections due to *Candida* species in patients hospitalized for mucosal or systemic diseases. The widespread application of FCZ or related azole antifungal is postulated to promote selection of resistant subpopulations by shifting colonization to more naturally resistant species, such as *Candida krusei* or *Candida glabrata.* Alternatively, azole-resistant subspecies have arisen *in vivo* and *in vitro* that shows changes in the target enzyme lanosterol 14-α-demethylase, in expression

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of multidrug efflux pumps, or in both (Mathema et al., 2001). In India, despite the numerous reports of isolation of azole resistant strains of *Candida* species from patients with refractory mucosal candidiasis, only a few longitudinal prospective studies have evaluated the antifungal susceptibility of *Candida* isolates recovered from HIV-infected patients receiving long-term therapy with azole (Gautam and Garg, 2013a). Drug-resistant *C. albicans* and non-*albicans Candida* species (NACs) in immunocompromised patients are the result of the selective pressure of currently used azoles and have been well recognized as a global problem in recent years (Gautam and Garg, 2013a). As plants can produce antimicrobial compounds to protect themselves from biotic attack, the search for antimicrobial agents with different chemical structure and biological mode of action from plant sources is an alternative option in the fight against these microbes (Lai and Roy, 2004). In the course of screening for active plant products against drug-resistant *Candida* species, we paid special attention on the effects of commonly available herbal medicinal plants. Here, we reported the drug-resistant oral *Candida* isolates from HIV-infected patients in a rural community in western Uttar Pradesh, India together with antifungal activity of eight herbal plants extracts against these isolates.

MATERIALS AND METHODS

Isolates

A total of 123 *Candida* isolates, which were previously isolated and identified from HIV-infected patients (Gautam and Garg, 2013b) in the Department of Microbiology, Chaudhary Charan Singh University, Meerut, India were included in this study. Out of 123 isolates, 91 were recovered from patients with oral lesions (Group 1) and 32 were from patients without oral lesions (Group 2). Informed consent obtained from participants and procedures were performed according to institutional board of ethical committee.

Antifungal susceptibility test

The three antifungal agents used for susceptibility testing of *Candida* species isolates were fluconazole (FCZ), itraconazole (ITZ), and amphotericin B (AMB) (Sigma-Aldric, USA). Fluconazole was dissolved in sterile distilled water while ITZ and AMB were dissolved in dimethyl sulfoxide (DMSO) (Hi-Media, India) to make stock solutions. Antifungal susceptibility test using broth microdilution (BMD) method was performed as per Clinical and Laboratory Standard Institute (CLSI) (CLSI M27-A3, 2008). A completely synthetic medium, RPMI 1640 (Hi-Media, Mumbai) supplemented with glutamine but without bicarbonate, was used. Medium was supplemented to final concentration of 20 g/L (2.0%) glucose for better growth of yeast isolates. The range of concentrations tested were 0.125 to 64.0 µg/ml for FCZ and 0.0313 to 16.0 µg/ml for ITZ and AMB. The BMD test was performed using sterile, disposable multiwell microdilution plates (96 U-shaped wells) (Tarson, Mumbai). Aliquots of 100 µl of each antifungal agent at a concentration two times the targeted final concentration were dispensed in the wells of the plates. The suspension of yeasts after 48 h of incubation onto Sabouraud's dextrose agar was prepared in sterile saline, adjusted spectrophotometrically at 530 nm to match the turbidity of a 0.5 McFarland standard and was diluted in RPMI 1640 in order to obtain a final concentration of 0.5×10^3 to 2.5×10^3 CFU/ml (CLSI M27-A3, 2008). A constant volume (100 µl) of the inoculum was aseptically added to each microdilution well containing 100 µl of serial dilution of antifungal agents so as to make final desired concentration. For the members of the azole drug the MIC was defined as the lowest drug concentration that resulted in 80% growth inhibition. For AMB, this value was defined as the value in which 100% growth inhibition was observed (Brito et al., 2010). The isolates were tested in duplicate. The breakpoints used as interpretive guidelines for *in vitro* susceptibility testing of *Candida* species were based on the M 27-A3 Document of the CLSI (CLSI M27-A3, 2008). Because of the lack of consensus about the definition of MIC breakpoints for AMB, arbitrary values suggested by a previous study were used (da Matta et al., 2007). Isolates with MICs ≤ 8 µg/ml for FCZ, ≤ 0.125 µg/ml for ITZ and ≤ 1 µg/ml for AMB were considered susceptible. Isolates with MICs from 16 - 32 µg/ml for FCZ and 0.25 - 0.5 µg/ml for ITZ were considered as susceptible in a dose-dependent manner (SDD). Isolates with MICs ≥ 64 µg/ml for FCZ, ≥ 1 µg/ml for ITZ and ≥ 2 µg/ml for AMB were considered resistant. Quality control strains including *C. albicans* (ATCC 90028) and *C. parapsilosis* (ATCC 22019) were also used. These strains were included with each batch of MICs testing and results were within acceptable ranges.

