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Assessment of Antifungal Activity of Some Medicinal Plants

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Abstract: This study reports for the first time the antifungal activities of acetone, hexane, dichloromethane and methanol leaf extracts of four plant species (*Acacia pennata*, *Anaphalis wightiana*, *Capparis pepiaria* and *Catunaregum spinosea*) from Belgaum district of Karnataka state, India. The antifungal activities were determined against pathogens (*Candida albicans*, *Kluyeromyces polysporous*, *Aspergillus niger*, *Aspergillus fumigatus*) representing yeasts, moulds and non-thermal dimorphic fungi. MIC values were determined by checking the growth after 24 and 48 h to determine the antifungal activity against the tested pathogens. Highest antifungal activity was observed with methanolic extracts of *Anaphalis wightiana* against all the tested pathogens with the MIC values ranging from 0.02 to 0.06. Methanolic extracts of *Acacia pennata*, *Anaphalis wightiana*, *Capparis pepiaria* have very strong antifungal activity against tested pathogens particularly *C. albicans* and *K. polysporus*. The overall results provide promising baseline information for the potential use of the crude extract of leaf in the treatment of fungal infections. An attempt has been made to highlight the promising plant species for further investigation as leads for new drug development.

Key words: Antifungal, Belgaum district, Karnataka state, MIC, India

INTRODUCTION

Plants used in traditional medicine may constitute an important source of new biologically active compounds. Utilization of plants for medicinal purposes in India has been documented long back in ancient literature (Tulsidas, 1631; Dradhbala, 1996). However, organized studies in this direction were initiated in 1956 (Rao, 1996). In India, plants are widely used by all sections of the population either directly as folk medicines or indirectly in the pharmaceutical preparations of modern medicines (Vedavathy *et al*., 1997; Warrier *et al*., 1997; Savithramma *et al*., 2007; Malabadi *et al*., 2005; Malabadi, 2005; Malabadi and Vijaykumar, 2005; Malabadi *et al*., 2007). In India more than 43% of the total flowering plants are reported to be of medicinal importance (Pushpangdan, 1995). Traditional healers throughout India uses plants for many medicinal purposes (Kurup *et al*., 1979; Chopra *et al*., 1956; Malabadi *et al*., 2007; Upadhyay *et al*., 2007; Kaushik and Dhiman, 2000). These include fever, asthma, piles, heart diseases, mensural problems, jaundice, sore throats, stomach and gastric problems, earache, pneumonia, headaches, gallstones, diarrhoea, dysentery, toothache, scorpion and snake bites, gastric ulcers, hookworm, colds, chest coughs, abdominal disorders, conjunctivitis, dysmenorrhoea, infertility in women, venereal diseases including syphilis, fattening babies, leprosy, nosebleeds, swelling caused by mumps, cleanse and urinary system, abdominal pains, backache, blennorrhagia, constipation and general weakness. Local healers from different areas of Belgaum district use different plants in various combinations to treat this affection (Malabadi *et al*., 2007). Single plants or combination of plants are given to the patients by these traditional healers depending on the severity of the disease, body nature, age and weight of the patient. These traditional healers usually move in groups, hunted animals and are very conservative in their established traditions. They possess a good knowledge of the medicinal uses of the plants. They have a number of superstitious beliefs related to different types of diseases and possesses a good knowledge in using herbal drugs (Malabadi *et al*., 2007). Some of these uses may be attributed to antifungal activity of extracts.

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Amphotericin B and azole group of antifungal agents are extensively used in the treatment of fungal infections (Masoko *et al*., 2007). Unfortunately, the widespread and incorrect use of these antifungals has led to the emergence of drug resistance in several common pathogenic fungi (Graybill, 1996; White *et al*., 1998). Due to this emergence of antibiotic resistant human pathogenic fungi, it is important to develop new antifungal agents. The field of ethanobotanical research has expanded greatly in recent years. Plants may yield valuable antimicrobials.

There is still a high mortality associated with some invasive fungal infections, especially those produced by filamentous fungi. Most antifungal agents are expensive and have serious side effects. Other sources of antifungal agents should also be investigated. Many ethanobotanists throughout world stressed the importance of investigating plants for new antifungal agents (Hostettmann *et al*., 2000; Chandrasekaran and Venkatesalu, 2004; Chamundeeswari *et al*., 2004; Bhatt and Saxena, 1979; Graybill, 1996; Adams *et al*., 2007; Kumar *et al*., 2006). The aim of this study is to investigate the antifungal activity of different leaf extract of newly reported medicinal plant species from Belgaum district of Karnataka state, India in order to determine which species have good potential as antifungal agents.

