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Inhibition Planktonic and Bio film Growth of Candida Albicans by Plant Extract Alone and in combination with Fluconazole

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Abstract

This study was to evaluate the efficacy of Methanolic extract of G. superba L in combination with Fluconazole against biofilm development and mature biofilms of C. albicans. Synergism between MEGS and Fluconazole combination against biofilm formation was evident with FICI of 0.187. Combination of MEGS and Fluconazole did not have synergistic potential against mature biofilm growth, evidenced in FICI of 0.916. MIC of standard Fluconazole was found to be 0.5 and >0.5 in biofilm development and mature biofilm respectively.

Key words: G. superba L; FICI; MTT; Germ tube assay

Introduction

Glory lily (Gloriosasuperba L.) is an important medicinal crop which belongs to family Liliaceae. It is commonly known with different vernacular names like Agnishikha, Agnimukhi and Garbhaghatini in Sanskrit; Bachnag, Languli and Karihari in Hindi; Supper lily, Tiger claw, Flame lily in English; and Kariannag in Marathi. Studies on the use of plant extracts for the control of diseases have shown the importance of natural chemicals (Phytochemicals) as possible sources of non-phytotoxic and easily biodegradable alternative fungicides and antibiotics. Against this background, the endangered medicinal plant Gloriosa superba was selected to evaluate its potential inhibitory effect against *Candida albicans*. Involvement of *Candida albicans* biofilms in clinical infections is a serious problem for immune compromised patients. Being a commensally it easily forms biofilms on host tissues as well as various prosthetic devices in the patient's body. It is reported to form biofilms on urinary catheters, intra-venous catheters, denture materials, central nervous system prostheses, artificial heart valves, joint prostheses, contact lenses, penile implants, and intrauterine devices as well as host tissue surfaces.[1]

Potential inhibitory effects of Gloriosa Superba on human pathogenic fungi

Man always been surrounded by countless microorganisms. The disease producing microbes are playing a very important role in human life. Pathogenic microorganisms are always trying to develop resistance to the various antimicrobial agents used for their control. Infectious diseases accounts for high proportion of health problems in the developing countries like India. [2]

Biofilm lifestyle of Candida albicans:

Biofilms are defined as structured microbial communities that are attached to a surface and surrounded by a self-produced extracellular matrix. In the early years, major focus was on bacterial biofilms, with a first model to study C. albicans biofilm development in vitro only emerging in 1994. Since then, ample model systems for the study of fungal biofilms have been developed and C. albicans biofilm formation has been characterized both in vitro and in vivo by several research groups ;In general, C. albicans biofilm formation is characterized by four stages: cell-wall protein-mediated adherence of yeast cells to a surface, growth of the attached yeast cells into a thin layer of cells, maturation of the biofilm through development of pseudo hyphae and hyphae and excretion of matrix material and dispersal of yeast cells from the biofilm possibly leading to colonization of distant places.

Management of Biofilm Infections:

Viewing bacteria from the perspective of multicellular behavior is altering our view of microbiology and of Koch's postulates. It is evident that 99.9% of organisms prefer attachment, and that bacterial cells have the ability to aggregate into particular three-dimensional assemblages. Biofilms have been recognized as being important in human disease and the number of biofilm-associated diseases seems to be increasing. It is important to understand the characteristics of the biofilm mode of growth and the various aspects of biofilm formation.[3]

