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Research paper

Efficacy of Kumaun Himalayan *Biota orientalis* Endl. leaves extracts against pathogenic fungi

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Abstract

An increasing demand for natural plant products has shifted the attention from synthetic to natural antifungal agents. This study was carried out to evaluate the antifungal activity of methanol, ethanol, chloroform, hexane and water extracts of *Biota orientalis* Endl. leaves, a Kumaun Himalayan gymnospermic plant. The antifungal potential of all extracts of *B. orientalis* were tested against seven different fungal strains (*Alternaria alternata, Colletotrichum falcatum, Fusarium oxysporum, Pyricularia oryzae, Sclerotinia rolfsii, Sclerotinia sclerotiorum* and *Tilletia indica*) using agar-well diffusion method. The ethanol extract was found most active against all the pathogens tested (Percent inhibition, 27-59%) followed by hexane extract (Percent inhibition, 31-58%) and methanol extract (27-57%) while chloroform and aqueous extracts were found totally inactive against all the tested fungal strains, only chloroform extract showed inhibitory activity against *S. rolfsii* (% inhibition, 58%). The inhibitory activity of these extracts was found very effective as compared to Clotrimazol, standard antifungal agent that was used as positive control against tested fungal strains.

Keywords: *Antifungal Activity; Biota orientalis; Kumaun Himalaya; Plant Extracts.*

1. Introduction

Plants host many active substances, and are the basis of both ancient folk remedies and modern pharmacopeia (Sanjust *et al.* 2008, Pardo *et al.* 2010). Structural modification of antimicrobial drugs are an effective means of extending the lifespan of antifungal agents such as the azoles (Jeu *et al.* 2003), antiviral agents such as the non-nucleoside reverse transcriptase inhibitors (De Clercq 2001), and various antibacterial agents including β-lactams and quinolones (Poole 2001). Broad empirical screening of chemical entities for antimicrobial activity represents an alternative strategy for the development of novel drugs (Sati *et al.* 2015, Kumar *et al.* 2016). Natural products have been rich source of anti-infective agents, yielding, for example, the penicillins in 1940, the tetracyclines in 1948 and the glycopeptides in 1955 (Silver & Bostian 1990).

Scientific efforts to discover new antifungal drugs are principally guided toward synthetic and natural products of plant origin (Marzouk *et al.* 2011). The extracts of many plants/herbs have been shown to exert biological activity *in vitro* or *in vivo*, justifying research on traditional medicine that focuses on the characterization of antifungal activity of these plants in India that have a diverse flora and a rich tradition for use of medicinal plants. Since plants produce a variety of compounds with antimicrobial properties, it is expected that screening programs for some under-represented targets, such as antifungal activity, may yield important compounds for antimicrobial remedies (Ahmad & Beg 2001, Sati & Joshi 2011).

Gymnosperms are almost a neglected group of plants in Himalayan region so as the antimicrobial activity of these plants have not been explored adequately so far (Sati & Kumar 2015, Sati *et al.* 2015). From the rich biodiversity of the Kumaun Himalayan region of India, *Biota orientalis* Endl., commonly known as "morpankhi" or "Green Giant" is taken for this study. It is a fastest growing evergreen shrub or small bushy tree, 15-25 feet high and 4m in diameter, much branched with dense cylindrical crown becoming thin and irregular at maturity, resinous and aromatic.

B. orientalis has different medicinal uses and pharmacological activities. It also has platelet activating factor (PAF) inhibitory effect related to pinusolid and pinusolidic acid (Yang & Han 1998), neuroprotective activity related to 90% methanol fraction of *B. orientalis* against glutamate-induced neurotoxicity (Koo *et al.* 2002), cytotoxic activity by isolated deoxy podophyllotoxin (a lignane) from *B. orientalis* leaves against HeLa cells (Kosuge *et al.* 1985) and antimicrobial activity because of essential oils from twigs by hydrodistillation method and fruits by steam distillation method (Hassanzadeh *et al.* 2001, Bagci & Digrak 1996). The present investigation is an attempt to evaluate the antifungal efficacy of leaves extracts of Kumaun Himalayan gymnospermous plants *B. orientalis*.

2. Materials and methods

2.1. Collection of plant material

Green leaves of *Biota orientalis* Endl. (Cupressaceae) were collected during the month of June from Nainital, Kumaun Himalaya, India and authenticated by the Department of Botany, Kumaun University, Nainital. A voucher specimen of plant species was submitted to the herbarium of Department of Botany.

