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# Effect of Euphorbia Pulcherrima Leaf and Inflorescence Extract on Various Cytomorphological Parameters of *Aspergillus fumigatus*

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***Abstract*—**Microorganisms can be removed, inhibited or killed by physical agents, physical processes or chemical agents but they have their inherent disadvantages such as increased resistance against antibiotics etc. Since, plants have endless ability to synthesize aromatic substances which act as the master agents for plant defense mechanisms against microorganisms, insects and herbivores. Thus, secondary metabolites or phytochemicals obtained from plants can be used as agents of disease control nowadays. In the present study effect of different concentrations of acetone fraction of leaves and alcohol fraction of inflorescence of *Euphorbia pulcherrima* on various cytomorphological parameters i.e. cell number, mycelium width, conidial size, conidiophore size etc. of *Aspergillus fumigatus* has been studied. Change in mycelium/ hyphal cell width, conidium size, conidiophore size etc. was measured with the help of a previously calibrated oculometer. To study effect on morphology, fungal mycelium along with conidiophore and conidia were stained with cotton blue and mounted in lactophenol and observed microscopically. Inhibitory action of the acetone extract of *Euphorbia pulcherrima* leaf on growth of *Aspergillus fumigatus* was investigated. Control containing extract free medium supported profuse growth of the fungus. Although decrease in growth was observed even at 3.95µg/ml but significant inhibition of growth was started at7.81µg/ml concentration of the extract. Complete inhibition was observed at 15.62µg/ml and above. Microscopic examination revealed that at 3.95, 7.81 and 15.62µg/ml extract concentration hyphal cell width was found to be increased from 1.44µm in control to 3.86, 5.24 and 8.98 µm respectively giving a beaded appearance to the mycelium. Vesicle size was reduced from 24.78x20.08µm (control) to 11.34x10.06µm at 3.95µg/ml concentration. At 7.81 and 15.62µg/ml concentration no phialides and sterigmata were observed. Inhibitory action of the alcohol extract of inflorescence on the growth of *Aspergillus fumigatus* was also studied. Control containing extract free medium supported profuse growth of the fungus. Although decrease in growth was observed even at 3.95µg/ml but complete inhibition was observed at 62.5µg/ml and above. Microscopic examination revealed that hyphal cell width of *Aspergillus fumigatus* was found to be increased from 1.67µm in control to 5.84µm at MIC i.e. at 62.5µg/ml. Vesicle size was reduced from 44.76x 24.22µm (control) to 11.36x 6.80µm at 15.62µg/ml concentrations. At 31.25 µg/ml and 62.5µg/ml concentration no phialides and sterigmata was found. Spore germination was completely found to be inhibited at 3.95µg/ml concentration. Similarly 92.87% reduction in vesicle size was observed at 15.62µg/ml concentration. It is evident from the results that plant extracts inhibit fungal growth and this inhibition is concentration dependent.

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## I. Introduction

MICROORGANISMS are able to reproduce tremendously within a relatively short time when they find favorable conditions such as abundant supply of nutrients, optimum temperature and pH etc. This is the reason most pathogens can survive and proliferate in human body. Extreme growth of the pathogens results in various diseases, thus in order to treat a disease it is very essential to inhibit the growth of microorganisms. Plants posses tremendous ability of producing secondary metabolites which have been used for plant defense mechanisms against microbes. Thus, plant extracts and their products are now being used as antimicrobial agents. Antimicrobial agents have multiple target sites in microbial cells for inhibition or destruction of microorganisms resulting in microbiostatic and microbicidal effect. The main target sites affected by the antimicrobials are generally cell wall, cell membrane and cytoplasm of microorganism including the metabolic activity and metabolites. Some antimicrobials act directly on membrane and affect the permeability as well as viability. The biological and molecular action of plant secondary metabolites induces various morphological and cytological changes in microorganisms. Thus, the inhibitory effect of plant extracts can be studied at macroscopic as well as microscopic level. The spores of *Aspergillus fumigatus* are dark greenish blue and are responsible for the colour of fungal colony. A model that explains the mechanisms, which control sporulation in *Aspergillus* sps., suggests that conidiophore formation in this genus or in filamentous fungi occurs in response to extrinsic or intrinsic signals. These signals activate specific transcription factor that control the expression of each other as well as conidiophores morphogenesis genes. Activation of heterotrimeric G protein stimulates vegetative growth and represses asexual and sexual reproduction and production of toxic secondary metabolites. Inactivation of this pathway by extracellular signals leads to conidiophores formation. The inhibitory effect of benzene fraction of stem and petroleum ether fraction may be due to activation of G protein signaling pathways [1]. Fischer (1995) has reported that expression of two genes brl A and aba A are specifically required for conidiation and spore formation in *Aspergillus nidulans*. Suppression of asexual reproduction by plant extracts may be due to inactivation of these two genes [2]. Clutter buck (1969)

reported that when nuclei fail to migrate from vesicle into metulae, development stops at the stage of secondary sterigmata bud activation [3].

