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COMPARATIVE BIO- EVALUATION OF VARIOUS MEDICINAL PLANT EXTRACTS AND THEIR *INVITRO* ANTIFUNGAL POTENCY AGAINST *CANDIDA GLABRATA*

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ABSTRACT
Current research on natural molecule and products primarily focuses on plants since they can be sourced more easily and be selected based on their ethno-medicinal uses. The present study was carried out to investigate for preliminary phytochemical screening and antimicrobial effect of methanol extracts of 10 ethanomedicinal plant extracts against *Candida glabrata*. Phytochemical techniques have a significant role in finding resources of raw materials for pharmaceutical industry. The presence of phytoconstitutents was identified by characteristic changes using standard procedure. The phytochemical screening of plant extracts showed the presence of alkaloids, flavonoids, steroids, glycosides, tannins, saponins, terpenoids, plobatannins and carbohydrates. Antifungal activity of the 10 plant extracts was tested against *Candida glabrata* by disk diffusion method. The MIC values of methanolic extracts of plant species were *Markhamia lutea* and *Adenanthera pavonina* (512 µg/ml), *Spathodea companulata* (256 µg/ml), *Phyllanthus acidus* and *Tecoma stans* (>1024 µg/ml). We report here for the first time, the potent antifungal activity of these five plants against *Candida glabrata.*

KEYWORDS: *Markhamia lutea*, *Adenanthera pavonina*, *Spathodea companulata*, *Phyllanthus acidus* and *Tecoma stans*, Antifungal activity, *Candida glabrata*, MIC.

INTRODUCTION
Since time immemorial man has been finding healing power in plants or natural products to promote and maintain good health. Plant synthesizes a wide variety of chemical compounds which can be grouped into primary and secondary metabolites .Many plants are major source of useful secondary metabolites of commercial importance and find use in number of pharmaceutical compounds (Sousa, M. et al 2006).. Flavonoids help the plants for the defence against infections (Dholvitayakhun A et al. 2012).

Tannins are used as antiseptic and also exhibit antimicrobial activity by precipitation of microbial proteins (Dorsch W Wagner.H 1991). Saponins produce inhibitory effect on inflammation and have the property of precipitating and coagulating red blood cells. Steroids have antibacterial activity. Alkaloids have antibacterial and antispasmodic activity (Stray., 1998, Okwu., 2004) and have the property of cytotoxicity (Nobori et al., 1994). Tannin-rich plant are used as healing agents in a number of diseases and for the treatment of diseases like leucorrhoea, rhinnorhoea and diarrhoea. Thus use of crude methanol extracts of some of these plants as an antimicrobial agents needs further extensive research for their better economic and therapeutic utilization.

In humans the frequency of fungal diseases has increased in the last few decades and are one of the main causes of morbidity and mortality worldwide. An increasing incidence of fungal infections has been noticed due to a growth in immunocompromised population such as transplant recipients, cancer and HIV/AIDS patients (Arshad Hussian., 2011). The expanding pool of high risk patients susceptible to the opportunistic pathogen indicated that the yeast genus *Candida* is the most invasive agent. The frequency of mucosal and systemic infections caused by C. glabrata has increased significantly following the widespread and increased use of immunosuppressive therapy together with broad-spectrum antimycotic therapy .In fact, depending on the site of infection, *C. glabrata* is often the second or third most common cause of candidiasis after *C. albicans*. *C. glabrata* infections can be mucosal or systemic and are common in abnormal hosts (e.g., immunocompromised persons or those with diabetes mellitus). (Pfaller et al., 1996, Schwab et al.,1997, Vanden-Bossche et al., 1992, Willocks et al.,1991, Wingard et al.,1993).

Due to the undesirable side effects, long term consequences of antibiotics and emergence of drug resistant strains search for various phytotherapeutic compounds is on rise that can be less toxic, cheaper as well as highly effective. Worldwide extensive studies have been carried out on antimicrobial potential of different plant extracts. Investigation of several plants for their phytochemical and pharmacological features has led to the isolation of some of the natural antimicrobials.