2.2. Preparation of the extract

Leaves of the plant were thoroughly washed with distilled water and dried at the room temperature (20±2°C). The dried material was powdered in an electric grinder. To prepare stock solution 50g of this powder was placed in a 500 ml conical flask mixed with 200ml of solvents (w/v, 50g/200ml). The mouth of flasks are

tightly plugged with non-absorbent cotton and tightly wrapped with aluminium foil to prevent evaporation. Solvents used for extraction were methanol, ethanol, chloroform, and hexane. All flasks were shaken on a rotary incubator shaker at 190-220 rpm for 24 h at 37° C. The mixtures were filtered through Whatman filter paper no.1 and the filtrate collected separately in a clean beaker. The extracts were evaporated, using steam bath to dryness at 30° C. The dry extracts were kept in sterile sample bottles and stored in the refrigerator at 4°C for further use.

2.3. Microorganisms used

Seven fungal strains (*Alternaria alternata, Colletotrichum falcatum, Fusarium oxysporum, Pyricularia oryzae, Sclerotinia rolfsii, Sclerotinia sclerotiorum* and *Tillatia indica*) were obtained from Plant Pathology Department, Pantnagar University, Pantnagar, which were previously isolated from diseased plant materials.

2.4. Antifungal screening

The fungitoxic activity of different extracts was tested against seven fungal strains employing the Agar well technique of Grover and Moore (1962). Potato Dextrose Agar (Hi Media, 39 gm of medium dissolved in 1000 ml of distilled water) was used. The medium was autoclaved at 120° C for 30 minutes. 20ml of PDA media was poured into the 90mm petri plates. After solidification of agar plates, the appropriate well was made on agar plate by using cork borer of size 7.0mm and 200 µl of the extract was added into each well. Mycelial disks (7mm diam.) of actively growing colonies of tested fungal strains were cut from the periphery of the culture plates and aseptically placed 2.5 cm apart from the wells in the assay plates (90mm diam.). A standard antibiotic clotrimazole was used as positive control in the experiment. The tests were performed in triplicates and these plates were incubated at 25 ± 2°C for 4 to 5 days.

2.5. Estimation of antifungal activity

The antifungal activity of the extracts was analyzed by measuring the radial growth of the test fungi on 4th day of incubation in 2 directions: R_1 (radius in opposite direction) and R_2 (radius in direction of the well filled with plant extract). Percent inhibition of radial growth was calculated as suggested by Fokkema (1973) and Perez (1990) and expressed as mean value with standard error of means (SEM).

$$Percent\ Inhibition = \frac{R_1 - R_2}{R_1} \times 100$$

3. Results

In the present investigation different extracts of *B. orientalis* were screened for antifungal activity against seven pathogenic fungi. The results are summarized in table 1. As shown in table 1 the hexane and chloroform extract showed low activity, while only ethanol and methanol extract had good antifungal activity. The aqueous extract was found totally inactive against all the pathogen tested. Hexane extract showed its activity in three tested organisms only, against *A. alternata* (percent inhibition, 58%) followed by *S. rolfsii* (47%) and *P. oryzae* (31%).

Chloroform extract was found active only against *S. rolfsii* by showing 58% inhibition. Ethanol extract showed a maximum inhibition 59% against *S. rolfsii*, followed by 58% against *A. solani*. A low level inhibitory activity was also observed against *P. oryzae* (47%), *F. oxysporum* (39%), *B. cineria* (37%) and *C. falcatum* (27%). The extract was found inactive against *T. indica* (Table 1). For methanol extract, the highest percent inhibition was recorded against *S. rolfsii* (57%). A moderate activity was observed against *P. oryzae* (45%) followed by *A. alternata* (42%), *F. oxysporum* (39%), *C. falcatum* (27%) and *B. cineria* (26%). The results indicate that *S. sclerotiorum* is the most resistant fungal strain as no extract was able to inhibit it (Fig. 1). It is interesting to note that in some cases the plant extract showed better activity than the positive control "Clotrimazol" which is a standard antifungal agent (Fig. 1).

Table 1: Antifungal Activity of Different Extracts of *B. orientalis*

	Percent Inhibition (%)*					
Fungal strains	H	C	E	M	Aq.	Clotr.
A. alternata	58±2.9	na	58±1.0	42±2.0	na	40±1.3
B. cineria	na	na	37±4.6	26±3.4	na	35±2.3
C. falcatum	na	na	27±6.6	27±2.3	na	40±3.0
F. oxysporum	na	na	39±1.2	39±3.4	na	45±1.0
P. oryzae	31±1.3	na	47±7.0	45±1.3	na	48±4.5
S. rolfsii	47±4.5	58±0.6	59±2.3	57±0.8	na	45±1.5
T. indica	na	na	na	na	na	48±1.0