Collection and processing of herbals

In vitro antifungal activities of eight herbal plants extracts were evaluated. These were garlic (*Allium sativum*), neem (*Azadirachta indica*), aloe (*Aloe vera*), *Calendula* (*Calendula arvensis*), *Citrus* (*Citrus sinensis*), mint (*Mentha spicata*), tea (*Camellia sinensis*) and ginger (*Zingiber officinale*). All were collected from crop fields and local market of Western Uttar Pradesh, India. The plant material was dried under shade and reduced to small pieces; plant material was grounded in electronic grinder to obtain fine powder. Dried herbal powder (100 g) was macerated with 1000 ml ethanol (95%) in 2000 ml Erlenmeyer flask. Flask was closed with aluminium foil and placed on orbital incubator shaker at 130 rpm for 5 days with intermittent agitation and soaking. After 5 days the mixture was filtered using Whatman's filter paper No. 1. Herbal extract was evaporated to semi solid state under vacuum at 40°C using a Buchi Rotavapour. Semi solid extract was dried in the crucible under a controlled temperature of 40°C to obtain solid extract. Prepared extract was stored in dark-coloured container at 4°C until used (Gautam and Garg, 2013a). All herbals were processed in same manner.

Antifungal activity test of herbal extracts

Agar well diffusion method

All extracts were first subjected to an antifungal susceptibility test for their growth inhibition zone diameter (in mm) following the previous agar well diffusion (AWD) method with a slight modification (Gautam and Garg, 2013a). In this method, Mueller-Hinton Agar (MHA) plates were prepared. Each plate was inoculated with test organism of 2.5×10^3 CFU/ml inoculum density using spread plate technique. Three wells at equidistance were cut aseptically with the help of sterile cork borer. Stock solution having 1250 mg/100 ml air dried powder of herbal extracts dissolved in DMSO was prepared. 100 µl of each herbal extract was dispensed into 6 mm precut wells of MHA plates. These plates were incubated at 37°C for 48 h and inhibition zone diameter was measured using Hi Antibiotic Zone Scale-C PW297 (Hi-Media, Mumbai). Data are presented in the form of inhibition zone diameter (mm) which is an average of two

independent replicates.

Minimum inhibitory concentration assay

To measure MICs of active herbal extracts, macro-broth dilution (MBD) method was used with some modifications (Gautam and Garg, 2013a). In this method, 12 sterilized glass tubes with caps were taken. Tube 1 was filled with 2 ml of double-strength culture medium (Sabouraud's Dextrose broth) containing twice the final active herbal extract concentration (6.25 mg/ml). Tubes 2 - 11 were filled with 1 ml of double strength Sabouraud's Dextrose broth. Tube 12 was considered as blank filled with distilled water. 1 ml amount was taken from tube 1 and diluted two-fold by transferring it to tube 2 with a micropipette. 1 ml sample was then removed from tube 2 and transferred to tube 3 and so on to tube 10. The last 1 ml of diluted herbal extract was then discarded. Thus, each tube 1-10 contained 1 ml of double-strength Sabouraud's Dextrose broth containing twice the final herbal extract concentrations. Each tube was inoculated with 1 ml of 5×10^3 CFU/ml yeast suspension which will give the required herbal extract concentration and inoculums density of 2.5×10^3 CFU/ml. The growth control tube (tube 11), which contained 1 ml of sterile drug-free medium, was also inoculated with 1 ml of the same inoculum suspension. Tube 12 was filled with 2 ml of sterile distilled water, from the lot used to prepare the inoculum as a sterility control for media. Final volume of each tube was 2 ml. All tubes were incubated for 24 to 48 h at 37°C after which the turbidity was observed and measured with the spectrophotometer. On the basis of optical density, the tube showing critical inhibitory points were selected and 100 µl samples from each MIC assay tube with growth inhibition was spread onto Sabouraud's Dextrose Agar (SDA) plate. Plates were then incubated at 37°C for 48 h to count number of colony forming units (CFU). CFU/ml was determined for each selected well along with control well. Data are presented in the form of MIC_{50}, MIC_{80} and minimum fungicidal concentration (MFC). The MIC_{50} was considered the lowest tested concentration with a 50% reduction in growth as compared to growth of positive control. The MIC_{80} was considered the lowest tested concentration with 80% reduction in growth as compared to growth of positive control. The highest dilution of the plant extract that retained its inhibitory effect resulting in no growth (absence of turbidity) of a microorganism is recorded as the MFC value of the extract.

Phytochemical constituents of plant extracts

Herbal extracts were subjected to qualitative chemical tests for identification of various categories of organic chemical constituents such as alkaloids, tannins, steroids, flavonoids, saponins, glycosides, terpenoids, carbohydrates, antiquinones and phenols. The phytochemical analysis was done according to standard methods (Roopashree et al., 2008; Obasi et al., 2010; Raphael, 2012). Briefly the methods are given below.

Test for alkaloids

Extracts were dissolved individually in dilute hydrochloric acid and filtered.

a) Mayer's test: Filtrates were treated with Mayer's reagent (potassium mercuric iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.
b) Wagner's test: Filtrates were treated with Wagner's reagent (iodine in potassium iodide). Formation of brown/reddish precipitate indicates the presence of alkaloids.

