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ANTIMICROBIAL AND MUTAGENIC PROPERTIES OF THE ROOT TUBERS OF *GLORIOSA SUPERBA* LINN. *(KALIHARI)*

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Abstract

The methanolic, aqueous and petroleum ether extracts of the root tubers of *Gloriosa superba* were studied for antibacterial, antifungal and mutagenic activities. Results showed that the petroleum ether extracts were highly active against the Gram-negative bacteria, *Escherichia coli*, *Proteus vulgaris* and *Salmonella typhi* at 50 µg/ml and active at 1000 µg/ml against Gram-positive bacteria. All extracts strongly inhibited the growth of *Aspergillus niger* and *Mucor* at 1000 µg/ml and the spore germination of all the fungi at 500 µg/ml. The tubers of *G. superba* were found to possess mutagenic properties by Ames *Salmonella* mutagenicity test due to the presence of the colchicines.

Introduction

An increase in the number of antibiotic resistant strains makes the discovery of new therapeutic agents critically important. Many of the currently used anti-infective and antineoplastic agents are natural products, initially isolated from plants (Poonkothai *et al*., 2005a; Poonkothai *et al*., 2005b) and algae (Raja, 2003; Raja *et al*., 2007a; Raja et al., 2007b). *Gloriosa superba* Linn., is one of the endangered species among the medicinal plants (Badola, 2002) which is a striking tuberous climbing plant with brilliant wavy-edged yellow and red flowers that appears from November to March every year (Rajak & Rai, 1990). It is one of the seven upavishas (semi-poisonous drugs) in the Indian medicine, which cure many ailments but may prove fatal on misuse (Joshi, 1993). The tuberous root stocks of glory lily, *G. superba* boiled with *Sesamum* oil is applied twice a day on the joints, affected with arthritis reduces pain (Singh, 1993). It is also used to treat intestinal worms, bruises, infertility, skin problem and impotence. The sap from the leaf tip is used as a smoothening agent for pimples and skin eruptions. The tuberous roots are useful in curing inflammation, ulcers, scrofula, bleeding piles, white discharge, skin diseases, leprosy, indigestion, helminthiasis, snake bites, baldness, intermittent fever and debility. It is also considered useful in promoting labor and expulsion of placenta. Seeds are used for relieving rheumatic pain and as a muscle relaxant (Nadkarni, 2002).

The toxins of *G. superba* have an inhibitory action on cellular division resulting in diarrhoea, depressant action on the bone marrow and alopecia. Usually all parts of the plant, especially the tubers are extremely poisonous (Angunawela & Fernando, 1971; Aleem, 1992) and causes vomiting, purging, stomach ache and burning sensation (Roberts *et al*., 1987). The glory lily, has been used for suicidal purposes in India, Burma and Eastern Africa due to presence of colchicine (Menis, 1989; Lewis & Elvin, 1997).

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The tubers contain colchicines, benzoic and salicylic acid, sterols and resinous substances-colchicines, 3-demethyl colchicine, 1,2-didemethyl colchicine, 2,3-didemethyl colchicine, N-formyl, N-deacetyl colchicines, colchicocide, gloriosine, tannins and superbine (Capraro & Brossi, 1984). In the world market they are considered as rich sources of colchicines and gloriosine (Srivastava & Chandra, 1975; Prajapati *et al*., 2003). The present study was aimed to investigate the antimicrobial and mutagenic properties of the tubers of *G. superba*.

Materials and Methods

Plant material: The fresh tubers of *G. superba* were collected during the month of November, 2007 from the Namakkal District, South India. The intact tubers were certified at Botanical Survey of India (BSI), Southern Circle using an authentic specimen.

Chemicals: All chemicals were purchased from Sigma USA, unless otherwise stated.

Preparation of extracts: The fresh tubers were thoroughly washed, air dried and powdered immediately. Three different extracts of the tubers were prepared with water, methanol and petroleum ether for 24 h at room temperature by constant shaking and filtered twice through Whatman no. 1 filter paper with the aid of a suction pump. For each of the extraction process 5 g of the ground tuber was taken. The filtrate was evaporated to dryness at 40-50°C. Then, the dried extracts were reconstituted in one ml of DMSO. The volume of DMSO used in the following antimicrobial assays was less than 2%.

Preparation of inoculum: Microorganisms grown in Muller Hinton broth at 37°C to a concentration of approximately 10^5–10^6 CFU/ml was used for the antimicrobial analysis.

Antibacterial screening: The antibacterial activity of the extracts was performed against five different bacteria viz., *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 9144), *Salmonella typhi* (ATCC 6539) and *Proteus vulgaris* (ATCC 6380) (Mansoor *et al*., 2007).

Disc-diffusion assay: The paper disc diffusion method for antibiotic susceptibility testing was used (Jaiarj *et al*., 1999). Sterile discs of 6 mm diameter were impregnated with 25 µl each of the petroleum ether, methanol and aqueous extracts of the tubers of *G. superba.* The respective solvents were used as the negative control discs. These discs were then dried at 37°C before use. The antibiotic chloramphenicol discs were used as positive control. Muller-Hinton agar (Himedia) plates were streaked with the test organisms of approximately 10^3-10^5 cells/ml.