*All the values are mean ± SEM of three determinations. H, C, E, M, Aq: Hexane, Chloroform, Ethanol, Methanol, Aqueous; Clotr.:Clotrimazol; na: not active

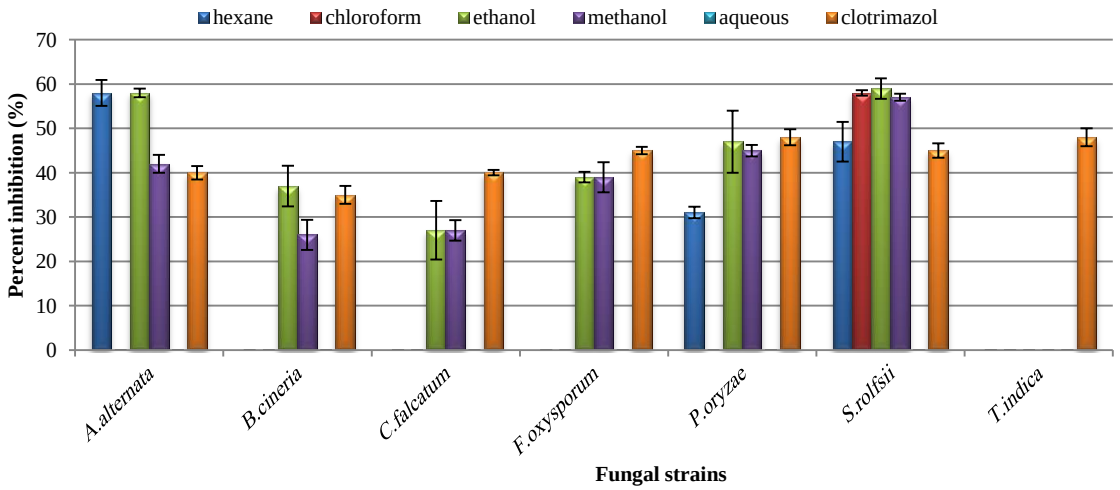

Fig. 1: Antifungal Activity of *B. orientalis* against Some Fungal Strains.

4. Discussion

Successful prediction of botanical compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Results of the antifungal assay carried out by agar well technique with aqueous and organic extracts of *B. orientalis* leaves were graphically represented in figure 1. The results of the present study indicate that the plant *B. orientalis* assayed possess antifungal properties (Plate 1). This explains the use of this plant in folk medicine for treatment of various diseases whose symptoms might involve fungal infections and underline the importance of the ethnobotanical approach for the selection of plants in the discovery of new bioactive compounds.

As evident from the literature *B. orientalis* is well documented for its use for remedies of various ailments (Ezzat 2001, Nickavar *et al.* 2003, Naser *et al.* 2005, Guleria *et al.* 2008, Sati *et al.* 2014). In folk medicine *B. orientalis* has been used to treat bronchial catarrh, cystitis, urine carcinomas, amenorrhea and rheumatism (Peng& Wang 2008). Biota plant ethanol fraction has been reported to produce protective potential against CCl_4 induced liver damage in rats (Dubey & Batra 2008). The antioxidant activity of aerial part of *B. occidentalis* ethanol fraction was evaluated by Dubey and Batra (2009). Essential oil of *B. oriantalis* has been widely used in steam bath. "THUJOIN" rich fraction separated from crude ethanolic extract of Biota had been reported to possess anticancer potential (Biswas *et al.* 2010).

