International Journal Of

Recent Scientific Research

ISSN: 0976-3031

Volume: 7(4) April -2016

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THE OFFICIAL PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH (IJRSR)
http://www.recentscientific.com/ recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 4, pp. 9863-9866, April, 2016

International Journal of Recent Scientific Research

Research Article

INVITRO ASSESSMENT OF ANTIFUNGAL ACTIVITY OF SELECTED BOTANICALS ON *CANDIDA TROPICALIS*

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ARTICLE INFO

Article History:

Received 05th January, 2015
Received in revised form 21st February, 2016
Accepted 06th March, 2016
Published online 28th April, 2016

Keywords:

Antifungal activity, *C.tropicalis*, *Flacourtia jangomas*, *Hydnocarpus pentandra* and *Acalypha hispida*

ABSTRACT

The augmentation of multidrug resistant strains of fungus owing to the indiscriminate use of existing antimicrobial drugs in the treatment of infectious diseases has spurred researchers in the field of ethno pharmacology to investigate for new antifungal agents from natural sources like plants. The present study was carried out with an objective to evaluate the antifungal activity of extracts of six plant species namely *Flacourtia jangomas*, *Acalypha hispida*, *Hydnocarpus pentandra*, *Euphorbia mili*, *Lucuma nervosa* and *Couroupita guianensis* against *Candida tropicalis*, an emerging Candida Non Albican species. The antifungal activity of methanolic extracts of different parts of the plant like leaf, flower, bark and root were evaluated using agar disc diffusion method. Significant antifungal activity was shown by *Flacourtia jangomas*, *Hydnocarpus pentandra* and *Acalypha hispida*. MIC values observed were 256µg/ml, 512µg/ml and >1024 µg/ml. Zone of inhibition of the extracts were compared with the standard Flucanazole. The phytochemical analysis carried out revealed the presence of carbohydrates, alkaloids, flavanoids, terpenoids, tannins, steroids, glycosides and saponins in most of the plants. Phlobotannins was present in only one extract and amino acids were not detected from any of the plant extracts under study. Scientifically supported by the results obtained in this study it is hoped that these plants can be used as a better and novel therapeutic agent to treat fungal diseases.

INTRODUCTION

Fungal infections have been in the rise destroying the peace of the healthy world for the past few decades. Despite knowing they are life threatening they are neglected pathogens. Immuno compromised patients are at a higher risk to these infections owing to their weakened immune system. The increased use of antibiotics and immunosuppressive drugs are a reason for the greater rate in the emergence of fungal infections as these drugs disrupt the normal bacterial colonization and by suppressing the immune system of the body create an environment in the body where fungi can thrive its best. They become multidrug resistant strains (MDR).The eukaryotic fungal cells have great similarities with mammalian cells, so this makes the therapeutic approach to these diseases very difficult.

In recent years significant change is observed worldwide where there is predominance of the non albican *Candida* sp. causing infections rather than the common *Candida albicans* (Devanaboyina Narendra, 2015). In India the epidemiological data showing the maximum number of nosocomial candidemia cases reveals the CAN sp. *Candida tropicalis* to be the most prevalent& dominant causative organism (Kothari, A. *et al*., 2009**).** In one of the studies, *Candida tropicalis* was the predominant cause of candidemia in catheterized ICU patients (Jain *et al*., 2011). The detrimental forces of fungi surpasses all human efforts leaving the world unhealthy still (M.Jain, 2011). Scientists and researchers are always on the alert seeking solutions for arresting the evergrowing infections caused by fungi (Gopal C. 2011). Plant world abounds in undiscovered & unexploited medicinal potentials for curing many chronic& incurable diseases. When safe, easy and less expensive remedies are near at hand in nature, researchers are turning their attention to it& continuing their effort to gift the world with new, affordable medicines with less side effects (Rauf A, 2014).

