Antagonistic Properties of Methanol Extract of *Elaeocarpus sphaericus* Leaves against *in vitro* Growth, Germ Tube Formation, Adhesion and Biofilm of *Candida* Species

P. GUPTA, A. RASTOGI, S. JOSHI, A. BHARDWAJ[1], K. MISRA[1] AND N. KUMAR*

Department of Biotechnology, Graphic Era (Deemed To Be University), 566/6, Bell Road, Clement Town, Dehradun-248 002, [1]Department of Biochemical Sciences, Defence Institute of Physiology and Allied Sciences, Lucknow Road, Timarpur, Delhi-110 054, India

Gupta *et al*.: Anticandida properties of *Elaeocarpus sphaericus* Roxb

The extracts of *Elaeocarpus sphaericus* (Roxb.) have shown antimicrobial activities against many pathogenic microbes including fungi. However, the effects of *Elaeocarpus sphaericus* have not been investigated against key virulence attributes of *Candida*. In the present study, methanol extract of *Elaeocarpus sphaericus* leaves was analysed against planktonic cells, hyphal growth, adhesion and biofilm formation of two most important fungal pathogens, *Candida albicans* and *Candida glabrata*. Methanol extract of *Elaeocarpus sphaericus* leaves was prepared by pressurized liquid extraction method. Inhibitory concentration of methanol extract of *Elaeocarpus sphaericus* leaves for both pathogens was determined by broth microdilution assay. Germ tube formation assay was performed to study the effect of the extract on hyphal transition. Effect of methanol extract of *Elaeocarpus sphaericus* leaves on adhesion and biofilm formation was investigated in microtiter

plate using 2,3-bis(2-methoxy-4-nitro-sulfophenyl)-2H-tetrazolium-5-carboxanilide reduction assay. IC_{50} values of the extract for *Candida albicans* and *Candida glabrata* were found between 0.625-1.25 mg/ml. Serum-induced germ tube development in *Candida albicans* was inhibited in presence of 5 mg/ml methanol extract. The extract remarkably inhibited *in vitro* adhesion and biofilm formation by *Candida albicans* and *Candida glabrata* in microtiter plates. The results of this study showed anticandida potential of methanol extract of *Elaeocarpus sphaericus* leaves.

Key words: *Elaeocarpus sphaericus*, *Candida albicans*, *Candida glabrata*, biofilm, adhesion, germ tube, XTT assay, broth microdilution

Candida is an opportunistic, commensal, human fungal pathogen, which causes infections ranging from superficial mucosal to deep invasive including systemic candidiasis, in immune-compromised patients[1,2]. *Candida albicans* and *C. glabrata* are two most common pathogenic species found in oral cavity, gastrointestinal tract and reproductive system of healthy humans[3]. Instances of *Candida* pathogenicity and virulence have been increased in the last two decades[4]. The trend of *Candida* caused infection is now shifting from *albicans* to non-*albicans Candida* species worldwide[5]. According to the report of Center for Disease Control, USA on antibiotic resistance threats in the United States (2013), *Candida* is developing resistance to available antibiotics and rate of occurrence of *C. glabrata* in clinical isolates is increasing alarmingly[6]. The ability of hyphal transition, adhesion and biofilm formation over biotic and abiotic surfaces (including various prosthetic, indwelling devices) contributes to the virulence and pathogenicity of *Candida*[7,8]. Reports on increasing resistance to existing drugs in the clinical isolates of the *Candida* and toxicity of the existing drugs have justified the need of a natural product with antifungal properties[9]. Plants have always been an important source of medicines for human beings since time immemorial[10,11]. Plant-derived products are deriving the interest of researchers towards their use in development of new generation drugs[12,13]. Hundreds of plant extracts have been reported so far to have anticandida properties[14]. *Elaeocarpus sphaericus* is a well-known plant with medicinal value from thousands of years in the Ayurveda, Unani and Siddha. Earlier, antimicrobial properties of the leaves of *E. sphaericus* have been investigated[15,16]. Few plant extracts have been reported to be effective against *Candida* biofilm also[17-19].

