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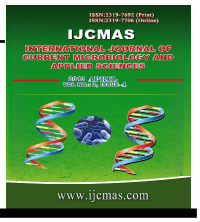

Original Research Article

Antifungal activity of *Euphorbia hirta* L. inflorescence extract against *Aspergillus flavus* – A mode of action study

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Keywords

Euphorbia hirta L; inflorescence; antifungal; cell membrane; Protein leakage.

A B S T R A C T

Euphorbia hirta Linn., a common garden weed belonging to the family Euphorbiaceae is proving to be a herb having a multitude of medicinal properties. Scientific proof for antifungal activities of medicinal plants usually stagnates with the studies of the respective plant parts against many fungi. Very few reports go further into it and verify the mode of action of these antifungal agents. In our study, we have proved that the ethyl acetate extract of the inflorescence of *E. hirta* exhibits possible antifungal activity targeting the cell membrane which could result in leakage of cellular proteins thereby estimated by the method. In this study the minimum inhibitory concentration of the extract was chosen.

Introduction

Euphorbia hirta L. is a pantropic herb found growing as a weed in tropical gardens. Commonly known as garden spurge (English) and Ammaan patcharisi (tamil), it has several clinical applications to its credit. It is proven as an antibacterial Vijaya *et al*., 1995; Vijaya and Ananthan , 1997) and antifungal agent with a number of published reports asserting this fact. Nonetheless, the mode of action study of the inflorescence of *E. hirta* L. against mould growth is lacking which is the reason this study is being published.

E. hirta L. has been proven to have wide pharmacological activities like antifungal, antibacterial, larvicidal and so on (Lanhers *et al.*, 1990; 1991). Suresh *et al*., (2008) has studied the antifungal potential of *E. hirta* L. ethanolic extract of leaves against *A. flavus*. Khan *et al*., (2011) reported the antifungal activity of *E. hirta* L. (methanolic extract) on *A. flavus*. Ethanolic extract of *E.hirta* L. showed an antifungal activity against *A. niger* using paper disc diffusion method (Mohamed *et al*., 1996). Bhaskara Rao *et al.*, (2010) have reported antifungal of *E.hirta* L. where they worked on the leaves. Yazdani *et al.*, (2012) have screened plants from Malaysia against *A. flavus* where they used the shoots of *E. hirta L*. Lagnika *et al.*, (2012) have studied the antifungal activity

of some plant extracts on *A. flavus* sporulation. Poor antifungal activity has been reported by Pieme *et al.,* (2008) for the methanol extract of *E. hirta* L.

Materials and Methods

Euphorbia hirta L. whole plant was collected from gardens in and around Chennai, Tamil Nadu, shade dried and the inflorescence part of the plant was separated and powdered before put to further use. A voucher specimen of the plant is deposited in the herbarium (Presidency College , Voucher No. 8413).

Preparation of extract

The powdered portion of the inflorescence (10g) was subjected to hexane treatment (to remove fats) followed by ethyl acetate (100 ml) for a day after which the solvent was evaporated and the residue weighed and solubilized in 50% of DiMethyl Sulfoxide (DMSO) for further use.

Preparation of inoculum

Aspergillus flavus strains were isolated from groundnuts and air and maintained in Potato Dextrose Agar (PDA). A spore load of 8.8 and 7.9 x 10^5 from food and air borne stains respectively was used as inoculum for further study. Briefly, 10 ml of sterile distilled water was poured in a plate covered with the strains. After one swirl, the broth was transferred to a sterile test tube. 0.01ml of Tween 80 was added to obtain uniform distribution of the spores. 0.1 ml of the suspension was loaded into Haemocytometer for performing spore count.

Microbroth Dilution Assay

96 well Microtitre plate was used for this assay. The ethyl acetate extract dissolved in 50% DMSO having a concentration of 1.82mg/ml was used as a neat dilution. Method of Perumal *et al*., (2012) was used to quantify the Minimum Inhibitory Concentration (MIC) of the extract.

Fluconazole and Amphotericin B were included as drug controls (10µg/ml). First three wells had the test sample in triplicate subsequently diluted from well B to well H, followed by Drug 1 (three wells) and Drug 2 (three wells). 50% DMSO (last three wells) was also included as solvent control along with positive media control of PDB.