Comparative efficacy of the extracts, with five synthetic fungicides such as bavistin, blitox, captan, dithane M-45 and thiram revealed that, complete inhibition of mycelia growth of all the test fungi were observed only in thiram even compared to test plants. Dithane M-45 recorded least activity. Highly significant inhibition of mycelia growth of *Curvularia lunata* was observed in methanol extract of *Decalepis hamiltonii*, *Mimosops elengi*, *Lawsonia inermis* and *Acacia nilotica* compared to blitox and dithane M-45 [4]. Farag *et* al. (2013) demonstrated that water extract of peppermint extract was the most effective completely inhibited spore germination and mycelial growth of *Fusarium oxysporum* at concentration of 2%, followed by rheum and garlic extracts which completely inhibited fungal conidiospore germination and mycelial growth on agar medium by the rate of 3% [5]. The present study was done with an aim to determine the effect of different concentrations of acetone fraction of leaves and alcohol fraction of inflorescence of *Euphorbia pulcherrima* on various cytomorphological parameters i.e. cell number, mycelium width, conidial size, conidiophore size etc. of *Aspergillus fumigatus*. Amongst the various fractions of *Euphorbia pulcherrima* leaf and inflorescence best activity was observed with acetone fraction of leaf and alcohol fraction of inflorescence against *Aspergillus fumigatus*. Hence, only these two extracts were used for further research.

## II. Materials and Methods

### *A. Extract Preparation*

*Euphorbia pulcherrima* leaves and inflorescence were shade dried at room temperature and finely ground in an electrical grinder. The ground material was passed through sieve No. 240 so as to obtain powder of mesh size 60, which was used to prepare extract. Reflux method of solvent extraction was used for separation of different organic constituents of dried and powdered leaf as well inflorescence [6], [7]. This method involves continuous extraction of powdered dried plant material in soxhlet apparatus with acetone and alcohol of leaves and inflorescence respectively. 40 gm dry leaves and inflorescence powder was kept in soxhlet extraction unit and extracted with 280 ml acetone and alcohol respectively till soluble fractions were extracted. Residue was dried and weighted to determine the percent extraction of the plant part.

### *B. Effect of Extract on Morphology of Test Fungi*

Change in mycelium/hyphal cell width, conidium size, conidiophore size etc. was measured with the help of a previously calibrated oculometer. To study effect on morphology, fungal mycelium along with conidiophore and conidia were stained with cotton blue and mounted in lactophenol and observed microscopically. Number of oculometer divisions superimposed on conidia or mycelium were counted and then multiplied by calibration factors to get final size of conidia or mycelium. Photographs were taken with the help of Olympus Trinocular Research Microscope BX-51.

## III. Results and Discussion

### *A. Effect of Acetone Extract of E. pulcherrima Leaf on Growth and Morphology of Aspergillus fumigatus*

Results of different concentrations of *E. pulcherrima* leaf acetone extract on cytomophology of *Aspergillus fumigatus* are presented in Table I. It was found that the fungi were inhibited by the extract and the inhibition was directly proportional to the increasing concentration of the extract. Concurrent to this, increase in mycelium width and decrease in spore/conidia size, size of vesicle etc. was also observed.

Table I shows inhibitory action of the extract on growth of *Aspergillus fumigatus*. Control containing exract free medium supported profuse growth of the fungus. Although decrease in growth was observed even at 3.95µg/ml but significant inhibition of growth was started at 7.81µg/ml. Complete inhibition was observed at 15.62µg/ml and above. Microscopic examination revealed that at 3.95, 7.81 and 15.62 µg/ml extract concentration hyphal cell width was found to be increased from 1.44 µm in control to 3.86, 5.24 and 8.98 µm respectively giving a beaded appearance to the mycelium. Vesicle size was reduced from 24.78x 20.08 µm (control) to 11.34x10.06 µm at 3.95 µg/ml concentration. At 7.81 and 15.62 µg/ml concentration no phialides and sterigmata were observed.