In the present study, the effect of methanol extract of *E. sphaericus* leaves prepared through pressurized liquid extraction was analysed against key virulence attributes of *C. albicans* and *C. glabrata* such as *in vitro* growth, germ tube formation, adhesion and biofilm formation.

C. albicans (strain SC5314) and *C. glabrata* (strains MTCC 3019) were obtained from the Institute of Microbial Technology (CSIR), Chandigarh, India. Strains were maintained on yeast-peptone-dextrose (YPD) agar plates and incubated at 37°. After 24 h, colonies were picked from the surface of agar plate and inoculated into YPD broth. YPD was prepared by dissolving individual components (yeast extract 1 %, agar 2 %, peptone 2 % and dextrose 2 %; procured from HiMedia) in distilled water. Biofilm and adhesion assays were performed in yeast nitrogen base (YNB) medium (0.67 % YNB w/o amino acids with ammonium sulphate supplemented with 2 % dextrose, pH 7.0; procured from Becton Dickinson) on the surface of pre-sterilized, polystyrene, flat bottomed 96-well microtiter plates (Make-HiMedia). Purpurine, amphotericin B (amp B), menadione and 2,3-bis (2-methoxy-4-nitro-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) were purchased from HiMedia.

The leaves of *E. sphaericus* Roxb. were collected from Dhradun and verified in the Department of Botany, HNBG University, Srinagar and a specimen sample was submitted to the herbarium of the University, vide voucher specimen number GUH20720. The methanol extract of leaves of *E. sphaericus* was prepared through the method of pressurized liquid extraction with an aim to get phenolic-rich extract[20,21]. Briefly, dried

***Address for correspondence**
E-mail: navinbajpai@gmail.com

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leaves were crushed to a fine powder and pressurized liquid extraction was performed in accelerated solvent extraction system equipped with a solvent controller unit (ASE350, DIONEX and Corporation Sunnyvale, CA, USA)[22]. Powdered sample (10 g) was loaded into the extraction cell of ASE with an equal amount of silica powder in 1:1 ratio. Absolute methanol was used as the solvent. A pressure of 1500 psi was applied and for each extraction, five cycles (5 min each) were performed. Evaporation of solvents was done in a rotavapor (Rota Vapor124, Bucchi, and Flawil, Switzerland). After evaporation, the plant extract was lyophilized and stored at 4° until further use. Dilutions of extract were prepared in 25 % dimethyl sulfoxide (DMSO) before use. Throughout this communication, methanol extract of *E. sphaericus* Roxb. was referred to as MES.

Inhibitory concentrations of MES against *C. albicans* and *C. glabrata* were determined using M27-A2 guidelines[23]. Briefly, log phase culture in YNB broth was inoculated in 96-well round bottom plant containing YNB medium with different dilutions of the extract (from 0.156 to 5 mg/ml). Final volume of assay system was kept 200 µl per well. The plates were covered with lid and were incubated at 35° for 48 h. The growth was measured by taking absorbance at 600 nm.

Germ tube formation was induced in a medium containing new born calf serum (10 % v/v) as an inducer of germ tube formation[24]. Overnight culture of *C. albicans* was diluted in fresh YPD broth and different components were mixed to make the volume 1.0 ml in each tube. Germ tube formation was induced by 10 % serum in two tubes, with and without MES (5 mg/ml). Two uninduced tubes, with and without MES, were kept as controls. All tubes were incubated at 37° with shaking for 4 h. After incubation cells were observed microscopically under phage contrast microscope (Olympus). Samples were analysed by negative stain before being photographed at 40X. Since *C. glabrata* does not form germ tube, therefore only *C. albicans* was used.