MATERIALS AND METHODS

Plant collections, drying and storage: Plants used in this study viz., *Acacia pennata* (Mimosaceae), *Anaphalis wightiana* (Astceraceae), *Capparis pepiaria* (Capparaceae) and *Catunaregum spinosea* (Rubiaceae) were collected from Belgaum district, Karnataka, India. The leaf part of all these plants were used for various medicinal purposes by the local traditional healers More information on the origin and references of these plants are presented elsewhere (Malabadi *et al*., 2007). Leaves were separated from stems and dried at room temperature. Most of the workers have tended to use dried material because there are few problems associated with large scale extraction of dried plants rather than fresh plant material (Eloff, 1998a; Malabadi, 2005). The dried plant material were milled to a fine powder and stored at room temperature in closed containers in the dark until further use.

Extraction procedure: Plant samples from each species were individually extracted by weighing four aliquots of 1 g of finally ground plant material and extracting with 10 mL of acetone, hexane, dichloromethane (DCM) and, methanol in centrifuge tubes. These tubes were vigorously shaken for 3-5 min in a shaking machine at high speed. After centrifugation at 3500 rpm for 10 min the supernatant was decanted into labelled containers. This process was repeated for 2 to 3 times to exhaustively extract the plant material and extracts were combined. The solvent was removed under a stream of air at room temperature before dissolving extracts in acetone to a concentration of 10 mg mL^{-1}, to quantify the assay.

Fungal test organisms: Four fungi were obtained from the mother cultures of fungi, Department of Botany, Karnatak University, Dharwad, India. These fungi represent different morphological forms of fungi namely yeasts (*Candida albicans*, *Kluyeromyces polysporous*) and moulds (*Aspergillus niger*, *Aspergillus fumigatus*). These are most common and important disease-causing fungi of animals and human beings. All the fungal strains were maintained on YM (Wickerhams medium) agar medium in accordance with Malabadi (1994) and Malabadi and Raghavendra (1994, 1995).

Antifungal assay: A serial dilution assay (Eloff, 1998b) was used to determine the Minimum Inhibitory Concentration (MIC) values for plant extracts using tetrazolium violet reduction as an indicator of growth. This method had previously been used only for antibacterial activities (Eloff, 1998b; McGaw *et al*., 2001; Malabadi *et al*., 2005; Malabadi, 2005; Malabadi and Vijaykumar, 2005). To apply it to measuring antifungal activities, a slight modification was made to suit fungal growth conditions (Masoko *et al*., 2007). Residues of the different extracts were dissolved in acetone to a concentration of 10 mg mL^{-1}. The plant extracts (100 μL) were serially diluted 50% with water in a 96-well microtitre plates (Eloff, 1998b). Fungal cultures were transferred into fresh YM agar broth and 100 μL of this added to each well. Amphotericin B was used as the reference antibiotic and positive control and appropriate solvent blanks were included as negative control. As an indicator of growth, 40 μL of 0.2 mg mL^{-1} of p-iodonitrotetrazolium violet (INT) (Sigma, USA) dissolved in water was added to each of the mircroplate wells. The covered microplates were incubated for 2 to 3 days at 35°C. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth after 24 and 48 h. MIC values are recorded in the Table 1-4. Here all the experiments are repeated for three times and the readings in the Table 1-4 represents the average of three independent experiments. When cells from wells showing no growth after 48 h were incubated in fresh growth medium, however fungal growth resumed. The inhibition therefore, appears to be

Table 1: MIC values in mg mL^{-1} of *Acacia pennata* plant species after 24 and 48 h incubation

		MIC values (mg mL^{-1})			
Organisms	Time (h)	Acetone	Hexane	DCM	Methanol
C. albicans	24	0.18	0.67	0.27	0.02
	48	0.18	0.68	0.27	0.02
K. polysporus	24	0.15	0.31	0.09	0.03
	48	0.15	0.30	0.10	0.06
A. niger	24	0.42	0.19	0.17	0.03
	48	0.41	0.21	0.17	0.03
A. fumigatus	24	0.04	0.24	0.04	0.05
	48	0.04	0.24	0.04	0.05

Table 2: MIC values in mg mL^{-1} of *Anaphalis wightiana* plant species after 24 and 48 h incubation

		MIC values (mg mL^{-1})			
Organisms	Time (h)	Acetone	Hexane	DCM	Methanol
C. albicans	24	0.13	0.47	0.37	0.02
	48	0.13	0.48	0.37	0.02
K. polysporus	24	0.17	0.21	0.02	0.03
	48	0.17	0.20	0.02	0.03
A. niger	24	0.22	0.49	0.07	0.06
	48	0.21	0.49	0.07	0.06
A. fumigatus	24	0.34	0.44	0.02	0.04
	48	0.34	0.44	0.02	0.04