The current trend of continuous search for plants with bioactive compounds that can be used to treat human fungal diseases and their proper documentation informed this study.

MATERIALS AND METHODS

Plant material: Fresh plant parts were collected from the region of Bangalore, Karnataka, India. The details of plant parts screened, their families and therapeutic use are given in Table 1.The plants were properly identified with the help of authentic literature and documented with their characteristic features. Fresh plant material were washed under running tap water, shade dried and then homogenized to fine powder and stored in airtight bottles with proper labelling for future use.

Extraction of plant material: Crude plant extract was prepared by Soxhlet extraction method. About 20gm of powdered plant material was uniformly packed into a thimble and extracted with 250ml of methanol for 24 hours or till the solvent in siphon tube of an extractor became colourless. After that the extract was taken in a beaker and kept on hot plate and heated at 30-40ºC till all the solvent got evaporated. Dried extract was kept in refrigerator at 4ºC for their future use in phytochemical analysis.

Qualitative phytochemical analysis

The extract was tested for the presence of bioactive compounds by using following standard methods.

Test for carbohydrates

Fehling's test: Equal volume of Fehling A and Fehling B reagents were mixed together and 2ml of it was added to crude extract and gently boiled. A brick red precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

Benedict's test: Crude extract when mixed with 2ml of Benedict's reagent and boiled, a reddish brown precipitate formed which indicated the presence of the carbohydrates.

Molisch's test: Crude extract was mixed with 2ml of Molisch's reagent and the mixture was shaken properly. After that, 2ml of concentrated H_2SO_4 was poured carefully along the side of the test tube. Appearance of a violet ring at the interphase indicated the presence of carbohydrate.

Iodine test: Crude extract was mixed with 2ml of iodine solution. A dark blue or purple coloration indicated the presence of the carbohydrate.

Test for proteins

Millon's test- Crude extract when mixed with 2ml of Millon's reagent, white precipitate appeared which turned red upon gentle heating that confirmed the presence of protein.

Ninhydrin test- Crude extract when boiled with 2ml of 0.2% solution of Ninhydrin, violet colour appeared suggesting the presence of amino acids and proteins.

Test for Alkaloids: 3 ml crude extract was stirred with 3 ml of 1% HCl on steam bath. Mayer and/or Dragondroff's reagent was then added to mixture. Turbidity of the resulting precipitate was taken as an evidence for the presence of alkaloid.

Test for Tannins

$FeCl_3$ Test- 2 ml of the crude extract was stirred with 2 ml of distilled water and few drops of $FeCl_3$ Solution were added. Formation of green precipitate was indication of presence of tannins.

Gelatin test- 1% gelatin solution containing 10% sodium chloride was added to the extract. Formation of precipitate indicated the presence of tannins and phenolic compounds.

Test for Saponins: 5 ml of crude extract was shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication of the presence of saponins.

Test for Phlobatannins: About 2 ml of aqueous extract was added to 2 ml of 1% HCl and the mixture was boiled. Deposition of a red precipitate was taken as an evidence for the presence of phlobatannins.

Test for Flavonoids: Alkaline reagent test-To 1 ml of aqueous extract, 1 ml of 10% lead acetate solution was added. The formation of a yellow precipitate was taken as a positive test for flavonoids.

Shinoda test-To leaf and bark (mixture) extracts, 5ml. 95% ethanol was added separately. Each mixture was treated with 0.5g magnesium turnings and few drops of conc. HCl. Pink colour, if produced, may confirm the presence of flavonoids.

Test for Terpenoids: **Salkowski's test-** The extract is treated with chloroform with few drops of concentrated sulphuric acid, shaken well and allowed to stand for some time, formation of yellow coloured lower layer indicated the presence of terpenoids.

Tests for glycosides: Liebermann's test- 2 ml of the crude extract was dissolved in 2 ml of chloroform and

then 2 ml of acetic acid was added in it. The solution was cooled well in ice. Sulphuric acid was then added carefully. A colour change from violet to blue to green indicates the presence of a steroidal nucleus (that is, a glycone portion of glycoside).