The prepared discs were placed on these plates with flamed forceps and gently pressed down to ensure contact. Plates were incubated at 37°C for 24 h. After the incubation time the inhibition zone diameters (including the 6mm disc) were measured. A reading of more than 6 mm indicated growth inhibition. All the assays were done in triplicate and the results were given in mean ± S.D.

Agar-well diffusion method: The agar-well diffusion assay was adopted (Smania *et al*., 1995) for the present assay. Each bacterial suspension was spread over the surface of

Mueller-Hinton agar (Himedia, India) plates containing 4 wells of 6 mm diameter. The wells were filled with 25 µl each of the extracts. The plates were incubated at 37°C for 24 h. The results were expressed in terms of the diameter of the inhibition zone (Saeed & Tariq, 2007). The respective solvents were used as negative control, and chloramphenicol as positive control. All the assays were done in triplicate and the results were given in mean ± S.D.

Minimum Inhibitory Concentration (MIC): The microplate method was followed for the determination of MIC of *G. superba* (Anon., 1999). One hundred microlitre of Muller Hinton broth was added to the first well followed by the addition of hundred microlitre of the extracts at 5000 µg/ml. Two fold dilutions of each extracts were made in the subsequent wells. One drop of the test organism approximately of 10^6–10^7 CFU/ml was added to the dilutions. After 18 hours of incubation at 37°C, the MIC was visually determined based on the turbidity of the medium. The MIC was regarded as the lowest concentration that did not give any visible growth of the bacteria. The experiment was repeated twice.

Antifungal activity: The test organisms *Aspergillus niger* (ATCC 6275), *A. terreus* (ATCC 16792), *Rhizopus oryzae* (MTCC 553) and *Mucor* species was isolated from the soil sample. All the fungal species were grown on potato dextrose agar (PDA). Tubers extracts dissolved in DMSO were added to 5 ml of sterile PDA with 1000 µg/ml. Control containing the solvent alone and positive control with nystatin were applied. The plates were inoculated with the discs obtained from actively growing margins of the fungal plates (7-10 day old cultures) and incubated between 28-30°C. Fungal growth was examined for 48 h (Hernández *et al*., 1999). The diameter of the fungal growth was compared with that of the control. The observed effects were recorded as follows: +++, 100% growth inhibition; ++, growth was inhibited above 50%; +, growth was inhibited less than 50%; -, no inhibition occurred.

Spore germination assay: All the extracts at 500 µg/ml were tested separately for the spore germination of the most sensitive fungi *Mucor* sp. and *A. terreus* (Rana *et al*., 1997). A loopful of spore obtained from 10 day old fungal cultures was mixed in 1 ml of sterile water. Twenty five microlitres of the spores were mixed with 10 µl of the extracts separately on glass slides and incubated in a moist chamber at 25 ± 2°C for 24 h. Each slide was fixed in lactophenol-cotton blue and observed the conditions under the microscope for spore germination.

Mutagenicity test: The mutagenicity of the tubers of *G. superba* was analyzed by the Ames test with *S. typhimurium* TA98 without metabolic activation (Ames *et al*., 1975). Each assay included 2 sets of plates with the overnight grown cultures were serially diluted and known amounts of these cultures were plated on nutrient agar, to determine the number of viable bacteria in the inoculum. The plates were incubated, inverted at 37°C overnight and the number of colonies counted. The spontaneous revertants in the inoculum were determined as above with the exception that 0.1 ml of the culture was added to the top agar before pouring on to the plates. The number of revertants induced by the test substances was determined by plating 0.1 ml of culture and 0.1 ml of 2-nitrofluorene appropriate concentration of on minimal agar plates supplemented with trace amounts of histidine and

biotin. The number of revertants mutated back by the standard mutagen was determined in the absence of S9 mix. After counting the number of viable bacteria in the inoculum, it was referred to as revertants per plate in subsequent results.

Results

The petroleum ether, methanol and aqueous extracts obtained from the tubers of *G. superba* were microbiologically evaluated and the results obtained are summarized in Table 1. All the extracts of the tubers of *G. superba* showed antimicrobial activity against all the Gram-negative bacteria as determined by both agar well and disc diffusion techniques. The Gram-positive bacteria, *B. subtilis*, *S. aureus* were inhibited by the extracts at concentrations of up to 1 mg/ml as determined by the minimum inhibitory concentration**.** A high inhibitory activity was observed against *E. coli* than the other two Gram-negative microorganisms. All the three extracts were significantly more active against *E. coli* and *P. vulgaris* (MIC 50 µg/ml) than *S. typhi* (MIC 100 µg/ml).

Percentage growth inhibition of four fungal species by *G. superba* tuber extracts is shown in Table 2. A hundred percent inhibition of *A. niger* was observed with all the extracts during the first 24 hours of incubation whereas a significant reduction was noted on the next 24 hours of incubation. The extracts inhibited the other three fungi tested at more than 50% which exhibited a lower activity on the next 24 hours of incubation. All the fungi were completely inhibited by the positive control, nystatin. The results obtained from the spore germination test against *A. niger* (the most sensitive organism tested by the antifungal screening test) reveals that a complete inhibition of the fungal spores was observed at 500 µg/ml concentration of the petroleum ether extract. A separate control was maintained simultaneously in presence of the respective solvents alone that did not inhibit the spore germination.

