Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Issue 2, 2012

Research Article

IN-VITRO ANTIFUNGAL ACTIVITY OF *HYBANTHUS ENNEASPERMUS* F MUELL

NAPOLEON ARUMUGAM*, SASIKUMAR KANDASAMY, M.SEKAR**

Pharmaceutical Chemistry Division, School Of Advanced Sciences, Vit University, Vellore, Tamil Nadu 632 014, India. **Ehrlich Laboratory, Chennai, India. Email: aanapoleon@vit.ac.in, neppharma@yahoo.co.in.

Received: 13 Dec 2011, Revised and Accepted: 10 Feb 2012

ABSTRACT

The aim of present investigation was to evaluate the antifungal efficacy of the different plant extracts of *Hybanthus enneaspermus*. The antifungal property of the petroleum ether, chloroform and methanol extracts of the plant *Hybanthus enneaspermus*, were tested against a group of fungi, viz., Aflatoxin producing fungi, *Aspergillus flavus, Aspergillus fumigatus* and human pathogenic fungi *Candida albicans* and *Candida tropicalis* by well diffusion method. The methanol extract exhibited significant antifungal activity on most of the fungal strains, viz. *Aspergillus flavus*, *Aspergillus fumigatus, Candida albicans and Candida tropicalis* followed by the petroleum ether extract and chloroform extract. This report is first of its kind showing antifungal activity of *Hybanthus enneaspermus.* Preliminary phytochemical screening revealed that the petroleum ether extract shows presence of both triterpenoids and steroids, chloroform extract shows presence of steroids only and methanolic extracts shows presence of steroids, alkaloids, flavonoids, saponins, phenolic compound, amino acid, protein and tannins.

Keywords*: Hybanthus enneaspermus*, Antifungal activity, Phytochemical, Fluconazole, *Aspergillus flavus, Candida albicans*

INTRODUCTION

Plants that are traditionally used in the treatment of bacterial and fungal infections or related ailments could be a good source for new safer drugs and could offer potential lead in the development of novel herbal medicines that are active against pathogenic micro organisms. Many plants are gaining importance due to fungi toxicity. Natural products of higher plants may possess a new source of antimicrobial agents with possibly novel mechanism of action. They are effective in the treatment of infectious diseases, while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials[1]. Numerous research works has been done aiming to know the different antimicrobial and phytochemical constituents of medicinal plants and in using them for the treatment of microbial infections (both topical and systemic applications) as possible alternatives to chemically synthetic drugs to which many infectious microorganisms have become resistant[2].

Hybanthus enneaspermus[3] F. Muell (Synonyms: *Ionidium enneaspermus* DC, Family: Violaceae) is native of high altitude Himalaya region and warmer parts of India from Delhi to Bengal and throughout Deccan Peninsula. It is reported to be used as a tonic, diuretic[4], anti-gonorrhoetic and demulcent. Roots are given in urinary infections and for bowel complaints of children[5]. The plant has been reported to have anti-inflammatory[6], antitussive[7], antiplasmodial[8], anticonvulsant[9], free radical scavenging activity and antidiabetic[10].

Hybanthus enneaspermus: A small, suffrutescent perennial herb 15-30cm high, with many diffuse or ascending branches, glabrous or more or less pubescent stem sparingly branched with woody base and spreading erect branches. Root spindle shaped, cylindrical, rough and light yellow in colour leaves linear or lanceolate, 4-5cm. by 3-8mm, subsessile, entire or with serrated margins; stipules gland tipped, subulate. Flowers red, axillary, solitary, pedicels shorter than the leaves, 6-12mm long, erect, slender; bracts small, above the middle of the pedicel. Capsules are about 6mm diameter, subglobose with ribbed seeds. Seeds ovoid, acute, longitudinally striate, yellowish white, about 1.5mm long. *Hybanthus* collected from sandy soil has been identified for possible cultivation in gardens for ornamental purposes. Their small sized body leaves and flowers are of different interesting conformation and colour which can attract many people.

The components of the plant dipeptide alkaloid (Aurantiamide acetate), a Triterpene (Isoarborinol) and β –Sitosterol are previously isolated from *Hybanthus enneaspermus.* Although chemical studies were shown for the presence of alkaloids, flavonoids, triterpene and steroids in this species, but no data are available concerning its detailed structural data[11] and its biological activities. *Hybanthus enneaspermus* is of considerable chemotaxonomic importance, as it constitutes the first reported of a Violaceae plant to elaborate a peptide alkaloid[12].