Tests for steroids: A red colour produced in the upper layer when 2 ml of organic extract was dissolved in 2 ml of chloroform and 2 ml concentrated sulphuric acid was added in it, indicates the presence of steroids.

Antimicrobial activity

Fungal strains: The test fungal strain *Candida glabrata* (MTCC3019) was used to evaluate the antimicrobial activity of various plant extracts. The stock culture for *Candida glabrata* was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India.

Culture Media: The media used for antifungal test was Sabouraud's dextrose agar/broth of Hi media Pvt. Mumbai, India. The fungal strain was inoculated separately in Sabouraud's dextrose broth and was incubated at 25 ºC for 48 hrs and the suspensions were checked to provide approximately 1-2 x 10^5 cells /mL.

Antifungal susceptibility test: The antimicrobial activity of the methanol extract (100µg/ml) was carried by disc diffusion method. 100µl inoculum of test culture was inoculated on Sabouraud Dextrose agar plates. Standard filter paper discs were individually impregnated with different plant extract (10µl, 100 mg/ml) and then placed into the agar plates which had previously been inoculated with the test microorganism. Disc fed with methanol served as negative control and flucanazole served as reference antimycotic drug. The plates were subsequently incubated at 35 ^{0}C for 24-48hrs. After incubation the growth inhibition rings were quantified by measuring the diameter of the zone of inhibition in millimetres.

The plants which exhibited potent antifungal activity against *Candida glabrata* in disc diffusion method were selected for determination of minimum inhibitory concentration (MIC). A broth microdilution assay was performed as per the standard NCCLS criteria. An inoculum was prepared from fungal cultures grown on Sabouraud Dextrose agar adjusted to 0.5-2.5 x 10^3 cells/mL. RPMI media inoculated with fungal culture was used as the control. Fluconazole which served as standard drug was taken at concentrations 8 – 512 µg/ml (two fold dilutions) in RPMI 1640 media and test compounds were taken at concentrations 16 - 1024 µg/ml (two fold dilutions) in RPMI 1640 media. A sterile 96 well microtiter plate was taken for the test. 90µl standard drug and test compounds of different test concentration with 10µl inoculum were taken separately in microtiter plate in triplicate. 90µl RPMI media without drug along with 10µl inoculum was used as control. The microtiter plate with treated fungal culture was then incubated for 48-72 hrs at 22^{o}C in Tecan Plate reader to evaluate the growth kinetics. MIC was determined as minimum concentration of drug giving 50% inhibition of growth compared to that in control.

RESULTS AND DISCUSSION

The phytochemical analysis of plant extracts using Methanol are shown in Table 1. Preliminary phytochemical analysis revealed presence of various phytocompounds in the methanol extracts such as alkaloids, flavonoids, steroids, glycosides, tannins, saponins, terpenoids, plobatannins and carbohydrates. Among the ten plants screened nine plants revealed the presence of alkaloids, seven plants showed the presence of tannins. Saponins were found in four plants. Six plants exhibited the presence of flavonoids and steroids, two plant species showed the presence of terpenoids and phlobatannins. Glycosides were found in eight plant species. The maximum secondary metabolites were revealed in Rhizome of *Curcuma aromatica* and minimum secondary metabolites were found in the flowers of *Spathodea companualata*.

Table 1: Preliminary Phytochemical analysis for the presence of various phytoconstituents.