Table 3: MIC values in mg mL^{-1} of *Capparis pepiaria* plant species after 24 and 48 h incubation

		MIC values (mg mL^{-1})			
Organisms	Time (h)	Acetone	Hexane	DCM	Methanol
C. albicans	24	0.08	0.47	0.47	0.02
	48	0.08	0.48	0.47	0.02
K. polysporus	24	0.05	0.41	0.18	0.04
	48	0.35	0.40	0.18	0.03
A. niger	24	0.12	0.39	0.13	0.03
	48	0.11	0.39	0.13	0.03
A. fumigatus	24	0.34	0.14	0.02	0.05
	48	0.34	0.14	0.02	0.05

Table 4: MIC values in mg mL^{-1} of *Catunaregum spinosea* plant species after 24 and 48 h incubation

		MIC values (mg mL^{-1})			
Organisms	Time (h)	Acetone	Hexane	DCM	Methanol
C. albicans	24	0.11	0.77	0.17	0.42
	48	0.11	0.77	0.17	0.42
K. polysporus	24	0.16	0.21	0.06	0.57
	48	0.16	0.21	0.06	0.57
A. niger	24	0.32	0.15	0.13	0.09
	48	0.31	0.15	0.13	0.09
A. fumigatus	24	0.02	0.11	0.07	0.41
	48	0.02	0.11	0.07	0.41

fungistatic rather than fungicidal at the levels tested (Masoko *et al*., 2007). Motsei *et al.* (2003) used a different technique to determine MIC values of medicinal plants traditionally used against *C. albicans* infections and it was not possible to distinguish between fungistatic and fungicidal activities.

RESULTS AND DISCUSSION

Four plant species were selected for antifungal activity screening based on their use in traditional medicinal treatments for both domestic animals and humans and availability in Belgaum district, Karnataka state, India (Malabadi *et al*., 2007). Success in isolating compounds from plant material is largely dependent on the type of the solvent used in the extraction procedure (Masoko *et al*., 2007). In this study, the plant material was extracted with four different solvents (acetone, hexane, DCM and methanol). Among all the solvents, methanol was the quantitatively the best extractant, extracting a greater quantity of plant material than any of the other solvents (data not shown). The results presented in the present paper can be considered as very promising in the prospective of new drugs discovery from plant sources. After evaporation of extracting solvents, the hexane, dichloromethane and methanol extracts were redissolved

in acetone because this solvent was not to be harmful towards bacteria (Eloff, 1998b). Of the four solvents used, methanol extracted more chemical compounds from the leaves of the plants tested, but the extract probably contained highly polar compounds and tannins that may not be that interesting for clinical applications. MIC values were determined by checking growth after 24 and 48 h to determine the end point (Table 1-4). The MIC values of most of the extract were in the order of 0.02 to 0.68 in case of *Acacia pennata* (Table 1). The acetone extracts of *Acacia pennate* were found very active against *A. fumigatus* indicating a strong antifungal activity towards this pathogen. On the other hand the rest of the extracts except methanol of *Acacia pennata* showed moderate antifungal activity towards all the tested organisms (Table 1). The methanol extract of *Acacia pennate* was found very active against all the tested pathogens with the MIC values ranging from 0.02 to 0.05 (Table 1). In case of *Anaphalis wightiana*, the MIC values were in the range of 0.02 to 0.49 (Table 2). Highest antifungal activity was observed with methanolic extracts of *Anaphalis wightiana* against all the tested pathogens with the MIC values ranging from 0.02 to 0.06. Rest of the plant extracts of *Anaphalis wightiana* showed moderate antifungal activity against all the tested organisms (Table 2). High MIC values were recorded with *Anaphalis wightiana* and *Acacia pennata* plant species with hexane (Table 1, 2). This might be due to the extracts tested are still in an impure form, or that active compound/s is present in very low concentrations (Malabadi, 2005).