The effect of MES on adhesion of *C. albicans* and *C. glabrata* was observed in a 96-well microplate (polystyrene surface)[25]. Log phase cell suspension was prepared in YNB medium at a concentration of 1×10^7 cells/ml. Hundred microliters of cell suspension was added to each well along with 100 µl of media containing different concentration of MES in a range of 0.156 to 5 mg/ml. Cells were allowed to adhere on the surface in presence of plant extract. Wells without MES were kept as control. The plates were incubated for 90 min at 37° at 100 rpm in an orbital shaker for adhesion. After incubation, wells were washed with phosphate buffer solution (PBS) to remove any planktonic cells. Density of adherence in each well was determined using XTT reduction assay, and percent metabolic activity was calculated to determine cell adherence. The effect of MES on *in vitro* adhesion is shown in terms of relative metabolic activity (RMA), which is percent metabolic activity at particular concentration of MES, assuming 100 % metabolic activity of the sample without MES.

Cells were allowed to develop biofilm on pre-sterilized polystyrene surface of 96-well microplate as per standard methodologies[26]. Briefly, log phase cells were suspended in PBS at a concentration of 1×10^7 cells/ml. Hundred microlitres of cell suspension was added to each well and plates were incubated at 37° for 90 min at 100 rpm in orbital shaker to allow attachment of cells on the surface. The wells were washed twice with PBS to remove non-adhered cells and the plates were observed under inverted light microscope for observing cell adherence. Afterwards, 200 µl of YNB medium with different concentrations of MES was added to each well, including one without MES (control) and plates were incubated at 37° for 48 h. After incubation, wells were washed twice with PBS to remove any planktonic cells and biofilm was observed using an inverted light microscope. Quantification of biofilm was performed by XTT reduction assay with slight modifications. The effect of MES on biofilm activity is shown in terms of RMA as mentioned above in adhesion assay.

XTT reduction assay was performed to measure the adhesion and biofilm development[26]. XTT measures the activity of mitochondrial dehydrogenase. The XTT solution (1 mg/ml) was prepared in PBS and then syringe filtered (0.22 µm pore size). Menadione solution (0.4 mM) was prepared in acetone. Prior to use, menadione was added to XTT to a final concentration of 4 µM. XTT-menadione solution (20:1) was added to each well and final volume of assay system was maintained by PBS at 200 µl. The plates were covered with lid and incubated in dark for 2 h at 37°. After incubation, supernatant (100 µl) was transferred to another fresh 96-well plate and optical density was measured at 450 nm wavelength.

All experiments were performed in triplicate and values presented are the average of three values along with standard deviation.

Emergence of pathogenic drug resistant strain is a complex problem today and *Candida* also belongs to the same category[6]. The virulence and pathogenesis of *Candida* is associated with its morphology and immune state of host[27]. The yeast to hypha transition in *C. albicans* is responsible for its pathogenicity. In this study, MES has shown complete inhibition of growth, hyphal transition, adhesion and biofilm development.

Phytochemical analyses of MES showed the presence of high contents of phenolics and flavonoids[16]. Phenols are organic compounds having substituted phenolic ring. Their antimicrobial activity is relative to the number and position of hydroxyl groups on the phenol group. These compounds produce their effect by inhibiting enzyme through oxidized compounds[28-32]. Flavonoids are the hydroxylated phenolic substances which are produced in plants during microbial infection and hence it is not surprising that they are potential inhibitors of microbial growth and work as antimicrobial agent either by making complexes with extracellular and soluble proteins or by disrupting fungal cell wall because of their lipophilic nature[33,34].

The effect of MES on planktonic growth of *C. albicans* and *C. glabrata* was determined by broth microdilution assay at different concentrations of the extract (fig. 1). As shown in fig. 1, growth of both pathogens decreased with the increasing concentration of the extract. Correlation coefficients, (R^2) were 0.951 and 0.977 for *C. albicans* and *C. glabrata,* respectively. IC_{50} values (concentration required for 50 % growth inhibition) of MES for *C. albicans* and *C. glabrata* were found to lie between 0.625-1.25 mg/ml. At 5 mg/ml of MES, both species were completely inhibited. In a previous study, minimum inhibitory concentration (MIC) of *E. sphaericus* leaves and dried fruit, against *Candida*, have been reported to be 1.5 mg/ml for chloroform extract and 4 mg/ml for ethanol extract in the broth microdilution method[35]. In another study, MIC of aqueous extract of *E. sphaericus* leaves was found to be 1 mg/ml against *C. albicans* in the agar well diffusion assay[15]. In the present study, MES showed better inhibition of *Candida* than extracts prepared in previous studies, probably due to the presence of high phenolics.