Plant Name	Plant part	Alkaloids	Tannins	Phlobatannins	Saponins	Flavonoids	Terpenoids	Glycosides	Steroids	Carbohydrates
Clitorea ternatea	Leaf	-	-	-	-	-	-	-	-	+
	Flower	-	-	-	-	-	-	+	-	+
Averrhoa bilimbi	Leaf	+	+	-	-	-	-	+	-	+
	Fruit	-	-	-	-	-	-	+	-	+
Phyllanthus acidus	Leaf	+	-	-	-	-	-	+	+	+
	Fruit	+	-	-	-	+	-	-	-	-
Tecoma stans	Leaf	+	-	-	-	+	+	+	+	+
	Flower	-	-	-	-	-	-	-	-	+
Curcuma aromatica	Leaf	+	+	-	-	+	-	+	+	+
	Rhizome	+	+	+	+	+	+	+	+	+
Anethum graveolens	Leaf	+	+	-	-	-	-	-	+	-
	Stem	+	+	-	+	-	-	-	-	+
Adhatoda vasica	Leaf	+	-	-	-	+	-	-	-	+
	Flower	-	+	-	+	-	-	+	-	+
Markhemia lutea	Leaf	+	-	-	-	-	-	+	-	+
	Flower	-	+	+	-	-	-	-	-	+
Spathodea companulata	Leaf	+	+	-	-	+	-	-	+	+
	Flower	-	-	-	-	+	-	-	-	+
Adenathera pavonina	Leaf	+	+	-	+	+	-	+	+	+
	Stem	+	+	-	+	-	-	+	+	+

The antifungal activity of plant extracts was determined by measuring the diameter of zone of inhibition recorded. The results of antifungal susceptibility test of the crude plant extracts are illustrated in Table 2.

Among the plants screened leaf of *Spathodea companulata* exhibited most remarkable antifungal activity against *Candida glabrata* with significant zone of inhibition. *Spathodea companulata* has been used for the treatment of various diseases in Ghanaian traditional medicine. Leaves of the plant are used for the treatment of dyspepsia and peptic ulcer, arthritis and fracture (Agbovie et al., 2002). The plant extract has also exhibited a broad spectrum antimicrobial activity against four strains of bacteria and yeast (Mensah et al 2003). *S. companulata* methanol extracts have exhibited antifungal activity against *C. albicans* (Ofori et al., 2009). The leaves of the plant are used for kidney diseases, urethral inflammations and as an antidote against animal poisons. Leaf extracts of the plant has been used to evaluate antimalarial activity against *Plasmodium falciparum* and antibacterial activity against *Staphylococcus aureus* which causes bovine mastitis (Dhanabalan et al., 2008).

Table2: Table showing the zone of inhibition values of different plant parts of the experimental samples against *Candida glabrata.* The values were expressed in mm ±s.e.

Plant Name	Plant part	Zone of Inhibition in mm
Phyllanthus acidus	Leaf	7.00 ± 0.00
Tecoma stans	Leaf	7.00 ± 0.00
Markhemia lutea	Flower	11.00 ± 0.25
Spathodea companulata	Leaf	12.00 ± 0.5
Adenathera pavonina	Stem	11.00 ± 0.5

Stem of *Adenanthera pavonina* and flower of *Markhamia lutea* also exhibited potent antifungal activity. Leaf of *Phyllanthus acidus* and *Tecoma stans* exhibited moderate antifungal activity against the test organism. *Phyllanthus* species has long been used in folk medicine in many countries as antimicrobial and antioxidants (IMS. Eldeen et al., 2010).

The flower extracts of *Tecoma stans* have promising potential for the development of antimicrobial and antioxidant agents. The plant also is a potential source of anti-inflammatory agent (Govindappa. et. al).

Crude extracts of remaining five plants viz *Clitoria ternatea, Averrhoe bilimbi, Adhatoda vasica , Curcuma aromatica, Anethum graveolens* did not show antifungal activity at a concentration of 100mg/ml. However negative results do not indicate absence of bioactive constituents nor is that the plant is inactive. In the crude extracts the concentration of active compounds may be present in insufficient quantity to exhibit activity with the dose levels employed (Taylor et al 2001).Thus, lack of activity can only be proven by using larger doses (Farnsworth,1993). Alternatively if the active principle is present in higher concentration there could be other antagonistically acting constituents or those negating the positive effects of the bioactive agents (Jager et. al; 1996).