Test for carbohydrates

Extracts were dissolved individually in 5 ml distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

a) Molisch's test: Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube. Formation of the violet ring at the junction indicates the presence of carbohydrates.
b) Benedict's test: Filtrates were treated with Benedict's reagent and heated gently. Orange red precipitate indicates the presence of reducing sugars.
c) Fehling's test: Filtrates were hydrolysed with diluted HCl, neutralized with alkali and heated with Fehling's A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

Test for glycosides

Extracts were hydrolysed with dilute HCl and then subjected to test for glycosides.

a) Modified Borntrager's test: Extracts were treated with ferric chloride solution and immersed in boiling water for about 5 min. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.
b) Legal's test: Extracts were treated with sodium nitropruside in pyridine and sodium hydroxide. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

Test for saponins

a) Froth test: Extracts were diluted with distilled water to 20 ml and this was shaken in a graduated cylinder for 15 min. Formation of 1 cm layer of foam indicates the presence of saponins.
b) Foam Test: 0.5 g of extract was shaken with 2 ml of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Test for phenols

Ferric chloride test: Extracts were treated with 3-4 drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Test for tannins

Gelatin test: To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

Test for Steroids

2 ml of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 ml H_2SO_4. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

Test for flavonoids

a) Alkaline reagent test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which

Table 1. Yeast distribution in different groups of the study population.

Species	Group 1* (n=91)	Group 2† (n=32)	Test group (n=123)
C. albicans	55 (60.4)	20 (62.5)	75 (60.9)
C. tropicalis	9 (9.9)	3 (9.4)	12 (9.8)
C. glabrata	6 (6.6)	2 (6.3)	8 (6.5)
C. parapsilosis	5 (5.5)	0	5 (4.1)
C. albicans + *C. tropicalis*	2 (2.2)	0	2 (1.6)
C. albicans +*C. krusei*	2 (2.2)	2 (6.3)	4 (3.3)
C. albicans + *C. dubliniensis*	3 (3.3)	0	3 (2.4)
C. famata	1 (1.1)	1 (3.1)	2 (1.6)
C. guilliermondii	1 (1.1)	2 (6.3)	3 (2.4)
Non *albicans Candida*	29 (31.9)	10 (31.3)	39 (31.7)

Data are shown in no. (%) of *Candida* spp. isolates; *, HIV-infected patients with oral lesions; †, HIV-infected patients without clinical signals of oral lesions.

becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

b) Lead acetate test: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

Test for terpenoids

a) Copper acetate test: Extracts were dissolved in water and treated with 3-4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

a) Salkowski's test: Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of concentrated sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour indicates the presence of triterpenes.

Statistical analysis

All the data were analysed in the worksheet of Statistical Package for Social Science (SPSS) software (Version 16.0) and analyzed accordingly.

RESULTS

A total of 123 *Candida* isolates, which were previously isolated and identified from HIV-infected patients (Gautam and Garg, 2013b) were included in this study. Out of 123 isolates, 91 were recovered from patients with oral lesions (Group 1) and 32 were from patients without oral lesions (Group 2). *C. albicans* was the most common isolate from both groups. The frequency of isolation of *Candida* isolates in Group 1 was: *Candida albicans* 68.1%. *Candida tropicalis* 12.1%, *Candida glabrata* 6.6%, *Candida parapsilosis* 5.5%, *Candida krusei* 2.2%, *Candida dubliniensis* 3.3%, *Candida famata* 1.1% and *Candida guilliermondii* 1.1%, while in Group 2 was: *C. albicans* 68.8%, *C. tropicalis* 9.4%, *C. glabrata* 6.3%, *C. krusei* 6.3%, *C. famata* 3.1% and *C. guilliermondii* 6.3% (Table 1).

Antifungal susceptibility testing

In vitro antifungal susceptibility results of 123 isolates are summarized in Table 2. The determined MIC ranges for Group 1 isolates were 0.125 - 128, 0.031 - 4.0 and 0.031 - 2.0 µg/ml for FCZ, ITZ and AMB, respectively. The determined MIC ranges for Group 2 isolates were 0.125-64, 0.031-1.0 and 0.031-1.0 µg/ml for FCZ, ITZ and AMB, respectively. Group 1 isolates demonstrated very high FCZ MIC_{50} in which two isolates of *C. glabrata* presented values of 128 µg/ml followed by ITZ MIC_{50} in which same isolates presented values of 4 µg/ml. Out of 123 isolates, 77 (62.6%) were susceptible to FCZ, 80 (65.1%) to ITZ and 113 (91.9%) to AMB. Only 10.6 and 17.3% of all isolates were dose dependent to FCZ and ITZ,, respectively. Among all isolates, 33 (26.8%) were resistant to FCZ, 27 (21.9%) to ITZ and 10 (8.1%) to AMB. Out of 84 *C. albicans* isolates, 25.0% were resistant to FCZ, 20.2% to ITZ and 5.9% to AMB while among 39 NAC isolates, FCZ resistance accounted for 30.8%, ITZ for 25.6% and AMB for 12.8%.