Effect of MES on germ tube formation was studied for *C. albicans* only because *C. glabrata* does not form true hyphae. As shown in fig. 2, serum-induced formation of germ tube was inhibited in the presence of 5 mg/ml MES (fig. 2), whereas serum-induced germ tube sample without extract showed significant germ tube formation (fig. 2). Uninduced controls did not show any germ tube induction (fig. 2). This inhibition may be due to presence of flavonoids and antioxidants, which have been earlier reported in this extract[20]. It has already been reported that the medicinal properties of plants are conferred due to presence of these secondary metabolites like flavonoids, phenolics, essential oils[36].

In vitro adhesions of *C. albicans* and *C. glabrata* were reduced to 50 % in the presence of MES between 0.156-0.312 mg/ml concentrations. At 5 mg/ml of MES, the adherences of *C. albicans* and *C. glabrata* were reduced to 29.53 and 16.81 %, respectively (fig. 3A). MES reduced biofilm formation by *C. albicans* and *C. glabrata* in concentration-dependent manner (fig. 3B). Biofilm formation was reduced to 50 % in *C. albicans* at a dose of 1.25-2.5 mg/ml of MES and in *C. glabrata* at 0.625-1.25 mg/ml of MES. MES at a concentration of 5 mg/ml reduced the biofilms of *C. albicans* and *C. glabrata* to 20-30 %.

This reduction in adhesion and biofilm formation of *C. albicans* and *C. glabrata* could be attributed to the presence of different secondary metabolites. Terpenes and terpenoids of different classes have been reported to reduce *Candida* biofilm in previous studies[37,38]. The leaf extracts of *Stryphnodendron barbatimam*, *Equisetum arvense*, *Glycyrrhiza glabra* and *Punica granatum* at a concentration of 50 mg/ml, reduced *C. albicans* biofilm by 27, 18, 24 and 26 %,

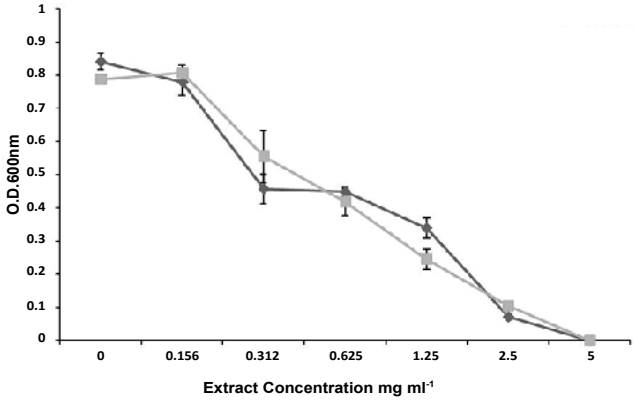

Fig. 1: Inhibitory effect of MES on *C. albicans* and *C. glabrata*
Broth microdilution assay was performed at different dilutions of MES in YNB media against the cultures and growth was measured through optical density at 600 nm after 48 h incubation at 35°. MES is methanol extract of *E. sphaericus* leaves. —♦— *C. albicans*; —■— *C. glabrata*