The extracts of *G. superba* showed frame shift (TA98) signs of mutagenic activity without exogenous metabolism. In addition, they enhanced the mutagenic activity of the mutagen 2-nitrofluorene used in assays with the TA98 strain, without exogenous metabolism (Table 3).

Discussion

A better agreement was found between the agar well diffusion, disc diffusion and MIC tests that were used to determine the antibacterial activity of *G. superba*. Gram-negative bacteria are more complex in structure than the Gram-positive bacteria; still *G. superba* showed a higher activity against the Gram-negative bacteria, *Escherichia coli, P. vulgaris* and *S. typhi*. In accordance with the diffusion tests, the Gram-negative were the most sensitive microorganisms with MIC at 50 µg/ml, whereas *B. subtilis* and *S. aureus* were found to have less pronounced effect at 1000 µg/ml.

There was a pronounced antifungal than the antibacterial activity with atleast more than 50% inhibition against all the fungi tested. The spore germination test screened against the most sensitive organism, *A. niger* confirmed the antifungal activity of the extracts. The extracts prepared from the tubers of *G. superba* are a source of different secondary metabolites which may act in synergy to produce an increased activity against microbes. The results from the above studies may justify the use of plant in the treatment of certain skin diseases, infected wounds and also abscess (Joshi, 1993; Singh, 1993).

Table 1. Antibacterial activity of *Gloriosa superba* by agar well (AD), disc diffusion (DD) and minimum inhibitory concentration (MIC).

Microorganisms	Antibacterial activity										
	PE[$]			ME[$]			AE[$]			CL	
	AD[*]	DD[*]	MIC[#]	AD[*]	DD[*]	MIC[#]	AD[*]	DD[*]	MIC[#]	AD[*]	DD[*]
B. subtilis	-	-	1000	-	-	1000	-	-	1000	25	27
E. coli	68.0±1.5	66.7±1.5	50	15.0±0.6	17.33±2.5	50	10.67±0.6	8±1	100	20	22
P. vulgaris	26.3±1.5	30.0±2.0	50	24.0±1.5	22.67±2.1	50	21.67±1.5	15±2	50	15	15
S. typhi	13.3±1.5	10.0±1.0	100	7.33±0.6	7.0±1.0	100	7.0±1.0	10±1	100	20	22
S. aureus	-	-	1000	-	-	750	-	-	1000	23	25

[$]CL – Chloramphenicol, PE – Petroleum ether, ME – Methanol and AE – Aqueous extracts; [*]Agar well (AD) and Disc diffusion (DD) tests expressed in mm; [#]MIC given in µg/ml; - no activity

Table 2. Antifungal activity of *Gloriosa superba*.

Microorganisms	Growth inhibition							
	PE[$]		ME[$]		AE[$]		Nystatin	
	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs	24 hrs	48 hrs
A. niger	+++	++	+++	++	+++	++	+++	+++
A. terreus	++	+	++	+	++	+	+++	+++
Mucor	++	+	++	+	++	+	+++	+++
Rhizopus Oryzae	++	+	++	+	++	+	+++	+++

[$]PE – Petroleum ether, ME – Methanol and AE – Aqueous extracts

+ = < 50% inhibition; ++ = 50% inhibition; +++ = 100% inhibition; - = no inhibition.

Table 3. Mutagenic and antimutagenic effects of *Gloriosa superba* in the *Salmonella* assay without metabolic activation.

Standard revertants (SR)	Number of His^+ revertants		
	SR + PE[$]	SR + ME[$]	SR + AE[$]
35	308	837	729
Standard mutagens (SM)	SM + PE[$]	SM + ME[$]	SM + AE[$]
268	382	563	527

[$]PE – Petroleum ether, ME – Methanol and AE – Aqueous extracts of tubers of *G. superba*

The results from the Ames test confirmed the mutagenic and antimutagenic nature of the tubers of *G. superba*. Previous experiments confirmed that the tubers of *G. superba* were found to contain mutagenic properties (Angunawela & Fernando, 1971). The chloroform, methanol and petroleum ether extracts of *G. superba* also showed very low ED50 value against P388 cell line (Mahidol *et al*., 1998). This may be due to the presence of highly active alkaloid compound, colchicine (0.1%-0.8%). It clearly exhibits very low ED50 value suggesting its potent cytotoxicity of the compound. Colchicine is still used to treat gout due to its anticancer properties. The mutagenic activity of colchicine was studied using the micronucleus, *Salmonella* and sperm abnormality assays (25-27). The application of ethanolic extraction of *G. superba* for metaphase chromosome preparation in mosquitoes showed a 100% metaphase rate (Choochote *et al*., 2001)**.** In our preliminary screening, the results clearly indicate that the antimicrobial and mutagenic activities of the extracts may be primarily contributed by the alkaloids like colchicine and its derivatives.

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