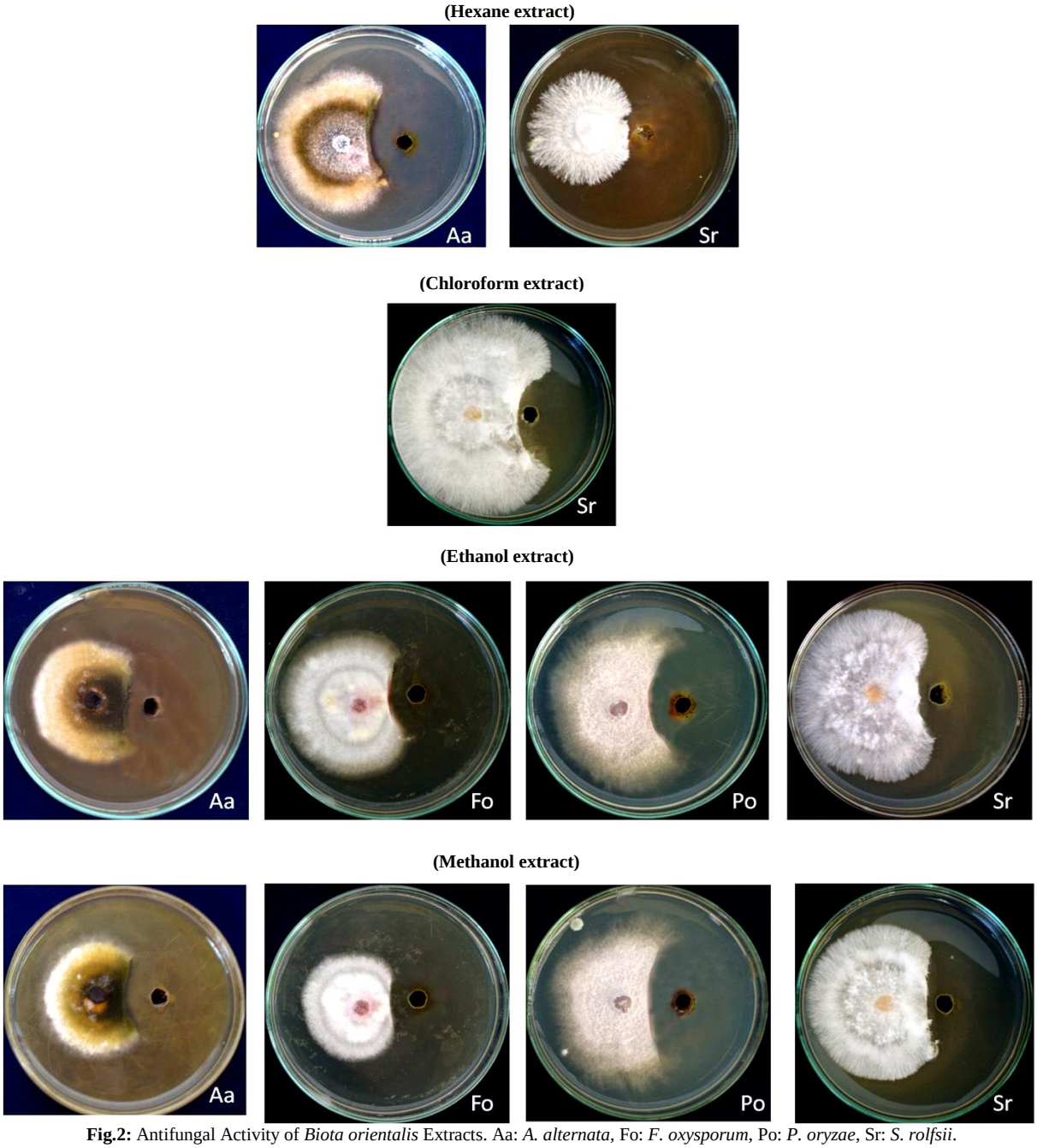

Fig.2: Antifungal Activity of *Biota orientalis* Extracts. Aa: *A. alternata*, Fo: *F. oxysporum*, Po: *P. oryzae*, Sr: *S. rolfsii*.

The pharcological effects of this plant has also been reported by some workers (Ezzat 2001, Nickavar *et al*. 2003, Naser *et al.* 2005, Guleria *et al.* 2008, Bissa *et al.* 2008, Bhan *et al.* 2011, Sati *et al.* 2014). Guleria *et al.* (2008) determined the chemical composition of this plant collected from north-western Himalaya and they found α-pinene, α-cedrol, caryophyllene, limonene, α- terpinolene and α- terpinyl acetate, active compound in leaf extract.

In the present investigation the antifungal activity of *B. orientalis* against 7 fungal strains showed a moderate activity by ethanol and methanol extracts were found effective against *A. altarnata*, *S. rolfsii*, and *P. oryzae*. No absolute inhibition was observed at 1000µg/ml concentration for fungal pathogen. However it is remarkable to note that ethanol and methanol extracts were found

more potential than the standard antifungal agent "Clotrimazol" used in present study (Figure 1).

Antifungal potential of *B. orientalis* was also evaluated by Guleria and Kumar (2006) and Guleria *et al.* (2008) but using their essential oils. In antifungal screening aqueous extract showed no activity against all the tested fungal strains (Table 1). This might be due to that substances which show activity are more soluble in organic solvents than aqueous medium and therefore, could not be presents in aqueous extract as suggested by Sati and Joshi (2010). Recently Srivastva *et al.* (2012), reviewed biological properties of *B. orientalis* and concluded that *B. orientalis* has the great potentiality against a number of health problem.

Relying upon the above, it is clear that the extracts of *B. orientalis* had very promising results against the tested fungal pathogenic strains in comparison to synthetic drug Clotrimazol. Thus the plant extracts are not only effective but also eco-friendly and requires more studies on this line.

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