Herbal activity against drug-resistant isolates

In vitro antifungal activity results of herbal plants extracts are summarized in Table 3. Using AWD method the highest growth inhibition zone diameter was recorded as 26±3.7 mm (mean) with garlic extract against all drug-resistant isolates, followed by neem extract which demonstrated 24.0±2.9 mm. *Aloe* and *Calendula* extracts demonstrated 18.2±3.5 and 17.5±3.2 mm, respectively. *Citrus* peel, Mint leaves and ginger extracts demonstrated inhibition zone diameters of 15.8±4.1, 13.3±2.9 and 13.6±1.6 mm, respectively, while tea leaves extract demonstrated only 11.1±2.2 mm.

Using MBD method, the lowest MIC_{50} value was recorded as 0.097 mg/ml with garlic and neem extracts each against all isolates tested while *Aloe* and *Calendula* extracts demonstrated MIC_{50} value of 0.390 mg/ml each. The isolates demonstrated low garlic extract, MIC_{50} in which 66.7% (22) presented values of 0.048 mg/ml. *Citrus* peel extract demonstrated MIC_{50} value of 0.390 mg/ml against all drug-resistant isolates while mint leaves and ginger extracts were comparatively less effective with MIC_{50} value of 3.125 mg/ml each. Tea leaves extract was effective only to inhibit 37.23% growth of drug-resistant isolates. Garlic and neem leaves extracts completely inhibit the growth of all drug-resistant isolates with MFC value of 0.390 and 0.781 mg/ml respectively, while 72.7%(24) isolates demonstrated low neem extract's MFC value of 0.390 mg/ml. *Aloe* leaves and *Calendula* extracts demonstrated MFC value of 1.562 mg/ml each. *Citrus* peel, mint leaves and ginger extracts were unable to inhibit complete growth of any of the drug-resistant

Table 2. Antifungal susceptibility of *Candida* spp. isolates using CLSI testing.

Test isolate	FCZ			ITZ			AMB*	
	S	SDD	R	S	SDD	R	S	R
Group 1 isolates								
C. albicans (62)	36 (58.1)	9 (14.5)	17 (27.4)	37 (59.7)	11 (17.7)	14 (22.6)	57 (91.9)	5 (8.1)
C. tropicalis (11)	7 (63.6)	1 (9.1)	3 (27.3)	8 (72.7)	1 (9.1)	2 (18.2)	11 (100)	0
C. glabrata (6)	2 (33.3)	0	4 (67.7)	2 (33.3)	0	4 (67.7)	3 (50.0)	3 (50.0)
C. parapsilosis (5)	5 (100)	0	0	5 (100)	0	0	5 (100)	0
C. krusei (2)	0	0	2 (100)	0	0	2 (100)	1 (50.0)	1(50.0)
C. dubliniensis (3)	3 (100)	0	0	3 (100)	0	0	3 (100)	0
C. famata (1)	1 (100)	0	0	1 (100)	0	0	1 (100)	0
C. guilliermondii (1)	1 (100)	0	0	1 (100)	0	0	1 (100)	0
Group 2 isolates								
C. albicans (22)	15 (68.2)	3 (13.6)	4 (18.2)	15 (68.2)	4 (18.2)	3 (13.6)	22 (90.9)	0
C. tropicalis (3)	3 (100)	0	0	3 (100)	0	0	3 (100)	0
C. glabrata (2)	1 (50.0)	0	1 (50.0)	1 (50.0)	0	1 (50.0)	2 (100)	0
C. krusei (2)	0	0	2 (100)	1(50.0)	0	1(50.0)	1(50.0)	1(50.0)
C. famata (1)	1 (100)	0	0	1 (100)	0	0	1 (100)	0
C. guilliermondii (2)	2 (100)	0	0	2 (100)	0	0	2 (100)	0
All isolates (123)	77 (62.6)	13 (10.6)	33 (26.8)	80 (65.1)	16 (17.3)	27 (21.9)	113 (91.9)	10 (8.1)

Data are shown in number (%) of *Candida* isolates; FCZ, fluconazole; ITZ, itraconazole; AMB, amphotericin B; S, susceptible; SDD, susceptible to dose dependent; R, resistant;*, no endpoint defined by CLSI.

Table 3. Growth inhibition zone diameters and MICs of the herbal extracts against 33 drug-resistant oral *Candida* isolates.

Herbal	Family	Part	GIZD* (mm)	MIC_{50} (mg/ml)	MIC_{80} (mg/ml)	MFC (mg/ml)
Garlic (*Allium sativum*)	Amaryllidaceae	Bulb	28.1 ± 3.7	0.097	0.195	0.390
Neem (*Azadirachta indica*)	Meliaceae	Leaves	24.0 ± 2.9	0.097	0.195	0.781
Aloe (*Aloe vera*)	Xanthorrhoeaceae	Leaves	18.2 ± 3.5	0.390	0.781	1.562
Calendula (*Calendula arvensis*)	Asteraceae	Petals	17.5 ± 3.2	0.390	0.781	1.562
Citrus (*Citrus sinensis*)	Rutaceae	Peel	15.8 ± 4.1	0.390	0.781	Nd
Mint (*Mentha spicata*)	Lamiaceae	Leaves	13.3 ± 2.9	3.125	Nd	Nd
Tea (*Camellia sinensis*)	Theaceae	Leaves	11.1 ± 2.2	Nd	Nd	Nd
Ginger (*Zingiber officinale*)	Zingiberaceae	Rhizome	13.6 ± 1.6	3.125	Nd	Nd