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F.jangomas plant parts are used as astringent, acrid, refrigerant, stomachic, diaphoretic, anti-inflammatory in curing skin diseases, jaundice & tumors. In India, dried leaves of *Flacourtia jangomas* are used to treat asthma (Shirona T.K., 2014)**.** *Couroupita guianensis* is considered as a plant with high therapeutic value as its mostly found as one of the active & important ingredients in preparations to cure scabies, scorpion poison, cold, stomach ache, gastritis and many other diseases **(**C.Alagesaboopathi *et al*., 2013). Different parts of cannon ball tree possess antimicrobial, antiulcer, anti inflammatory, antioxidant, antinociceptive, antihelminthic, anticancer properties (S.K.Gousia *et al*., 2013). Lucuma nervosa has natural healing abilities &is utilized as an antiinflammatory, antifungal, antibiotic & skin booster for centuries. *Euphorbia milii* plays a role in folk medicine where its parts are used as a cure for cancer and also to cure warts (S Okorondu, 2009). Traditionally *A.hispida* leaves poultice is used for leprosy (Mc.Laughlin JL.*et al*., 1999). The different plant parts of *Acalypha hispida* have been used in traditional remedies as laxative, diuretic, expectorant & in the treatment of leprosy and kidney ailments (Sahoo M.R., 2014). It is also used to treat wounds and ulcers. *H.pentandra* is a medicinal plant belonging to the family Flacourtiaceae whose seed oil has been used to treat leprosy, as an anti inflammatory agent & also in the local application in rheumatism, sprains and chest infections (T.Sivakumar, 2012). In this study the plant samples were screened for the phytochemical constituents using organic solvent method. The extracts were then used for evaluation of antifungal activity by MIC method. Percent inhibition in the activity was calculated for the evaluation process.

MATERIALS AND METHODS

Collection of plants*:* Healthy, disease free parts (Leaves, Bark, Flower, Root) of the 6 plants; *C.guianensis, F.jangomas, L.nervosa, E.mili, A.hispida & H.pentandra* were collected from Mavelikara, Alappuzha district. These were shade dried and ground to a fine powder using grinder mixer and stored in air tight containers. The plants were identified taxonomically and authenticated.

Test organism: The test organism *Candida tropicalis* (MTCC no.184) was collected from MTCC, IMTECH Chandigarh.

Preparation of Extract: 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 The extract was later 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.

Phytochemical Analysis

Test for Alkaloids: *3* ml aqueous 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: FeCl3 Test: About 2 ml of the aqueous extract was stirred with 2 ml of distilled water and few drops of FeCl3 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 each extract. Formation of precipitate indicated the presence of tannins and phenolic compounds.

Test for Saponins*:* 5 ml of aqueous 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 (Magnesium Hydrochloride reduction 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 organic 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 by Disk Diffusion Method: Fungal spore suspension was prepared from the Sabouraud dextrose agar plates. The spores were picked up using sterile loop and suspended into a 1ml of sterile distilled water. The suspension was further diluted until the cell concentration was 1-2 x 10^5 cells/mL. The methanol extracts (100mg/ml) of the test compounds were used for the experiment. Sterile methanol was used as control. To the Sabouraud Dextrose agar plates 100µl of inoculum was inoculated and spread plated using a sterile cotton swab. 6mm of Whatmann N 1 filter paper discs prepared were soaked in the respective extracts overnight. Flucanazole (10mg/ml) was used as positive control. The test compounds and standard disks were placed on Agar plates and were incubated at 35 ^{0}C for 24-48 hrs and observed for zone of inhibition around the disk.

MIC determination by micro broth dilution technique: Fungal spore suspension was prepared from the Sabouraud dextrose agar plates. The spores were picked up using sterile loop and suspended into a 1ml of sterile distilled water. The suspension was further diluted until the cell concentration was 0.5-2.5 x

10^3 cells/mL. Fluconazole (8-512µg/ml) dissolved in RPMI 1640 media was used as positive control. The extracts of about 16 - 1024 µg/ml dissolved in RPMI 1640 media was used for the treatments. RPMI media was used as negative control. Sterile 96 well plate was sued as the source for the experimentation. 90µl of the drug or test compounds of different test concentration was added to the wells along with 10µl inoculums. 90µl RPMI media alone with 10µl inoculums was added to the well which serves as the negative control. Treated Fungal cultures are incubated at 22^oC and 35^0C respectively. All the treatments were done in triplicates. The plates were incubated at $35\ ^0C$ for 48-72 hrs and the contents were measured for the absorbance at 492nm in Tecan Plate reader. MIC values were determined basing on the concentration of drug.