In the present investigation, phytochemical screening and efforts were made to find out the antifungal activity of different extracts of *H.enneaspermus* on Aflatoxin producing fungi, *Aspergillus flavus, Aspergillus fumigatus* and human pathogenic fungi *Candida albicans* and *Candida tropicalis* by well diffusion method.

MATERIALS AND METHODS

Plant material collection

The plant of *Hybanthus enneaspermus* (family: Violaceae) for present studies were identified by a Mr. Prabhakaran, Horticulturist in VIT University. The entire plant and plant powder used for present studies were collected from the Sri Vinayaga Herbals –Madurai. The powdered plant materials (shade dried) were used for the extraction and isolation purpose.

Preparation of extracts

The dried powdered plant material (500gm) of *Hybanthus enneaspermus* were extracted petroleum ether (60-80°C) by using Soxhlet's apparatus for 9hrs and the solvent was removed after the completion of extraction process, the extracts were concentrated by distillation under vaccum. The petroleum ether marc was air dried before extracting with chloroform and the chloroform extracts were concentrated by distillation under vaccum. The same chloroform marc was taken and air dried and it is extracted with methanol. Methanolic extracts were concentrated by distillation under vaccum. Solvents were removed from all the three extracts under vacuum and a semisolid mass was obtained. The extracts were stored in a refrigerator.

Preliminary phytochemical studies

Preliminary phytochemical studies were carried out for the presence of saponins, tannins, flavonoids, anthraquinones, terpenoids and alkaloids using various color reactions to identify the nature of phytoconstituents presence[13, 14](Table 1).

Antifungal Assay

Preparation of test solution

Accurately weighed dried extracts were dissolved in Dimethyl Formamide (DMF) in sterile test tubes to obtain concentration of 2mg/mL; 4mg/mL and 5mg/mL of the each extract (Petroleum ether, Chloroform and Methanolic extract) and subjected to

antifungal screening. 0.5gm of plant powder mixed with 20ml of Potato Dextrose Agar (PDA) medium constitute 25mg/ml. the control (a) contained only 20mL of PDA medium, control (b) contained 200mg of Bavistin (fungicide) added to 20mL of PDA medium with (10mg/ml).

Media

The culture media used for antifungal testing were Potato Dextrose Agar (PDA) of HiMedia Pvt. Ltd., Mumbai, India.

Microorganisms Used

Aspergillus flavus, Aspergillus fumigates, Candida albicans and Candida tropicalis

Inoculum Preparation

The fungal stock cultures were maintained on Potato Dextrose Agar slant, which were stored at 4°C. Each microorganisms maintained on PDA agar base were used to assess the antifungal activity of the plant extracts. Three or five similar colonies were selected and transferred to 50ml Potato Dextrose broth cultures were incubated for 48h at 25°C. Inoculum of the test organisms were prepared by inoculating a loopful of organism into sterile saline from 24hrs old cultures on PDA agar slants incubated at 25°C. The turbidity was matched with 0.5 McFarland standards[15] which correspond to 1.5 X 10^8 CFU/mL. One mL of this suspension was diluted aseptically to 10mL with sterile saline to give culture density of about 10^7 CFU/mL.

Antifungal Susceptibility Test (AST)

Antibiotic sensitivity test was conducted against standard antibiotics Fluconazole (1000µg/ml) for all fungal species by well diffusion method. Stock solutions of standards were prepared in DMF and diluted with same to obtained required standard concentration. Antifungal susceptibility test was performed by cup plate method as described under antimicrobial screening, where in the one cups were filled with 0.1mL (100 µl) of respective stock solutions of antibiotics with micropipette.

Preparation of standard drug (Fluconazole)

20 Fluconazole tablets (each tablet containing 150mg of Fluconazole[16]) are taken and weighed. The tablets are crushed in a mortar and the weight of powder equivalent to 100mg of Fluconazole is taken in a 100ml beaker and 50ml methanol is added to it. The solution is stirred well for 10mins and filtered. The residue is washed twice with 20ml methanol each. The filtrate is taken in a 100mL volumetric flask and the volume is made to 100mL with methanol. The resultant solution contains 1000µg/mL of solution.