*Growth inhibition zone diameter; MIC_{50}, minimum inhibitory concentration inhibiting 50% growth; MIC_{80}, minimum inhibitory concentration inhibiting 80% growth; MFC, minimum fungicidal concentration; Nd, not detected.

isolates resulting to, no MFC value. Figure 1 summarizes the comparative antifungal activity analysis of all herbal extracts tested against 33 drug-resistant oral *Candida* isolates.

Qualitative assay of herbal extracts for phytochemical constituents

Phytochemical assay results indicated the presence of carbohydrates (reducing sugars, hexose sugars, non-reducing polysaccharides gums and mucilages), alkaloids and flavonoids as the main constituents in herbals tested (Table 4).

DISCUSSION

A variety of antifungal agents are now available for the treatment of *Candida* infections. However, worldwide reports indicated that pathogenic isolates of *C. albicans* have relatively high potentials for developing resistance

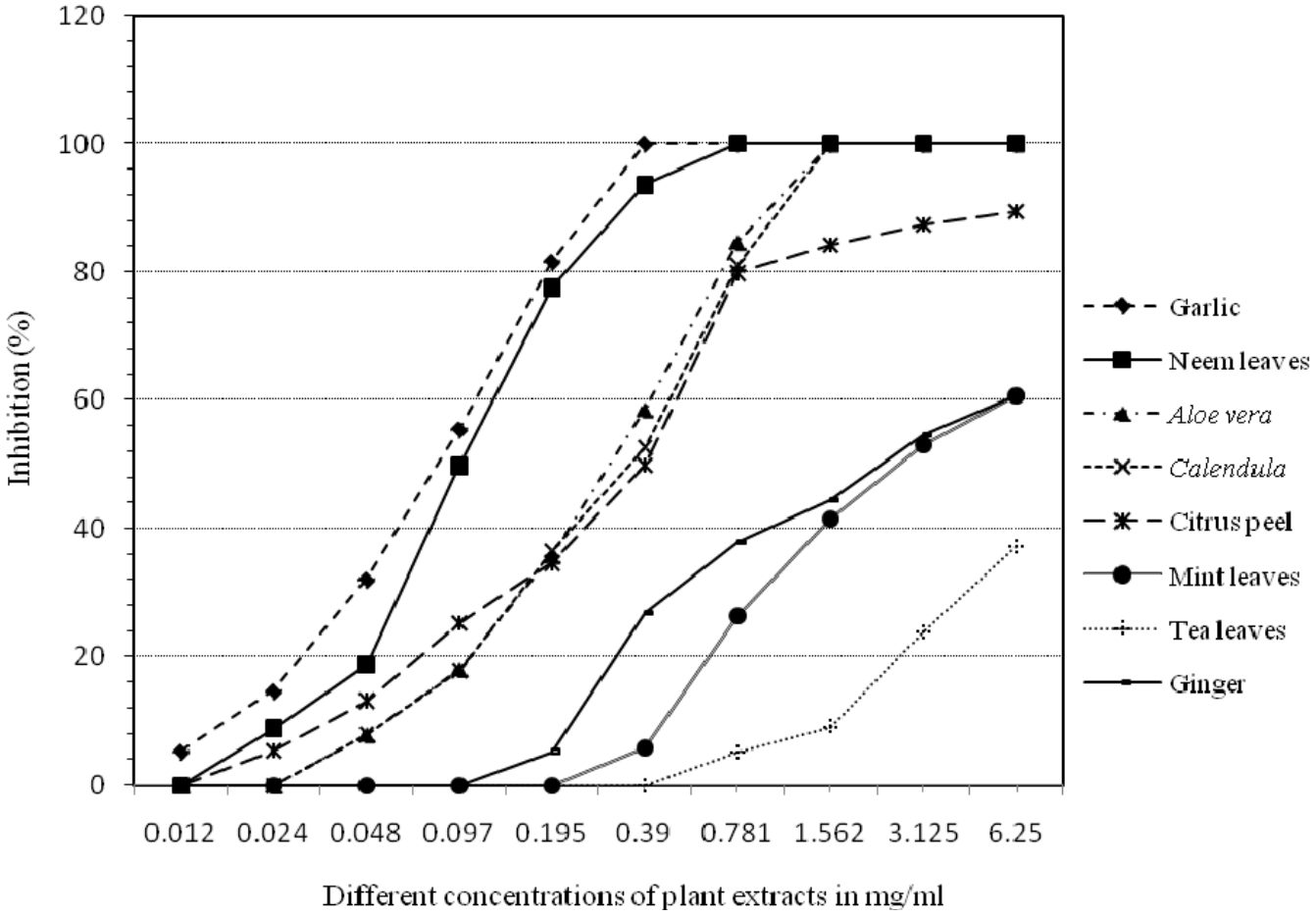

Figure 1. Comparative analysis of herbal extracts activity against drug-resistant oral *Candida* isolates.