The MIC of methanolic extracts of *Markhamia lutea* and *Adenanthera pavonina* was 512µg/ml whereas in case of *Spathodea companulata* a lower concentration of 256µg/ml inhibited the growth of *Candida glabrata.* Methanolic extracts of *Phyllanthus acidus* and *Tecoma stans* exhibited antifungal activity at a concentration greater than 1024 µg/ml.

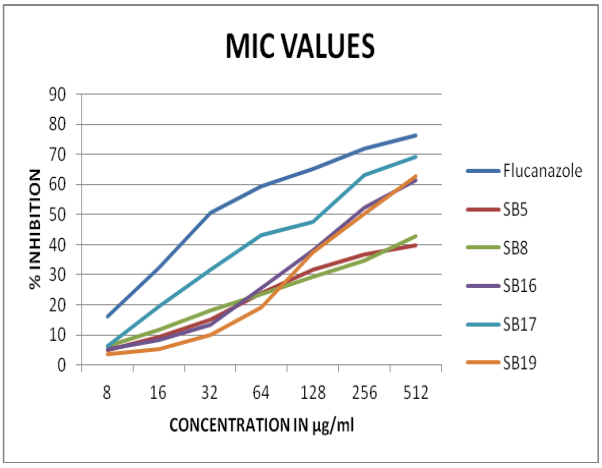

Fig 1: Graph showing the MIC values of the plant extracts against *Candida glabrata.* Flucanazole is used as positive control. SB5: *Phyllanthus acidus; SB8: Tecoma stans; SB16: Markhemia leutea; SB17: Spathodea companualata; SB19: Adenanthera pavonina.*

Our data provide, for the first time, substantive evidence on the antifungal activity of *Adenanthera pavonina, Spathodea companualata, Markhamia lutea, Phyllanthus acidus* and *Tecoma stans* against *Candida glabrata.* The finding from this work may add to the overall value of the medicinal potential of these plants.

CONCLUSION

Candidemia is the most important of the invasive mycoses. Emergence of non- albicans infections have been widely reported in recent years. Candida glabrata currently ranks second or third as the causative agent of superficial (oral, oesophageal, vaginal or urinary) or systemic candidal infections which are often nosocomial. Mucosal and systemic infection caused by *Candida glabrata* have increased significantly especially in HIV

infected population due to increased use of immunosuppressive agents. Due to their innate resistance to azole antimycotic therapy, *Candida glabrata* infections are difficult to treat and are thus responsible for high mortality rate in compromised, high risk hospitalized patients (Paul et al., 1999). Despite the existence of potent antifungal agents, continuous emergence of resistant strains impose the need for a permanent search and development of new drugs(Silver,1993).Plants produce diverse arrays of phytochemicals which are useful in the development of new drugs. The presence of antimicrobial substances in the higher plants is well established (Srinivasan, 2001).