10ml of the solution is then taken and it is evaporated to dryness in a Round Bottom flask. The dried contents are then dissolved in 5ml of Di-methyl formamide (DMF) and poured in a sample vial. The RB flask is washed two times each with 2ml of DMF and finally the volume of the sample vial is made into 10ml with DMF. The DMF solution contains 1000µg/mL of Fluconazole.

Antifungal Screening

Well Plate Diffusion Method

The plant extracts were tested for antifungal activity by cup plate (well plate diffusion[17]) method using four bacterial cultures. 0.1mL of fungal suspension was thoroughly mixed with 25mL of sterile molten PDA agar and poured in presterilized petri plates and set aside. After congealing the seeded agar was punched out with sterile cork borer in order to make three cups (6mm) at a spaced out position in the petri plate.

The entire three well were filled with 0.1mL (100 µL) of the Petroleum ether extract at three different concentrations 2mg/mL, 4mg/mL and 5mg/mL. This procedure is repeated for other extract like chloroform and methanol extract. Culture control and DMF as solvent control were also maintained. These agar plates were set aside at room temperature for one hour for diffusion and then incubated at 25°C for 48 hours. After incubation, the diameter of zone of inhibition was measured in millimeter (mm) and the value was recorded.

RESULT AND DISCUSSION

In the present investigation, the methanolic extract showed maximum inhibitory effect on the four fungal strains, viz. *Aspergillus flavus*, *Aspergillus fumigatus, Candida albicans and Candida tropicalis* followed by the petroleum ether extract and chloroform extract (Table 2). The results indicated that as the concentration of the plant extract increased, the zone of inhibition produced also found to be increased. All the extracts showed high to moderate zone of inhibition against four fungal strains when compared to the standard drug.

This report is first of its kind showing antifungal activity of *Hybanthus enneaspermus.* The results of this study support the use of these plants for human and animal disease therapy and reinforce the importance of the ethnobotanical approach as a potential source of bioactive substances

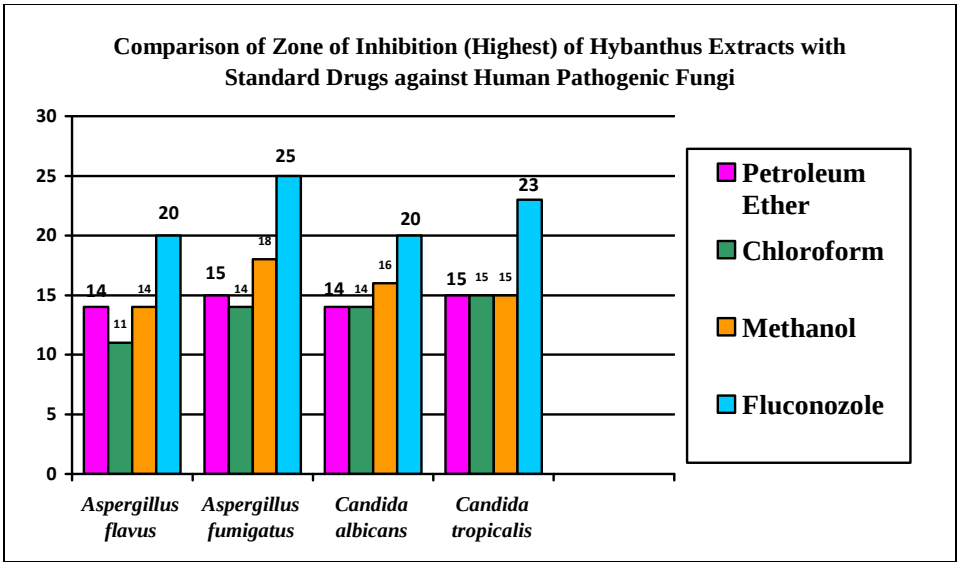

Fig. 1: Comparison of zone of Inhibition (highest) of *Hybanthus* extracts with standard drugs against human pathogenic fungi.