Table 4. Phytochemical constituents of plant extracts.

Variable tested	Garlic	Neem leaves	Aloe leaves	Calendula flower	Citrus peel	Mint leaves	Tea leaves	Ginger
Alkaloids	+	+	+	+	-	+	-	-
Tannins	-	-	+	-	+	+	+	-
Steroids	+	+	-	-	+	+	-	-
Flavonoids	+	+	+	+	+	+	+	+
Saponins	-	-	-	+	+	-	-	-
Glycosides	-	+	-	-	+	-	-	-
Terpenoids	+	+	+	-		+	-	+
Carbohydrates	+	+	+	-	+	-	-	-
Phenols	-	-	-	-	+	-	-	+

(+) Indicates present; (-) indicates absent.

(Espinel-Ingroff et al., 1998). Relatively, high resistance of *C. albicans* and non-*albicans Candida* to common antifungal agents tested was observed in the present study. Although triazole agents appear to be highly effective initially, the increase of resistance to them has been reported (Ellepola and Samaranayake, 2000; Pfaller and Diekema, 2007).

Antifungal resistance

Two different populations of individuals were studied, HIV-infected patients with oral lesions (symptomatic) and without oral lesions (asymptomatic). Isolates from symptomatic patients showed higher MIC range in comparison with isolates from asymptomatic patients. All

these isolates were recovered from patients who had previously received same antifungal agent. This feature could be due to a secondary resistance produced by exposure to the drug. In past few decades, there have been numerous reports of *Candida* infections in India (Basu et al., 2003; Gugnani et al., 2003; Bharathi and Rani, 2011; Jain et al., 2011; Gautam and Garg, 2013b, c). Several studies have also reported FCZ resistance in *C. albicans* strains isolated from HIV-infected patients with oral candidiasis (Mane et al., 2010).

In the present study, 21 of 33 FCZ resistant, 17 of 27 ITZ resistant and 5 of 10 AMB resistant isolates were *C. albicans* while remainders were NACs isolates. NACs isolates showed higher resistance as compared to *C. albicans*.

Among 33 drug-resistant isolates, 80.8% (27) isolates (seventeen *C. albicans*, two *C. tropicalis,* five *C. glabrata* and three *C. krusei*) presented resistance to both azole tested. These findings suggest the possibility of a cross-resistance phenomenon between these antifungal agents. Active flux and alteration in drug target are considered to be the most important mechanisms of azole resistance.

Although the breakpoint concentrations for AMB are not clearly defined, a strain is considered to be resistant when it represents an MIC ≥ 2 µg/ml (Luque et al., 2008). According to this criterion, only 10 isolates were found resistant to AMB and this feature is difficult to analyse from a clinical point of view. These isolates were recovered from patient, who had been previously treated with FCZ. These results agree with previous reports on the existence of strains resistant to AMB which had previously been in contact with azoles. The azoles affect the cellular membrane and this could be a factor in a selection of mutants (Carrillo-Muñoz et al., 1997). Most of the resistant isolates to AMB were recovered from immunocompromised individuals and this could be related to effects on cellular membrane of yeast, in relation to the multiple treatments received for these patients (Luque et al., 2008).

Herbal sensitivity

In this study, eight herbal extracts preparations were tested against drug-resistant oral *Candida* isolates. In the present study, garlic and neem leaves extracts exhibited highest anticandidal activity against all the drug-resistant isolates tested with MFC value of 0.390 and 0.781 mg/ml, respectively. Three *C. glabrata* isolates which were resistant to all the three drugs tested were completely inhibited with garlic and neem extracts with MFC value of 0.390 and 0.781 mg/ml, respectively. It has been proven over and over that garlic is an effective anti-fungal agent. Its compounds are very active against *C. albicans* and NACs isolates (Jafari et al., 2007; Bokaeian et al., 2010). In this study, *A. vera* and *Calendula* extracts were also found effective. In the present study, we confirm the inhibitory effect of Garlic, Neem, *Aloe* and *Calendula* extracts against drug-resistant *Candida* spp. isolateswhich has been described before by Agarry et al. (2005), George et al. (2009), Mahmoud et al. (2011), Doddanna et al. (2013) and Padhye et al. (2013). The observed growth inhibition zones and MICs showed that the isolates exhibited susceptibility. This indicates that garlic, neem, *Aloe* and *Calendula* extracts have broad spectrum of antifungal activity. The MFCs of garlic and neem extract reported in this study are in consonance with previous anticandidal studies on pathogenic and emerging drug-resistant *Candida* species such as *C. albicans*, *C. glabrata* and *C. krusei* (Gautam and Garg, 2013a). Although, *Citrus* peel extract was unable to inhibit the complete growth of any of the isolate tested but its MIC_{50} and MIC_{80} value of 0.390 and 3.125 mg/ml, respectively and intermediate growth inhibition zone diameter showed its anticandidal efficacy against drug-resistant isolates. Mint leaves extract were found comparatively less effective (Doddanna et al., 2013). Tea leaves extract was unable to demonstrate signification reduction in growth of *Candida* isolates as previously reported by Doddanna et al. (2013).