Plant profile

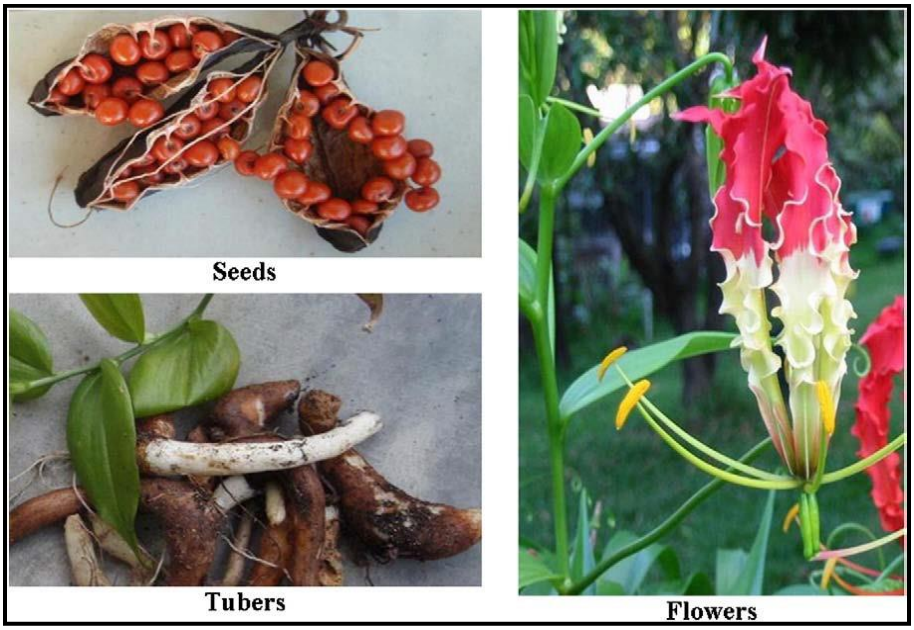
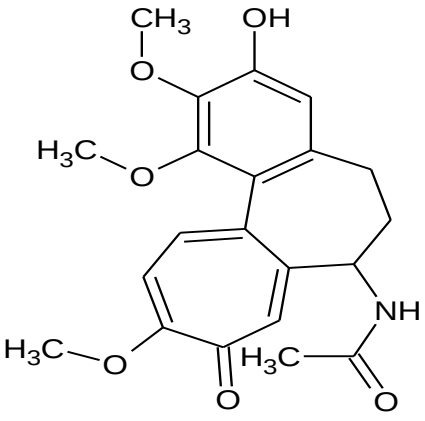


Figure No 1.Images of *Gloriosa superba L.* plant parts

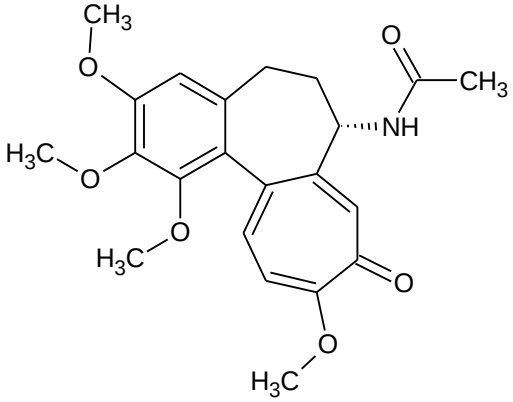
Gloriosa superba L. (Family Colchicaceae), popularly known as "Bachnag" in Hindi, is an ornamental and medicinal plant. It is regularly planted for its high colchicine content especially in tubers and seeds in tropical countries and is found in the South Africa, tropical Africa, India and South-eastern Asia. *G.superba* has a wide range of ecological habitats; like forest-savanna boundaries, thickets, hedges, open forests, grasslands and bush lands. Various parts of Gloriosa are used as medicine for many diseases and disorders. The juice of leafs is used to kill lice in hair, while its tuber contains bitter principles likesuperbin and gloriosine, which are responsible for tonic and purgative properties in small doses. Other constituents like colchicine and colchicoside are used as antidote against snakebite. Tuberous root of this plant shows anti-inflammatory, alterative, anthelmintic and antileprotic activities. Gloriosa is used as ayurvedic medicine in Africa and south-east asia. [4, 5]