### *B. Effect of Alcohol Extract of E. pulcherrima Inflorescence on Growth and Morphology of Test Fungi*

Results of different concentrations of *E. pulcherrima* inflorescence alcohol extract on growth and cytomophology of test fungi are presented in Table II. It was found that the fungi were inhibited by the extract and the inhibition was directly proportional to the increasing concentration of the extract. Concurrent to this increase in mycelium width and decrease in spore/conidia size, size of vesicle etc. was also observed.

Results denote the inhibitory action of the extract on the growth of *Aspergillus fumigatus*. Control containing extract free medium supported profuse growth of the fungus. Although decrease in growth was observed even at 3.95µg/ml but complete inhibition was observed at 62.5µg/ml and above. Microscopic examination revealed that hyphal cell width of *Aspergillus fumigatus* was found to be increased from 1.67 µm in control to 5.84 µm at MIC i.e. at 62.5 µg/ml. Vesicle size was reduced from 44.76x 24.22 µm (control) to 11.36x 6.80 µm at 15.62 µg/ml concentration. At 31.25 µg/ml and 62.5 µg/ml concentration no phialides and sterigmata was found. Spore germination was completely found to be inhibited at 3.95 µg/ml concentration. Similarly 92.87% reduction in vesicle size was observed at 15.62 µg/ml concentration.

TABLE I
EFFECT OF LEAF EXTRACT OF *EUPHORBIA PULCHERRIMA* ON MORPHOLOGY OF *ASPERGILLUS FUMIGATUS*

| S. No. | Extract Concentration (µg/ml) | Hyphal cell width (µm) * | % Increase in hyphal cell width | Vesicle size (µm)* | % Reduction in vesicle size | Spore size (µm) * | % Reduction in spore size |
|---|---|---|---|---|---|---|---|
| 1. | Control (Extract Free) | 1.44±0.03 | - | 24.78x 20.08 ±0.98 | - | 4.98±0.24 | - |
| 2. | 3.95 | 3.86±0.02 | 62.69 | 11.34x 10.06 ±0.32 | 77.07 | NF | - |
| 3. | 7.81 | 5.24±0.21 | 72.51 | NF | - | NF | - |
| 4. | 15.62 | 8.98±0.24 | 83.96 | NF | - | NF | - |

* All values are average of 3 replicates NF= Not Formed

TABLE II
EFFECT OF INFLORESCENCE EXTRACT OF *EUPHORBIA PULCHERRIMA* ON MORPHOLOGY OF *ASPERGILLUS FUMIGATUS*

| S. No. | Extract Concentration (µg/ml) | Hphal cell width (µm) * | % Increase in hyphal cell width | Vesicle size (µm) * | % Reduction in vesicle size | Spore size (µm) * | % Reduction in spore size |
|---|---|---|---|---|---|---|---|
| 1. | Control (Extract Free) | 1.67±0.06 | - | 44.76x 24.22 ±0.89 | - | 6.44±0.89 | - |
| 2. | 3.95 | 1.88±0.03 | 11.17 | 32.06x 14.73 ±0.30 | 56.43 | NF | - |
| 3. | 7.81 | 2.5±0.07 | 33.2 | 21.71x 10.75 ±0.64 | 78.47 | NF | - |
| 4. | 15.62 | 3.76±0.02 | 55.58 | 11.36x 6.80 ±0.48 | 92.87 | NF | - |
| 5. | 31.25 | 4.24±0.13 | 60.61 | NF | - | NF | - |
| 6. | 62.5 | 5.84±0.06 | 71.4 | NF | - | NF | - |

*All values are average of 3 replicates NF= Not Formed

It is evident from the results that plant extracts inhibit bacterial and fungal growth and this inhibition is concentration dependent. It is concluded from the results that as the concentration of the extract increased, colour of the fungal colony also changed and gradually the colony lost all colour and become cream or whitish coloured. It may be due to reduction of sporulation or inhibition of fungal pigments.

Several workers have reported that as the concentration of extract increased, growth of bacteria and fungi decreased [8]. Antimicrobial agents have multiple target sites in microbial cells for inhibition or destruction of microorganisms resulting in microbiostatic and microbicidal effect [9]. The main target sites affected by the antimicrobials are generally cell wall, cell membrane and cytoplasm of microorganism including the metabolic activity and metabolites. Seed oil of *Pongamia pinnata* and *Azadirachta indica* are antibacterial in nature and the activity is mainly due to inhibition of cell membrane synthesis in the bacteria [10]. Rhayour *et al*. (2003) reported that essential oils of *Origanum compactum* and clove, containing two phenolic compounds namely thymol and eugenol are responsible for both cell wall and membrane damaging of the treated bacteria [11].