Table 1: Phytochemical Investigation of the different extracts of *Hybanthus enneaspermu*

S.No	Phytochemical Test	Petroleum Ether	Chloroform	Methanol
1	Triterepenoids	**+**	**-**	**-**
2	Steroids	**+**	**+**	**+**
3	Alkaloids	**-**	**-**	**+**
4	Flavonoids	**-**	**-**	**+**
5	Phenolic Compounds	**-**	**-**	**+**
6	Tannin	**-**	**-**	**+**
7	Saponin	**-**	**-**	**+**
8	Amino acid	**-**	**-**	**+**
9	Protein	**-**	**-**	**+**

Table 2: Antifungal activity of plant extract of *H.enneaspermus* at 25°C ±2°C

Plant Extract	Concentration mg/mL	Microorganisms (zone of inhibition in mm)			
		Aspergillus flavus	***Aspergillus fumigatus***	***Candida albicans***	***Candida tropicalis***
Fluconazole	1	20	25	20	23
DMF	-	-	-	-	-
Petroleum ether	2	8	13	9	11
	4	10	14	12	14
	5	14	15	14	15
Chloroform	2	8	11	10	10
	4	10	13	12	11
	5	11	14	14	15
Methanol	2	10	12	10	10
	4	11	15	12	12
	5	14	18	16	15

ACKNOWLEDGEMENT

The authors wish to thank the Chancellor and Vice chancellor and Colleagues of VIT University, Vellore, TN, India for their constant encouragement and providing necessary facilities to carry out this study.

REFERENCES

1. Sowmya G. Shetty, Vinayachandra, Syed Hidayathulla, K.R. Chandrashekar. Antimicrobial activity and phytochemical screening of *Pterospermum reticulatum* wight & arn. International Journal of Pharmacy and Pharmaceutical Sciences. 2011: 3(5): 35-37.
2. Surekha Shukla, Sanjukta Chatterji, Deepak Kumar Yadav, Geeta Watal. Antimicrobial efficacy of *raphanus sativus* root juice. International Journal of Pharmacy and Pharmaceutical Sciences. 2011: 3(5): 89-92.
3. Krithikar.K.R, Basu.B.D (1975). Indian Medicinal Plants, International Book Distributors**,** Dehra Dun, 212-213.
4. Chopra.R.N, Nayar S.L, Chopra I.C (1956). Glossary of Indian medicinal plants (CSIR, New Delhi), 129-130.
5. Khare. C.P (2007). Indian medicinal plants, Springer International Edition, 329-330.
6. Boominathan.R, Parimaladevi.B, Mandal. S.C, Ghoshal. S.K. Anti-inflammatory evaluation of *Ionidium suffruticosam* Ging. in rats**.** Journal of Ethnopharmacology. 2004: 91:367-370.
7. Boominathan.R, Parimala Devi. B, Mandal. S.C. Evaluation of antitussive potential of *Ionidium suffruticosam* Ging. (Violaceae) extract in albino mice. Phytotherapy Research. 2003: 17: 838-839
8. Weniger.B, Lagnika.L, Vonthron Senecheau.C, Adjabimey.D, Gbenou.J, Moudachirou.M, Brun. R, Anton. R, Sanni. A. Evaluation of ethnobotanically selected Benin medicinal plants for their *in vitro* antiplasmodial activity. Journal of Ethnopharmacology. 2004: 90**:** 279.
9. Hemlatha. S, Wahi. A.K, Singh.P.N, Chansouria.J.P.N. Anticonvulsant and free radical scavenging activity of Hybanthus enneaspermus: A preliminary screening. Indian Journal of Traditional Knowledge.2003: 2 (4): 383-388.
10. Irfan Ali Khan, Atiya Khanum (2005). Herbal Therapy for Diabetes Volume –I, Ukaaz Publications. Hyderabad, 36-37.
11. Warnhoff. E.W (1970). Foertschritte der chemie organischer naturstoffe, Volume 28, (Springer-Verlag, Berlin), 162-203.
12. Raveendra Retnam, John De Britto. Antimicrobial activity of a medicinal plant *Hybanthus enneaspermus* (Linn.) F.Muell. Journal of Natural Product radiance. 2007:6: 366-368.
13. Kokate.C.K, Purohir.A.P, Gokhale.S.B (2001). Pharmacognosy, 17th edition, Nirali Prakashan Publication, 607-611.
14. Harborne. J.B (2001). Phytochemical Methods, a guide to modern technique of plants, 3rd Edition, Springer Private Ltd, New Delhi, 40-100.
15. Finegold.S.M, Baron.E.J. (1986). In Finegold and Baron E.J, edited. Diagnostic Microbiology, 7th edition. The C.V Mosby Co. St. Louis, 176-177.
16. Indian Pharmacopoeia, Volume II, (2010), Indian Pharmacopoial Commission, Ghaziabad, India, 1354-55.
17. Perez. C, Pauli. M, Bazer.P. An antibiotic assay by the well agar method. Acta Biologiae et Medicine Experimentalis. 1990: 15: 113-115.