To determine which plants can be used for further testing and isolation, not only MIC is important, but also of the total activity (Eloff, 1998a). Because the MIC value is inversely related to the quantity of antifungal compounds present, an arbitrary measure of the quantity of antifungal compounds present was calculated by dividing the quantity extracted in mg from 1 g leaves by the MIC value in mg mL^{-1}. This total activity value indicates the volume to which the biologically active compound present in 1 g of the dried plant material can be diluted and still kill the fungi (Eloff, 1998b). In other plant species such as *Capparis pepiaria*, the MIC values were in the range of 0.02 to 0.48 (Table 3). Highest antifungal activity was observed with methanolic extracts of *Capparis pepiaria* against all the tested pathogens with the MIC values ranging from 0.02 to 0.05. Rest of the plant extracts of *Capparis pepiaria* showed moderate antifungal activity against all the tested organisms (Table 3). Acetone, hexane and DCM extracts of *Catunaregum spinosea* showed the higher antifungal activity against *A. fumigatus* with the MIC values in the range of 0.02 to 0.11 (Table 4). Methanol extract of *Catunaregum spinosea* also found moderate activity against all the tested pathogens (Table 4).

On the basis of information from the local traditional healers from Belgaum district, the leaf juice of *Catunaregum spinosea* taken internally twice a day in loose motion and therefore, showed antidysentric property, the leaf juice of *Capparis pepiaria* was used for treating the skin infections, swollen parts to reduce edema in animals, the leaves of *Acacia pennata* were used as vegetable for bone strength and leaf juice of *Anaphalis wightiana* were also useful during skin infections (Malabadi *et al.*, 2007). Present study to some extent confirmed the medicinal properties of these plants. Some other workers determined antifungal activities and MIC using different plant species. Their MIC values were generally high. Delaporte *et al.* (2004) used *Tillandsia streptocarpa* to test antimicrobial activity on *C. albicans* (MIC>0.5 mg mL^{-1}), Chandrasekaran and Venkatesalu, (2004) have found that seed extracts of *Syzgium jambolanum* were effective against different pathogens, *C. albicans*, *C. neoformans*, *A. fumigatus* and *M. gypseum* with the MIC values of 0.62, 0.25, 0.125 and 0.25 mg mL^{-1}, respectively. Chamundeeswari *et al.* (2004) found antifungal activity of *Trewia polycarpa* root extracts on *C. albicans*, *A. niger*, *C. neoformans* and *Penicillum* sp. Alcoholic extracts had mild antifungal activity with MIC values of 0.25, 0.25, 0.125 and 0.13 mg mL^{-1}, respectively. When comparing the MIC values with our data it is clear that extracts of *Acacia pennata* (Mimosaceae), *Anaphalis wightiana* (Astceraceae), *Capparis pepiaria* (Capparaceae) and *Catunaregum spinosea* (Rubiaceae) have substantial activity against fungal pathogens. Methanolic extracts of *Acacia pennata*, *Anaphalis wightiana Capparis pepiaria* have very strong antifungal activity against tested pathogens particularly *C. albicans* and *K. polysporus*.

Both *C. albicans* and *K. polysporus* are very dangerous for human beings and may be controlled by using the tested plant species in the form of herbal drugs. It is thought that *C. albicans* and *K. polysporous* invades human tissues. *C. albicans* is a non-thermal dimorphic yeast. Its ability to switch from yeast cells to hyphae is considered to be important for the interactions of *C. albicans* with its host (Cutler, 1991). Both yeast cells and hyphae are present in the host commensal growth and during infection. Hypha are important factor for fungal virulence factor that promotes invasion of cells into mucosa, allowing candial cells to resist macrophage and neutrophil engulfment (Yang, 2003). Generally the action mechanism of saponins may lie in the damage to

the membrane and leakage of cellular materials, ultimately leading to cell death (Yang, 2003). This activity has been documented in a number of saponins and the damaging effect have been shown against variety of fungal pathogens including *C. albicans*, *S. cervisiae*, *C. neoformans* (Lalitha and Venkataraman, 1991). The results of the present work indicates that all the tested plants species possess substantial antifungal properties. If there are no synergistic effects and the antifungal compounds comprise 0.1% of the mass, the antifungal compound may have an MIC values of 0.02 to 0.06 mg mL^{-1} compared to MIC values of 0.2 to 0.6 mg mL^{-1} of amphotericin B for these pathogens.

The results of this study support several of traditional medicinal uses of plant species from Belgaum district, Karnataka state, India. Hence it is very necessary for the isolation and identification of compounds responsible for the antifungal activity. The antifungal mechanisms associated to each group of chemical to which the isolated compounds belong, may explain the inhibition potency of the tested samples. This study also provide an important basis for the use of methanolic extracts of all the extracts of tested plant species for the treatment of infections associated to the pathogens used in this study and could be useful for the development of new antifungal drugs. Present results allow us to conclude that the crude extracts of the plants exhibited significant antifungal activity and properties that support folkloric use in the treatment of some mycosis, corroborating the importance of ethanopharmacological surveys in the selection of plants for bioactivity screening. Therefore, bioactivity screening of compounds is very much essential for the future study for the development of new antifungal drugs.

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