Leakage of cell electrolytes due to damage to cell membrane eventually leads to cell death. This may be one of the causes for decrease in cell numbers of test organisms. Reduced uptake of nutrients by damaged cell membrane will lead to decreased metabolic activities such as assimilation and synthesis of structural and functional metabolites. Thus, reduction in metabolic activity may be responsible for reduction in cell size. Impairment of membrane results in dissipation of two components of proton motive force, the pH gradients (ΔpH) and electrical potential (Δψ) [12], [13]. The detrimental effects on proton motive force are strongly correlated with leakage of specific ions [14], [15]. Essential oil of tea and mint as well as carvacrol can cause leakage of cellular material. The dissipation of ion gradients leads to impairment of essential processes in the cell and finally to cell death. Therefore decrease is viable number of bacterial cells could be due to action of essential oil and diterpenoids [16].

There was a remarkable decrease observed in growth and reproduction of all test fungi following treatment with acetone extract of leaf and alcohol extract of inflorescence of *Euphorbia pulcherrima*. Tsai *et al.* (1999) suggests that some fungal pigments are natural products and associated with development of reproductive structures. Similarly, dark brown pigment called melanin is formed by oxidative polymerization of phenolic compounds and synthesized during spore formation. In pathogenic fungi, pigments not only contribute to the survival of the fungal spores but also act as an important virulence factor [17]. Tsai *et al*. (1999) reported that disruption of alb L genes which was responsible for colour of fungal spores creates a pigment less conidial phenotypes and leads to a significant reduction in fungal infection of murine model. A similar mechanism may be responsible for the inhibitory effect of acetone fraction of leaves and alcohol fraction of inflorescence [17].

Abnormal filamentation in the fungus has been also reported after treatment with plant extract [18], [19]. Soylu *et al.* (2006) reported antifungal effect of various essential oils and observed morphological alteration in hyphae such as cytoplasmic coagulation, vacuolation, hyphal shrivelling and protoplast leakage [20]. Inhibitory effect of garlic and souring rush extract, two plant products; bio-clean and citronella oil on spore germination, hyphal growth and infection of *Phytopthora infestans* on potato leaves was evaluated [21]. They reported that highest inhibition of *Phytopthora infestans* was obtained with Bio-clean and garlic extract. Bianchi *et al.* (1997) investigated that the aqueous extract of *Allium sativum* caused morphological alterations on the hyphae of *Pythium*

*ultimum*, *Rhizoctonia solani*, *Colletotrichum lindemuthianum* and *Fusarium solani* [22].

These morphological alterations are thought to be produced by various secondary metabolites. Secondary metabolites are target specific and their biochemical and molecular target are mainly proteins such as receptors and enzymes [23]. These secondary metabolites are antimicrobial in nature hence all morphological alterations may be related to effect of secondary metabolites on enzymatic reactions regulating cell wall synthesis as well as disruption of cell membrane. Flavonoid, proanthyocynidins, tannins, quercetin have ability to inactivate microbial adhesions, enzymes, cell envelope, transport proteins etc. [24]. Flavonoids may also disrupt microbial membrane [25]. Therefore change in the morphology of hyphae could be due to the loss of integrity of the cell wall. Consequently plasma membrane permeability might be affected, which could explain the changes in morphology and size of mycelia and sporangia [26]. All these adverse effect on fungal mycelia and hyphae may be responsible for the decrease in growth and reproduction of fungus. TEM images of Aqueous Garlic Extract treated *Aspergillus flavus* cells of the same age showed singular accumulation of osmophil bodies immediately under plasma membrane which became rough and irregular with continuous folding in cytoplasm and detachment of fibrillar layer was also observed. Disappearance of surface ornaments due to presence of massive vacuolation of cytoplasm and spaces filled with amorphous material. Hyphal tips treated with Aqueous Garlic Extract, besides the increase in spaces filled with amorphous material, showed a strong thickening of cytoplasmic membrane that was partially detached from cell wall, whereas the control revealed highly dense cytoplasm and was enveloped with tightly attached cell wall and cytoplasmic membrane. Some hyphal tips were found disintegrated [27].

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