Spore Count Assay

100 µl of spores of *A. flavus* with the standard spore load mentioned above was mixed with the MIC dilution of the extract (100µl). After incubation for 24 h at room temperature, spore counts were performed using haemocytometer (Lagnika *et al*., 2012). The Percentage Inhibition was calculated by the formula:

$$\textbf{PI \%} = \textbf{A - B / A x 100}$$

where A refers to the control spores and B refers to the test (extract treated) spores.

Protein Leakage Study

A spore load of 6.33 x 10^5 for air borne strain and 5.99 x 10^5 for the food borne strain was used for the assay. 50 ml of the spore suspension was mixed with equal volume of the MIC dilution of the plant extract and protein leakage estimated using the method of Lowry *et al*., (1951), at regular time intervals of 25 min (air borne strain) and 20 min (food borne strain) at 660 nm.

Results and Discussion

Microbroth Dilution Assay

The micro broth diluton assay revealed the MIC of the ethyl acetate extract as 606 µg. Drug 1 (Fluconazole) exhibited MIC of 3.33 µg whereas Drug 2 (Amphotericin B) showed inhibition at 1.66 µg (Plate 1). The cultures from well C-H exhibited morphological variations as observed under the phase contrast microscope. Only vegetative growth was observed in the wells at C. The slide mount of plant extract treated wells showed hyphal growth with many chlamydospores. The presence of chlamydospores dwindled after wells F-H, only vegetative growth was seen. No reproductive bodies were observed even in DMSO control well which indicates that DMSO cannot be fully relied as inert solvent in such biological assays (Plates 2a - 2e). This indicates that the plant extract may play some, if not an insignificant role in affecting the hyphal morphology.

Spore Count Assay

The inhibition index of the *A. flavus* spores was calculated to be 40 % for the extract treated air borne spores and 64 % for food borne spores. In contrast, the drug controls revealed slightly lesser values *i.e*., 34 % for Drug 1 and 35 % for Drug 2 (air borne) and 45 % for drug 1 and 56 % for drug 2 (food borne strain) (Table 1)

Protein Leakage Study

The Lowry *et al*.,(1951) method of protein estimation of the (extract and drug control) treated food borne *A. flavus* culture at regular time intervals of 20 min revealed some changes in the leakage pattern. Maximum leakage was observed after 80 min of incubation with plant extract. Both the drug controls showed very less leakage of protein when compared with the test but when compared with the control at 80 min incubation, drug 1 and 2 had some significant increase in OD values even if it was at the negative range (Fig. 1). From the results of airborne fungal strain of *A. flavus* (Fig. 2), significant amount of protein leakage is evident after 2 h 15 min of incubation with the plant extract.

The Drug 1 showed its maximum release after 1 h 50 min that was considerably lesser than that of extract treated culture. Maximum leakage by Drug 2 was evident after 2 h 15 min of incubation with the OD value at a lower value than the test and Drug 1.

From the results, it is clear that the ethyl acetate extract of *E. hirta* L. does have some inhibitory effect on the *A. flavus* strains isolated form food and air. Even though the MIC of drug controls is significantly low, it is assumed that the drugs are in their purest form and the plant extract is at its crudest form which is why the MIC of extract is higher than the drug controls.

Nonetheless, our MIC values of extract are much lower than the previous reported results that were in milligram levels, (Dhole *et al.,* 2011; Bhaskara Rao *et al.*, 2010; Sharma *et al.*, 2010; Geeta Singh and Padma Kumar, 2011; Momoh *et al.*, 2011). Though the effect of the extract and the drug controls did not reveal much by way of morphological changes, one cannot deny that DMSO (50%) has some, if not a profound effect on the morphology of the fungal strains. Therefore, its use for biological assays has to be reconsidered.

Table.1 Spore Count Assay using ethyl extract of *E. hirta* L. Percentage Inhibition of spores

Type of spores	Control	Test	Drug 1	Drug 2	Test PI	Drug 1 PI	Drug 2 PI
Air borne	880	520	580	570	40%	34%	35%
Food borne	790	280	430	340	64%	45%	56%

Plate. 1 Microtiter plate showing the MIC results of ethyl acetate extract on *A. flavus*