Phytochemistry

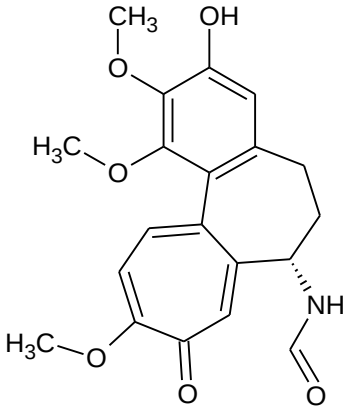
Colchicine is a major alkaloid of *Gloriosa superba* and it is also known as methyl ether of colchicines. Colchicine is an amino alkaloid derived from the amino acids phenylalanine and tyrosine. The tubers of Gloriosa superba contains 0.9 % of colchicine while colchicine is present as 0.33-0.41%, 1.18%, 0.08% in other plant parts like stem, flower and ovary respectively. All parts of this plant contain colchicine hence they are extreamly toxic if ingested orally. Seeds and tubers of *Gloriosa* contain higher concentration of colchicine than Colchicum genera. Seeds contain 2-5 times more colchicine than tubers.



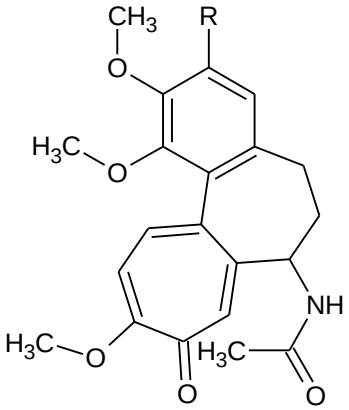
3-demethylcolchicine

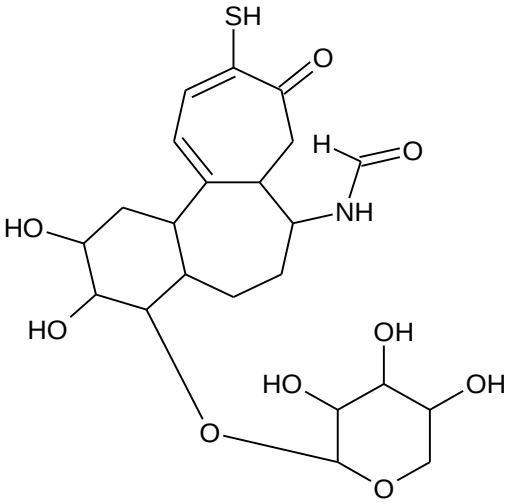


Colchicine

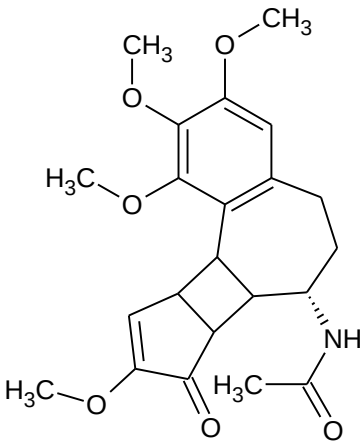


3-demethyl-N-formyl-NdeacetylcolchicineColchicoside





Thiocolchicoside



Lumicolchicine

Figure No.2.Natural and semi synthetic derivatives of *Gloriosa superba* L.

Traditional uses

Tribes have considerable information about the use of many plants and plant part as a medicine, according to World Health Organization (WHO) 80% of people worldwide for their primary healthcare needs depend on traditional medicine. India have a long history of use of plants as source of medicine in 'Ayurveda' plant products are used for treating various diseases and disorder. *Kalihari* is one of the important herb which is used for medicinal applications by tribal people. *Gloriosa superba* L. is listed as *one of the seven upvishas*in the Indian system of medicine which cures many diseases and disorders but may prove fatal on misuse.[6]

In vitro studies

Antimicrobial activities

n-Butanol extract of tubers possess excellent antifungal activity against *Candidaalbicans* and *Candidaglabrata*, *Trichophytonlongifusus* while chloroform fraction showed activity against *Microsporumcanis*, *Staphylococcus aureus*. Aqueous, methanol and petroleum

ether extract of rhizomes demonstrated inhibition against gram negative *bacteria E coli, Proteus vulgaris* and *Salmonella typhi* at 500 µg/ml and at 1000 µg/ml against Gram positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*. Dichloromethane: methanol (1:1, v/v) extract of tuber caused complete inhibition of *Staphylococcus aureus, Streptococcus faecalis, Saccharomyces cerevisiae*and *Candidaalbicans*. [7]