RESULTS AND DISCUSSION

Any part of the plant like bark, flower, leaves, root, fruit contain the natural phytoconstituents which may be responsible for their bioactivity. A preliminary phytochemical screening is very important inorder to detect these bioactive compounds which can further give way to the development of new, improved & safe therapeutic agents of plant origin for the treatment of infectious diseases. The performed qualitative studies of the 24 extracts revealed that carbohydrates was present in almost all samples (in 23 extracts) followed by alkaloids (14), flavonoids (13), steroids (12) & glycosides (11). In addition to this terpenoids (in 10 extracts), tannins (9) & saponins (7) were also found. Phlobotannins was present in only in one extract and aminoacids were not present in any of the extracts. The results of phytochemical analysis of the extracts of the 6 plants is shown in table 1.

plant extracts 11 extracts showed antifungal activity. Out of the 4 plant parts in each of the 6 plants, 3 parts of *Acalypha hispida* showed significant activity against *C.tropicalis*. In other 3 plants *Hydnocarpus*, *Flacourtia*, & *Euphorbia* 2 parts each showed antifungal activity & in 2 plants *Couroupita guianensis* & *lucuma nervosa* one part showed antifungal activity.

The most significant or highest antifungal activity was shown by *F.jangomas* flowers. Methanol extract of *C.guianensis* showed broad spectrum of antibacterial and antifungal activity when compared with aqueous extract (Kavitha R.*et al*., 2011).

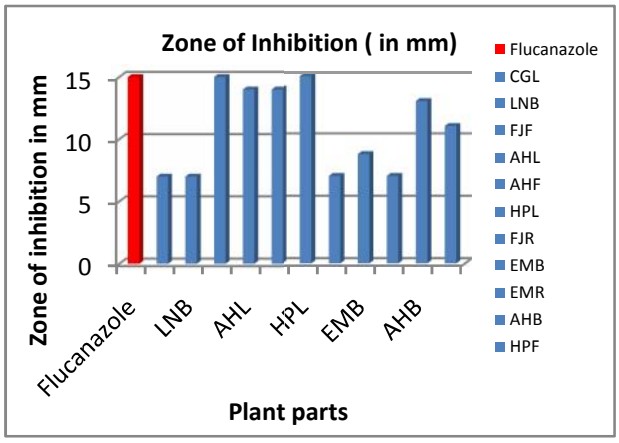

Fig 1: Graph showing the zone of inhibition values of the plant extracts against *C.tropicalis.* All the values were expressed in mm. CGL: *Couroupita guinensis* leaves; LNB: Lucuma nervosa bark; FJF: Flacourita jangomas flower; AHL: Acalypha hispida leaves; AHF: Acalypha hispida flowers;

Table 1 Phytochemical constituents of the plant samples. L: Leaves; B: Bark; F: Flower; R: Root.

Phytoconstituents	*C.guianenensis*				*F.jangomas*				*L.nervosa*				*E.mili*				*A.hispida*				*H.pentandra*			
	L	B	F	R	L	B	F	R	L	B	F	R	L	B	F	R	L	B	F	R	L	B	F	R
Carbohydrates	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Amino acids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alkaloids	-	-	-	+	+	-	-	+	+	+	+	+	+		-	-	+	+	+	-	+	-	-	-
Tannins	-	-	-	+	+	-	-	-	+	-	-	-	-	+	-	-	-	+	-	-	+	-	+	+
Phlobatamines	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Saponins	-	-	-	-	-	-	-	+	-	-	+	+	-	-	-	-	-	-	-	+	-	+	-	-
Flavanoids	-	-	-	-	-	-	-	+	+	-	+	-	+	+	-	-	+	+	+	-	+	+	+	+
Terpenoids	-	-	-	+	+	-	+	-	+	-	-	-	-	+	-	-	-	+	-	+	+	-	+	+
Glycosides	+	-	+	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	-	-	+	-	+	+
Steroids	-	-	-	+	+	-	-	+	+	-	-	+	-	-	-	-	-	+	+	+	+	+	+	-