Figure.1 Protein leakage study on airborne *A.flavus*

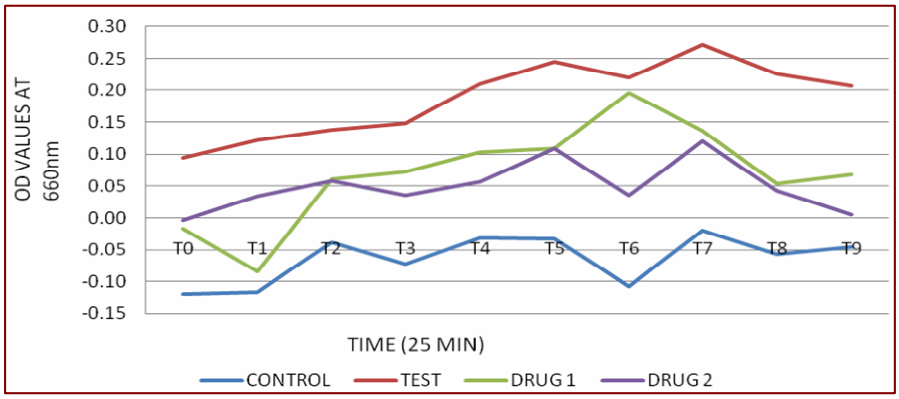


Figure.2 Protein leakage study on Food borne *A.flavus*

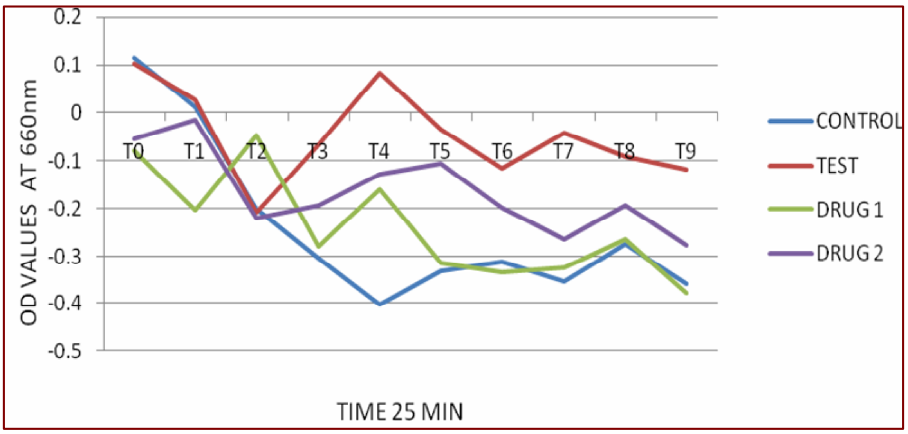
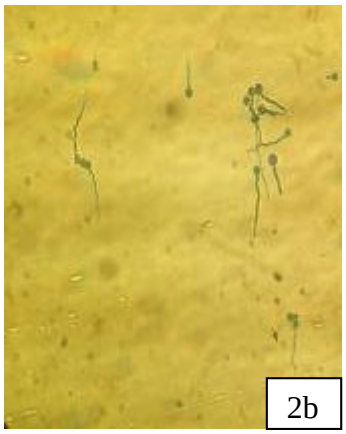


Plate. 2 Hyphal morphology at the MIC dosage – from well B
(as observed in phase contrast microscope)

Control hyphal morphology

2a

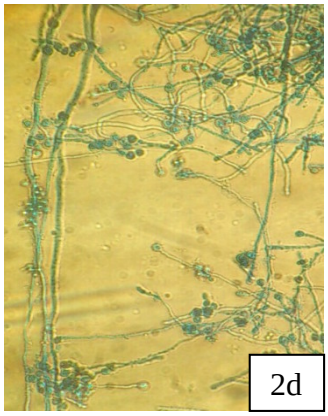
Plant extract treated hyphal morphology from well B



2b

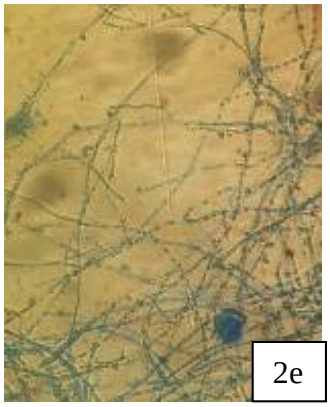
Fungal hyphal morphology of Drug 1 (2c) and Drug 2 (d) treated wells B and C respectively below MIC