The herbal extracts offer several advantages such as unlimited availability and possibility of minimal problem of drug resistance. The present study showed the presence of tannins, alkaloids and flavanoids as main constituents of the ethanolic herbal extracts. A correlative relationship has been reported between the phytochemicals such as tannins and flavonoids and the free radical scavenging activity and antimicrobial activity (Kaur et al., 2010). Tannins and flavanoids have therapeutic uses due to their anti-inflammatory, anti-fungal, antioxidant and healing properties (Thiago et al., 2008). An added advantage of using herbals to treat yeast infections is that no clinical strains of *C. albicans* have been known to be resistant to herbal therapy.

Conclusion

The high percentage of antifungal resistant against *Candida* isolates in the present study could be due to the fact that many of these patients had received previous treatments with these drugs. Therefore, azole prophylaxis should be considered only in special cases. Our study, suggest to avoid indiscriminate use of antifungal agents, which may ultimately decrease the incidence of candidiasis caused by resistant and non-*albicans Candida* species. The results of this study provided scientific justification for the use of neem, garlic, *Aloe* and *Calendula* extracts in health products and herbal remedies against multidrug-resistant candidiasis. Therefore, complementary and alternative medicine practices with herbal extract including neem, garlic, *Aloe* and *Calendula* as a means of decreasing the burden of

drug resistance and reducing the cost of management of diseases would be of clinical and public health importance in any country.

Conflict of interests

The author(s) have not declared any conflict of interests.

REFERENCES

Agarry OO, Olaleye MT, Bello-Michael CO (2005). Comparative antimicrobial activities of *Aloe vera*gel and leaf. Afr. J. Biotechnol. 4 (12):1413-1414

Basu S, Gugnani, HC, Joshi S, Gupta N (2003). Distribution of *Candida* species in different clinical sources in Delhi, India and proteinase and phospholipase activity of *Candida albicans* isolates. Rev. Iberoam. Micol. 20(4):137-140.

Bharathi M, Rani AU (2011). Pathogenic fungal isolates in sputum of HIV positive patients. J. AIDS and HIV Res. 3:107-113.

Bokaeian M, Nakhaee A, Moodi B, Farhangi A, Akbarzadeh A (2010). Effects of garlic extract treatment in normal and streptozotocin diabetic rats infected with *Candida albicans*. In. J. Clin. Biochem. 25 (2):182-187.

Brito GNB, Inocêncio, AC, Querido, SMR, Jorge, AOC, Koga-Ito, CY (2010). *In vitro* antifungal susceptibility of *Candida* spp. oral isolates from HIV-positive patients and control individuals. Braz. Oral. Res. 25(1):28-33.

Carrillo-Muñoz AJ, Tur C, Estivill D, Montsant L, Carceller A, Hernández-Molina JM (1997). Resistencia in vitro al fluconazol e itraconazol en aislamientos clínicos de *Candida* sp. y *Cryptococcus neoformans*. Rev. Iberoam. Micol. 14:50-54.

Chunchanur SK, Nadgir SD, Halesh LH, Patil BS, Kausar Y, Chandrasekhar MR (2009). Detection and antifungal susceptibility testing of oral *Candida dubliniensis* from human immunodeficiency virus-infected patients. In. J. Pathol. Microbiol. 52:501-504.

Clinical and Laboratory Standard Institute (2008). Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard- Third Edition M27-A3. National Committee for Clinical Laboratory Standards, Wayne, Pennsylvania, USA.

da Matta DA, de Almeida LP, Machado AM, Azevedo AC, Kusano EJU, Travassos NF (2007). Antifungal susceptibility of 1000 *Candida* bloodstream isolates to antifungal drugs: results of a multicenter study conducted in Sao Paulo, Brazil, 1995-2003. Diagn. Microbiol. Infect. Dis. 57:399-404.

Doddanna SJ, Patel S, Sundarrao MA, Veerabhadrappa RS (2013). Antimicrobial activity of plant extracts on *Candida albicans*: An *in vitro* study. In. J. Dent. Res. 24(4):401-405

Ellepola AN, Samaranayake LP (2000). Antimycotic agents in oral candidiasis: An overview: Treatment of oral candidosis. Dent. Update. 27:165-170.

Espinel-Ingroff A, Brachiesi F, Hazen KC, Martinez-Suarez JV, Scalise G (1998). Standardization of antifungal susceptibility testing and clinical relevance. Med. Mycol. 36:68-78.

Gautam RK, Garg AP (2013a). Drug resistance and antifungal activity of garlic and neem extracts on *Candida* spp. isolates from HIV-infected patients. Int. J. Adv. Pharm. Res. 4(2):1426-1433.

Gautam RK, Garg AP (2013b). Evaluation of risk factors and prevalence of oral candidiasis in HIV-infected patients with and without oral lesions in Western Uttar Pradesh, India. J. Infec. Dis. Photon. 112:168-175.