The methanol extracts of parts of 6 plants were tested against the pathogenic microbe, *Candida tropicalis*. *C.tropicalis* is an emerging CNA sp. *Lactobacillus acidophilus*, *C.albicans* & *C.tropicalis* etc are some other microbial species that knowingly cause several oral diseases such as dental caries, endodontic infections, periodontal diseases and oral candidiasis (Akpan *et.al*., 2002). The results of antifungal activities in terms of zone of inhibition (mm) is presented in the table 3. Flucanazole was used as a reference standard. Out of the 24

HPL: *Hydnocarpus pentandra* leaves; FJR: Flacourita jangomas root; EMB: *Euphorbia mili* bark; EMR: *Euphorbia mili* roots; AHB: Acalypha hispida Bark; HPF: *Hydnocarpus pentandra* flowers. All the values are average of triplicates.

Extracts of *Flacourita jangomas* flower, Acalypha hispida leaves, Acalypha hispida flowers and Acalypha hispida flowers showed highest inhibition zones when compared to positive control. The zone of inhibition. The decreasing order of

antifungal activity of extracts of the plant parts are given as below: F.jangomas flowers>H.pentandra leaves> A.hispida leaves >A.hispida flowers> A.hispida bark >H. pentandra flowers> E. mili bark >C.guianensis leaves, L.nervosa bark> F.jangomas root, E.mili root.

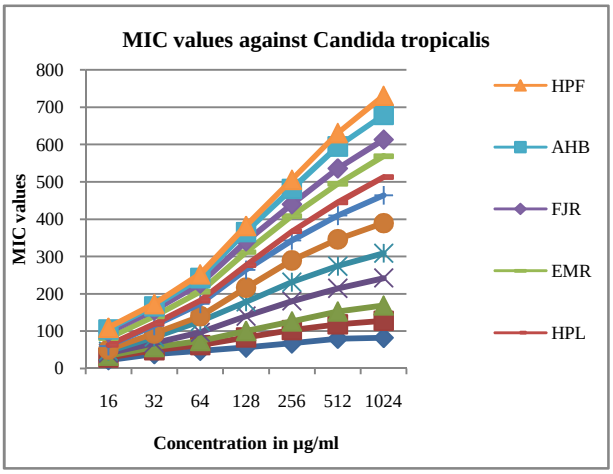

Fig 2: Graph showing the MIC values of the plant extracts against extracts against *C.tropicalis.* all the values were expressed in mm. CGL: *Couroupita guinensis* leaves; LNB: Lucuma nervosa bark; FJF: Flacourita jangomas flower; AHL: Acalypha hispida leaves; AHF: Acalypha hispida flowers; HPL: *Hydnocarpus pentandra* leaves; FJR: Flacourita jangomas root; EMB: *Euphorbia mili* bark; EMR: *Euphorbia mili* roots; AHB: Acalypha hispida Bark; HPF: *Hydnocarpus pentandra* flowers. All the values are average of triplicates.

Significant antifungal activity was shown by *Flacourtia jangomas, Hydnocarpus pentandra* and *Acalypha hispida*. MIC values observed were 256µg/ml, 512µg/ml and >1024 µg/ml. Zone of inhibition of the extracts were compared with the standard Flucanazole. The MIC values of *Hydnocarpus pentandra* Leaves, *Acalypha hispida* Leaves, *Acalypha hispida* flowers and *Flacourtia jangomas* flowers were all 256µg/ml respectively.

CONCLUSION

The plants used in the study has scientifically proved their antifungal efficacy against *Candida tropicalis* which is an emerging resistant strain that ranks second or third causative agent of many candidal infections. The obtained results in this study present the first report on the antifungal activities of *C.guianensis, F.jangomas, L.nervosa, E.mili, A.hispida & H.pentandra* against *Candida tropicalis*. This confirms antifungal effectiveness and therapeutic applications of the examined plants. Further studies which aim at the isolation and structure elucidation of the active constituents from these plants have been planned. Significant antifungal activity was shown by *Flacourtia jangomas, Hydnocarpus pentandra* and *Acalypha hispida*. MIC values observed were 256µg/ml, 512µg/ml and >1024 µg/ml. The phytochemical analysis carried out revealed the presence of carbohydrates, alkaloids, flavanoids, terpenoids, tannins, steroids, glycosides and saponins in most of the plants.

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TSSN 0976-3031