2c



2d

50% DMSO treated hyphal morphology



2e

Results of the protein leakage study does indicate some changes in the membrane integrity that could have led to the higher OD values for the test (plant extract treated fungal strain). Protein leakage from *A. flavus* isolated from air revealed a constant increase in the OD values from T0 to T7 where the value is at its highest for the test. Values for drug 1 and drug 2 show marginal increase and values for both are low when compared to extract at time T7. This leads to the assumption that the extract results in a higher leakage than the drug controls. Interestingly, the strain from food revealed lesser level of leakage when compared to that from air. The extract treated sample reveals a high of 0.042 at T4 (80min) whereas drug 1 and 2 gave out very low value at the same time interval of exposure. This could be due to cellular adaptations to the changing environment in the food based niche that may have led to a much integrated cellular membrane structure than that observed in the strain isolated form air. This study can definitely be replicated with clinical isolates which are bound to give better results since they are more susceptible to antifungal agents than environmental isolates since it is understood that environmental strains have much higher adaptation physiologies that make them survive harsh conditions.

References

Bhaskara Rao, K. V., L. Karthik, E.K. Elumalai, K. Srinivasan and Gaurav Kumar. 2010. Antibacterial and antifungal activity of *Euphorbia hirta* L. Leaves: A comparative study. J. Pharm. Res. 3: (3) 548-549.

Dhole, J A., N.A. Dhole K.D. Lone and Bodke, S. S. 2011. Preliminary Phytochemical Analysis and Antimicrobial Activity of Some Weeds collected from Marathwada Region. J.Biol.Sci. (5): 902-907.

Geeta Singh., and Padma Kumar. 2011. Extraction and antimicrobial activity of alkaloids of *Euphorbia hirta*. J. Pharm.Res.4(4): 1228-1230.

Khan A M., R.A. Qureshi, S.A. Gillani and Ulla, F. 2011. Antimicrobial activity of selected medicinal plants of Margalla Hills, Islamabad, Pakistan. J. Med.Plant. Res. 5 (18): 4665-4670.

Lagnika, L., M.H.T. Fantodji and Sanni, A. 2012. Phytochemical Study and Antibacterial, Antifungal and Antioxidant Properties of *Bridelia Ferruginea* and *Pteleopsis Suberosa*, Int. J. Pharm. Sci. Res. 3 (7): 2130-2136.

Lanhers, M. C., J. Fleurentin, P. Dorfman, F. Mortier and Pelt, J.M. 1991. Analgesic, antipyretic and anti-inflammatory properties of *Euphorbia hirta*. Planta Medica. 57: 225-231.

Lanhers, M.C., J. Fleurentin, P. Caballion, A. Rolland, P. Dorfman , R. Misslin and Pelt, J.M. 1990. Behavioral effects of *Euphorbia hirta* L: sedative and anxiolytic properties. J. Ethnopharma. 291-89

Lowry, O.H.H., N.I. Rosebrough, A. L. Farr, and Randall, J. R. 1951. Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193:265-275.

Mohamed, S. , S. Saka, S.H. ElSharkawy, S.M. Ali and Muid, S. 1996. Antimycotic screening of Malaysian plants against plant pathogens. Pest. Sci. 47: 259-264.

Momoh, A. R. M., O.B. Idonjije, O. Okhai, O.I. Iribhogbe, A.A. Momoh, H.O. Otamere, C.N. Ekhator, P.O. Okolo and Oseghale, D. A. 2011. The antifungal effects of extract of *Euphorbia hirta* on selected pathogenic fungi. New York Sci. J. 4: (8) 77-79.

Perumal, S., S. Pillai, L.W. Cai, R. Mahmud and Ramanathan, S. 2012. Determination of Minimum Inhibitory Concentration of *Euphorbia hirta* (L) extracts by Tetrazolium Micro-plate Assay. J. Nat. Prod. 5: 68-76.

Pieme, C. A., J.P. Dzoyem, F.A. Kechia, F.X. Ethoa and Penlap, V. 2008. *In-vitro* antimicrobial activity of extracts from some Cameroonian medicinal plants. J. Biol.Sci.8 (5): 902-907.

Vijaya, K., and Ananthan, S. 1997. Microbiological screening of Indian medicinal plants against enterophathogens. J. Alter. Complemen. Med. 1: 15-18.

Vijaya, K., R. Nalini and Ananthan, S. 1995. Antibacterial effect of the aflavin, polyphenon 60 and *Euphorbia hirta* L. on *Shigella* spp. – a cell culture study. J. Ethanopharma. 49: 115-118.

Yazdani, D., M.A. Zainal Abidin, Y.H. Tan, S. Kamaruzaman and Jaganath, I. B. 2012. J. Med. Plant. Res. 6 (42): 5464-5468.