REFERENCES

1. Agbovie, T., Amponsah, K., Crentsil, O. R., Dennis, F., Odamtten, G.T., OfosuheneDjan, W. Conservation and sustainable use of medicinal plants in Ghana- Ethanobotanical Survey., 2002.
2. Arshad Hussian., Aleza Rizvi., Shadma Wahab., Iffat Zareen, Saba Ansari., Md.Sarfaraj Hussain. Antibacterial screening of the bark of Adenathera pavonina(L.). International Journal of Biomedical Research., 2011; 2(2): 110-112.
3. Dhanabalan R. Doss A., Jagadeeswari M., Karthic R.,Palaniswamy M., Angayarkanni. J Ethanobotanical Leaflets., 2008; 12: 811-19.
4. Dholvitayakhun A et al. "Antibacterial activity of three medicinal Thai plants against Campylobacter jejuni and other foodborne pathogens". Natural Product Research., 2012; 26(4): 356–363.
5. Dorsch W, Wagner H. New antiasthmatic drugs from traditional medicine. Int Arch Allergy Appl Immunol., 1991; 94(1-4): 262-5.
6. Farnsworth, N. R. Biological approaches to the screening and evaluation of natural products. In: Rasoanaivo P, Ratsimamanga-Urverg S (Eds) Biological Evaluation of Plants with Reference to the Malagasy Flora,Madagascar, 1993; 35- 43
7. Govindappa, M., Sadanada,T. S., Channabasava, R., Vinay, B. Raghavendra. *Invitro* anti-inflammatory, lipoxygenase, xanthine oxidase and acetylcholinesterase inhibitory activity of Tecoma stans(L.) Juss. Ex Kunth. International Jounal of Pharma and Bio Sciences., 2011; 2(2): 275-285.
8. Jager,A.K.,Hutchings,A., van Staden,J. Screening of Zulu medicinal plants for prostaglandin synthesis inhibitors. J. Ethanopharmacology., 1996; 52: 95-100.
9. Mensah, A. Y., Houghton, P. J., Woode E. Fungi, Friends or Foes? Ghana Pharm. J, 2004; 28: 22-36.
10. Nobori, T., Miurak, K., Wu, D.J., Takabayashik, L.A, Carson,D.A. Deletion of cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature, 1994; 46: 753-756.
11. Ofori-Kwakye, K., Kwapong, A.A., Adu, F. Antimicrobial activity of extracts and topical products of Spathodea campanulata for wound healing. African J. Trad., 2009; 6(2): 168 – 174.
12. Okwu, D.E., Okwu, M.E. Chemical composition of Spondias mombin linn. plant parts. J. Sustain. Agric. Environ., 2004; 6(2): 140-147.
13. Paul L. Fidel, JR., Jose A. Vazquez.,Jack D. Sobel. Candida glabrata: Review of Epidemiology, Pathogenesis, and Clinical Disease with Comparison to C. albicans., Clinical Microbiology Reviews.,1999; 12: 80-96.
14. Pfaller, M. A. Nosocomial candidiasis: emerging species, reservoirs, and modes. Clin. Infect. Dis., 1996; 22: S89–S94.
15. Schwab, U., F. Chernomas, L. Larcom, and J. Weems. Molecular typing and fluconazole susceptibility of urinary *Candida glabrata* isolates hospitalized patients. Diagn. Microbiol. Infect. Dis., 1997; 29: 11–1.
16. Silver,L.L. Discovery and development of new antibiotics: the problem of antibiotic resistance. Antimicrob. Agents Chemother., 1993; 37: 377-383.
17. Sousa, M.; Ousingsawat, J.; Seitz, R.; Puntheeranurak, S.; Regalado, A.; Schmidt, A.; Grego, T.; Jansakul, C. et al. "An Extract from the Medicinal Plant Phyllanthus acidus and Its Isolated Compounds Induce Airway Chloride Secretion: A Potential Treatment for Cystic Fibrosis". Molecular Pharmacology., 2006; 71(1): 366–76.
18. Stray, F. The Natural Guide to Medicinal herbs And Plants. Tiger Books International, London, 1998; 12-16.
19. Taylor, J. L. S., Rabe, T., McGraw, L.J., Jager, A.K., van Staden, J. Towards the scientific validation of traditional medicinal plants. Plant Growth Regul., 2001; 34: 23-37.
20. Vanden-Bossche, H., P. Marichal, F. C. Odds, L. LeJeune, and M. C. Coene. Characterization of an azole-resistant Candida glabrata iso- late. Antimicrob. Agents Chemother., 1992; 36: 2602–2610.
21. Willocks, L., C. L. Leen, R. P. Brettle, D. Urquhart, T. B. Russell, and L. J. Milne. Fluconazole resistance in AIDS patients. Antimicrob. Agents Chemother., 1991; 28: 939.
22. Wingard, J. R., W. G. Merz, M. G. Rinaldi, C. B. Miller, J. E. Karp, and R. Saral. Association of Torulopsis glabrata infections with fluconazole prophylaxis in neutropenic bone marrow transplant patients. Antimicrob. Agents Chemother., 1993; 37: 1847–1849.