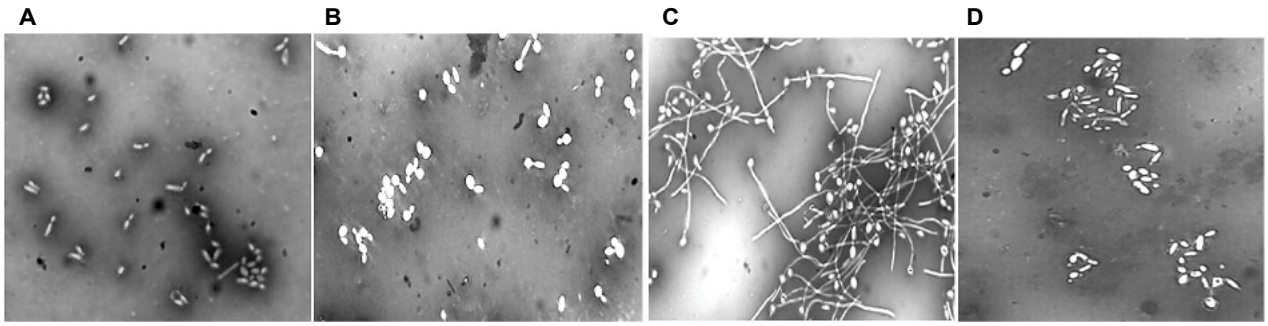

Fig. 2: Effect of MES on germ tube formation by *C. albicans*
Germ tube was induced by 10 % serum in different tubes, with and without MES and photographs were taken after 4 h incubation at 37°. A. uninduced cells; B. uninduced cells with MES; C. induced cells; D. induced cells with MES. MES is methanol extract of *E. sphaericus* leaves.

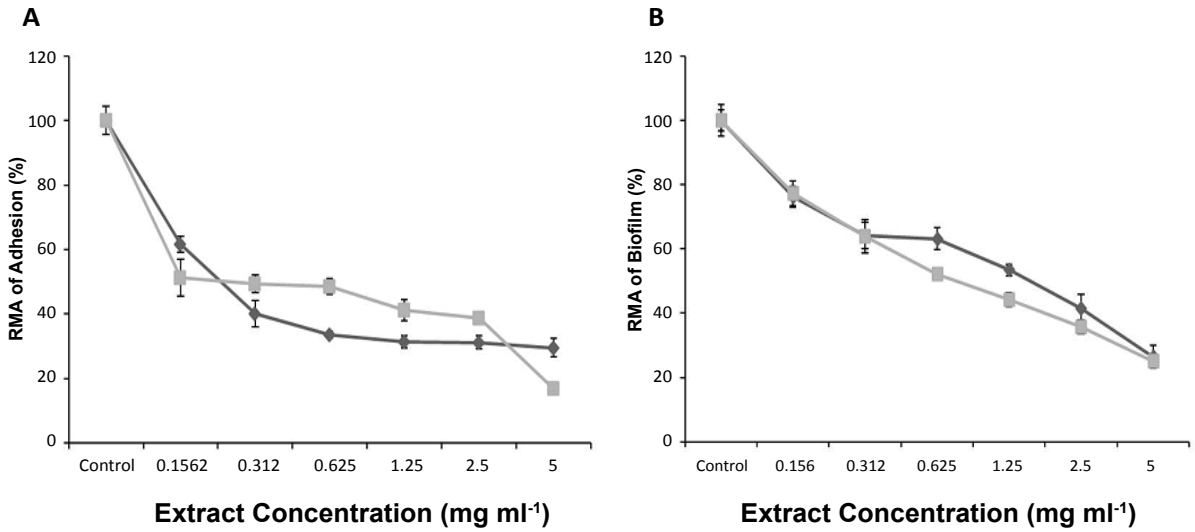

Fig. 3: Effect of MES on adhesion and biofilm formation by *C. albicans* and *C. glabrata*
Adhesion and biofilm formation was quantified by XTT assay and relative metabolic activities are shown at y-axis which are percent metabolic activities at different concentrations of MES, assuming 100 % activity of the sample without MES. MES is methanol extract of *E. sphaericus* leaves —♦— *C. albicans*; —■— *C. glabrata*

respectively[18]. The extract prepared in present study showed better result against *C. albicans* biofilm, when compared to previous reports.

This study has demonstrated anticandida properties of methanol extract of *E. sphaericus* leaves against plaktonic form, germ tube formation, adhesion and biofilm formation of two potent human pathogens, *C. albicans* and *C. glabrata*. These findings have further elaborated the medicinal value of *E. sphaericus* Roxb. that might help in translating the extract into a promising therapeutic agent to treat *Candida* infections.

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Conflict of interest:

Authors declare no conflict of interest.

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