Anti-malarial

Bagavan and co-workers reported antimalerial activity of ethyl acetate and methanol extracts of leaf of *Gloriosa superba* which showed moderate inhibition of *Plasmodium falciparum*. This extract was also found effective against chloroquine resistant strains like Dd2 and INDO.[8, 9]

In-vivo activities

Anti-venom

The tubers are found useful against snakebite since from ancient time and it is mentioned in Ayurveda. This plant is also suggested as anti-venom by saperas community of Khetawas, Jhajjar district, Haryana, India. This plant has been named as *"BACHNAG"* in Hindimeans protection from snake/remedy for snakebite.

Anti-inflammatory

Mathur*et al.* reported anti inflammatory activity of methanol and aqueous extract or tubers of *Gloriosa superba*. This study revealed dose dependant activity in carrageenan induced animal models. *Gloriosa superb* possess good anti-inflammatory activity due to the presence of colchicine in extracts.

Toxicity

Colchicine is the active principle responsible for toxicity of this plant. The chances of poisoning are higher from over dosing in traditional medicine preparations which are used to treat ailments.[10]

Antioxidant

The reactive oxygen species (ROS) contains oxygen and unpaired electrons and make them chemically ractive molecule eg. superoxideanion radicals (O2•−), hydroxyl radicals (OH•), hydroperoxyl radicals(HOO•), peroxyl (ROO·) and non-free radical species such ashydrogen peroxide (H2O2), ozone (O3), and singlet oxygen (1O2).

Materials and Methods

Collection, authentication and preparation of extracts of Gloriosasuperba L. tubers

The tubers of *G. superba*L. Were collected from Shree Baidyanath Ayurved Bhavan Pvt. Ltd, Nagpur, of Maharashtra State of India. The tubers were sliced into round shaped pieces then ground into a powder using a blender. The powder was subjected to solvent extraction 25 gm of powder was extracted in 250 mL of solvent for 5 h. two different solvents, (AR), ethyl acetate (AR), methanol (AR) were selected on the basis of increasing polarity. [11]

Culture, media, chemicals and culture conditions

The strain was maintained on Yeast-Peptone-Dextrose (YPD) agar slants at 4° C. A single colony from the yeast extract-peptone-dextrose (YPD) agar plates was inoculated in 50 ml of YPD broth (pH 6.5), in a 250 mL Erlenmeyer flask. The flasks were incubated at 30° C on an orbital shaker at 120 rpm for 24 h. Cells from the activated culture were harvested by centrifugation for 5 min at 2000 *g* speed.

Biofilm formation

Candida albicans bio films were developed on polystyrene surface of 96-well plates as per standard methodologies. A cell suspension of 1×10^7cells.mL^{-1}was prepared in PBS and 100 µL was inoculated in each well. In the adhesion phase, plates were incubated at 37° C for 90 minutes to allow attachment of cells on the surface. Non-adhered cells were removed by washing the wells with sterile PBS, two to three times. 200 µL of the RPMI-1640 medium was added to each well and the plates were incubated at 37° C for 48 h to allow biofilm formation.[12]

Biofilm quantitation by MTT assay

Germ tube assay by using micro titer plate

Germ tube assay in which firstly YPD medium were prepared and in which 20% serum is added. After that 24hr.activated culture is harvest and centrifuge then wash cell three times in sterile dist. Water then cells keep side for starvation for 1hr.After the starvation cell density is calculated 1 x 10^5 in -1 or -2 dilution take slide and 20 micro.