Gautam RK, Garg AP (2013c). Microbiological and clinical assessment of *Candida* carriage in different clinical samples from HIV-infected and non infected patients. Sch. J. App. Med. Sc. 1(2):69-75.

George D, Bhat SS, Antony B (2009). Comparative evaluation of the antimicrobial efficacy of *Aloe vera* tooth gel and two popular commercial toothpastes: an *in vitro* study. Gen. Dent. 57(3):238-241

Gugnani HC, Becker K, Fegeler W, Basu S, Chattopadhya D, Baveja U (2003). Oropharyngeal carriage of *Candida* species in HIV-infected patients in India. Mycoses. 46:299-306.

Jafari AA, Bafghi AF, Anvari MH, Zarrinfar H (2007). *In vitro* synergism effect of an aqueous garlic extracts against six commonly clinical isolated *Candida* species. Iranian J. Pub. Health. 36(1):1-2.

Jain PA, Kulkarni RD, Ajantha GS, Shubhada C (2011). A comparative evaluation of oral *Candida* carriage in HIV-infected individuals and HIV seronegative healthy individuals in north Karnataka. J. Biosci. Tech. 2:232-237.

Kaur J, Xavier R, Marimuthu, Leng KM, Rajasekaran A, Kathiresan S, Sreeramanan S (2010). Preliminary investigation on the antibacterial activity of mango (*Mangifera indica* L: Anacardiaceae) seed kernel. Asian Pacific. J. Trop. Med. 3(9):707-710.

Lai PK, Roy J (2004). Antimicrobial and chemopreventive properties of herbs and spices. Curr. Med. Chem. 11:1451-1460.

Luque AG, Biasoli MS, Tosello ME, Binolfi A, Lupo S, Magaro HM (2008). Oral yeast carriage in HIV-infected and non-infected populations in Rosario, Argentina. Mycoses. 52:53-59.

Mahmoud DA, Hassanein NM, Youssef KA, AbouZeid MA (2011). Antifungal activity of different neem leaf extracts and the nimonol against some important human pathogens. Braz. J. Microbiol. (2011) 42:1007-1016

Mane A, Panchvalli S, Bembalkar S, Risbud A (2010). Species distribution and antifungal susceptibility of oral *Candida* colonizing or infecting HIV infected individuals. Letter to Editor, In. J. Med. Res. pp 1.

Mathema B, Cross E, Dun E, Park S, Bedell J, Slade B (2001). Prevalence of vaginal colonization by drug-resistant *Candida* species in college-age women with previous exposure to over-the-counter azole antifungals. Clin. Infect. Dis. 33:23-27.

Merçon M, Tuboi SH, Batista SM, Telles SR, Grangeiro JR, Zajdenverg R (2009). Risk-based assessment does not distinguish between recent and chronic HIV-1 infection in Rio de Janeiro, Brazil. Braz. J. Infect. Dis. 13:272-275.

Moura MDG, Grossman SMC, Fonseca LMS, Ramos-Jorge ML, Mesquita RA (2010). Risk factors for oral candidiasis in Brazilian HIV-infected adult patients. Braz. J. Oral. Sci. 9(4):470-474.

Moura MDG, Grossmann SMC, Fonseca LMS, Senna MIB, Mesquita RA (2006). Risk factors for oral hairy leukoplakia in HIV-infected adults of Brazil. J. Oral Pathol. Med. 35:321-326.

Obasi NL, Egbuonu ACC, Ukoha PO, Ejikeme PM (2010). Comparative phytochemical and antimicrobial screening of some solvent extracts of *Samanea saman* pods. Afr. J. Pure and Appl. Chem. 4(9):206-212.

Padhye S, Rai S, Narula N, Dekate S (2013). Screening of antifungal activity of some essential oils and antifungals on clinical fungal isolates from diabetic patients Res. J. Pharm. Biol. Chem. Sc. 4(2): 16-21.

Pfaller MA, Diekema DJ (2007). Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. 20:133-163.

Raphael E (2012). Phytochemical constituents of some leaves extract of *Aloe vera* and *Azadirachta indica* plant species. Glob. Adv. Res. J. Env. Sc. Toxicol. 1(2):14-17.

Roopashree TS, Dang R, Rani SRH, Narendra C (2008). Antibacterial activity of anti-psoriatic herbs: *Cassia tora*, *Momordica charantia* and *Calendula officinalis*. Int. J. App. Res. Natur. Prod. 1(3):20-28.

Thiago ASA, Nelson LA, Elba LCA, Ulysses PA (2008). A new approach to study medicinal plants with tannins and flavanoids contents from the local knowledge. J. Ethnopharmacol. 120:72-80.

Thompson GR 3rd, Patel PK, Kirkpatrick WR, Westbrook SD, Berg D, Erlandsen J (2010). Oropharyngeal candidiasis in the era of antiretroviral therapy. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 109(4):488-495.

Vazquez JA, Peng G, Sobel JD, Steele-Moore L, Schuman P, Holloway W (2001). Evolution of antifungal susceptibility among *Candida* species isolates recovered from human immunodeficiency virus-infected women receiving fluconazole prophylaxis. Clin. Infect. Dis. 33:1069-1075.