Chequerboard format for determination of FICI for the combination of drug and plant molecules against planktonic and biofilm growth

Dilutions of individual drug and plant molecules as well as their combinations were prepared in a chequerboard format as per standard methodology. A two dimensional array of serial concentrations of test compounds was used for preparation of dilutions of the drugs. 100 µl of cell suspension was added to each well and the micro plates were incubated at 35 °C. After 48 h of incubation, absorbance was read using micro plate reader at 620 nm. MIC for growth was determined as the concentrations of antifungal drugs where 50 % reduction in the absorbance compared to that of control was obtained. FIC indices were calculated using formula:

$\Sigma FIC = FIC_A + FIC_B$. Where,

FIC_A = (MIC of drug A in combination / MIC of drug A alone)

FIC_B = (MIC of drug B in combination / MIC of drug B alone)

When the value of $\Sigma FIC \leq 0.5$, it is the synergism and when $\Sigma FIC > 4$ it is known as the antagonism. A ΣFIC result of >0.5 but ≤ 4 is considered as indifference (Johnson *et al.*,2004). This was followed for planktonic as well as biofilm growth forms of *C. albicans.*[14, 15]

Microscopic analysis

Biofilms were observed under an inverted light microscope (Metzer, India). Photographs were taken by Labomed microphotography system (Labomed, Korntal, Germany) at × 200 magnification. [16]

Statistical analysis

Results were presented as mean with standard deviations obtained from three different observations. Values in the control and treatment groups for various molecules were compared using Student's t -test. A value of $P < 0.05$ was considered statistically significant.[17]

Discussion

Candida albicans form biofilms on host cells followed by maturation of biofilms, these biofilms of *Candida albicans* are resistant to most of the currently available antifungal drugs. Biofilm resistance is because of regulation of drug efflux pumps, up regulation of target gene expression and alteration in membrane sterol composition etc. Additionally some toxic effects of anti fungal agents also contribute to failure of therapy

hence the search for phytochemicals with activity against *Candida* biofilms is the prime area of interest. *G.superba* has many traditional uses like antidote against snake bite, rheumatism, gout, traditional wound healer, Abortifacient, pneumonia and skin diseases. In this study, biofilm communities of *C. albicans* formed on polystyrene plates were found to be completely resistant to the widely prescribed drug, fluconazole.[18,19] Cells in developing biofilm were 500 times more resistant to fluconazole than that of the planktonic cells. Antifungal resistance exhibited by biofilm is a multifactorial phenomenon; hence, targeting it with more than one molecule can be a good strategy.[20, 21, 22.]

Biofilm inhibitory activities observed were at concentrations less than that of the standard drug, fluconazole. Results showed Methanol extract-fluconazole combinations were synergistic also against biofilm formation. Presence of low concentrations of methanol and ethyl acetate extract (-- and --µg ml^{-1}) dramatically lowered down the fluconazole concentrations required to prevent biofilm formation. [23, 24]

Conclusion

The solvent extracts were not very effective against planktonic growth. However, these were noted to exhibit anti-biofilm activities. Particularly, methanol extract possess potential to inhibit formation of drug resistant biofilms by C. albicans. Use of Methanol extract alone or in combination with available antifungal drugs would be a novel approach against drug resistant biofilms of C. albicans. It will be useful to avoid side effects associated with high dosages and long term usage of the conventional antifungal drugs during anti- biofilm therapy. Combinations of MEGS with fluconazole were analyzed against biofilm growth of C.albicans, for first time. Result of the study suggested synergistic activity of MEGS against biofilm development. MEGS acts as a chemo sensitizers for fluconazole activity to prevent biofilm formation. MEGS may be mediating their activity through repression of specific gens importance in biofilm formation like, CSH1, ALS1, ALS3 and HWP1 which need to be analyzed further through gene expression studies. Also antibiofilm efficacy of these molecules alone and in combination with fluconazole must be confirmed in animal models. The comprehensive information obtained in this study gives insight into development of plant extract molecule as a potential therapeutic strategy for prevention and eradication of biofilm associated Candida infection.

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