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Antimicrobial activity of flavonoids from *in vitro* tissue culture and seeds of *Gossypium* species

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Abstract

Flavonoids extracts of seeds and callus tissues of three species of Gossypium (fam. Malvaceae) were screened against Bacillus cerus, Escherchia coli, Mycobacterium smegmatis, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, S. epidermidis, Trichoderma viride and Candida albicans adopting disc diffusion method. Results were compared with the zone of inhibition produced by commercially available standard antibiotics. Maximum activity was observed in flavonoid fraction of callus tissue as compared to seeds. Flavonoids extracts did not show any activity against Candida albicans.

Keywords: *Gossypium*, seeds, tissue culture, flavonoids, antimicrobial activity.

Introduction

Gossypium (cotton) is a good candidate not only for fiber, food and feed but also for the productions of various types of secondary metabolites like flavonoids, steroids, antifertility compounds which makes plants resistant against drought, salinity and pathogen. The wide distribution of antibiotic principles has comprehensively been discussed [1]. It was followed by survey of 174 papers by Nickell [2] which covered the distribution of antibiotics in 147 plant families. Many studies suggested that flavonoids of plants belonging to various families exhibit antimicrobial activity against Gram-positive, Gram-negative bacteria and fungal pathogen [3, 4, 5]. Tissue cultures have also been screened for their antimicrobial substances [6, 7, 8, 9, 10, 11, 12, 13, 14]. Flavonoids, present at high levels in most plant seeds grains play vital roles in defense against pathogens and predators and contribute to physiological functions such as seed maturation and dormancy. They have many biological effects including anti-allergic, anti-inflammatory, anti- hepatotoxic, anti-ulcer, anti-viral and anti-spasmolytic and are of interest in the investigation of disease processes and as potential new drugs [15, 16, 17, 18].

The present work deals with the screening of flavonoid fractions, extracted from seeds and callus tissues of *Gossypium arboreum* L. CINA 343, *G. herbaceum* L. G.Cot.23 and *G. hirsutum* L. LRK 516 for antimicrobial activity against *Bacillus cerus, Escherichia coli, Mycobacterium smegmatis, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, S. epidermidis, Trichoderma viride* and *Candida albicans*.

Materials and methods

Plant material:

The seeds of *Gossypium arboreum* L. cultivars CINA 343, *G. herbaceum* L. G.Cot.23 and *G. hirsutum* L. LRK 516 used as test material were obtained from the Central Institute for Cotton Research (CICR) Nagpur.

Establishment of callus culture:

Seeds were soaked in 12-15% H_2SO_4 for 2 min in order to remove fibers from the surface of cotton seeds and washed with tap water to remove acid. Delinted seeds were dipped in 2% bevestin for antifungal treatment followed by washing with sterile distilled water. These seeds were immersed in 0.2% $HgCl_2$ for 2 min and washed with sterile distilled water under laminar flow. Seeds were germinated aseptically on solidified MS/MSB {combination of MS [19] salts and B_5 vitamins [20]} medium at 25± 1ºC and photoperiod of 16 hrs light and 8 hrs dark. Hypocotyl segments of 0.5 cm length excised from 7-10 days old seedlings were used as explants in this study.

Explants were aseptically transferred on MS/MSB medium supplemented with different concentrations of auxin: 2, 4- dichlorophenoxy acetic acid (2, 4-D; 0.1 – 5.0 mg/l), indole-3-acetic acid (IAA; 0.1 – 5.0 mg/l), naphthalene acetic acid (NAA 1.0 – 7.5 mg/l) and cytokinins: kinetin (Kn 1.0 - 5.0 mg/l). Callus tissues were maintained for 12 months by frequent subculturing at 26 ± 1ºC, 55 % relative humidity under light condition (3000 Lux). All the media used throughout this study were supplemented with 3% sucrose and 0.7% agar. The pH was adjusted to 5.80 ± 0.02 with 1N NaOH or 0.1 N HCl before autoclaving at 121°C and 15 lb psi for 20 min. The seeds and six weeks old callus tissue (maximum growth index) of all the cotton cultivars were used in antimicrobial screening.

Preparation of flavonoid extract:

The dried, powered seeds and callus tissue were separately soxhlet extracted [21] in 80% ethanol for 24 hours. Each of the extract was concentrated and re-extracted with petroleum ether (fraction I), ethyl ether (fraction II) and ethyl acetate (fraction III) in succession. Ether extract was rejected due to its being rich in fatty substance. The ethyl ether fraction was analyzed for free flavonoids while the ethyl acetate fraction was hydrolyzed to cleave glycosides by refluxing with 7% H_2SO_4 for 2 hours. The mixture was filtered, the filtrate extracted with ethyl acetate, neutralized with 5% NaOH, then dried *in vacuo* and analyzed for bound flavonoids. The free and bound flavonoid fractions were used for antimicrobial activity against bacteria and fungus.

Test microorganisms:

The test microorganisms used were *Bacillus cerus* (NCIM 2156), *Staphylococcus aureus* (NCIM 2654) *Staphylococcus epidermidis* (NCIM 2493), *Mycobacterium smegmatis* (NCIM 5138), *Pseudomonas aeruginusa* (NCIM 5032), *Proteus vulgaris* (NCIM 2027), *Salmonella typhimurium* (NCIM 2501), *Escherichia coli* (NCIM 2027), *Trichoderma viride* (NCIM 1221) and *Candida albicans* (NCIM 3466).

The growth medium used for *B. cerus, S. aureus, S. epidermidis, P. aeruginusa, Proteus vulgaris, Salmonella typhimurium, E. coli*, was nutrient broth (0.5% peptone, 0.3% yeast extract and 0.3% NaCl pH adjusted to 7), for *M. smegmatis* M pheli medium (0.5% KH_2PO_4, 0.006% $MgSO_4$, 0.25% sodium citrate, 2% glycerol and 0.5% asparagine pH adusted to 7.8), for *Trichoderma viride* Sabouraud's liquid medium (0.1% peptone and 0.4% dextrose pH adjusted to 5.6) and for *Candida albicans* MGYP liquid medium (0.3% malt extract, 1% glucose, 0.3% yeast extract, 0.5% peptone, pH 6.4-6.8). The microorganisms were

allowed to grow at a temperature 35 – 37°C [10]. The inoculum was prepared by adjusting the concentration of microorganisms at 40% transmittances for bacteria and 65% for fungi, using UV-VLS spectrophotometer-119 set at 630 nm.

Reference antibiotics known to be effective against each of the test microorganism in their established doses were used as reference for comparison of antimicrobial activity of the test samples. These were chloromphenicol (25µg) for gram positive bacteria (*Bacillus cerus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *and Mycobacterium smegmatis*), ampicillin (10µg) for gram negative bacteria (*Pseudomonas aeruginusa*, *Proteus vulgaris*, *Salmonella typhimurium*, and *Escherichia coli*) and Fluconazole (25µg) for fungi (*Trichoderma viride* and *Candida albicans*).

Testing for antimicrobial activity:

Petri plates were pre-seeded with 15 ml of growth agar medium and 1.0 ml of inoculum [10, 13]. Paper discs of 6 mm diameter, which absorbs about 0.1 ml of test samples, isolated substances and known quantity of standard reference antibiotic were used. The inoculated plates were kept at 5 ºC for 40-45 minutes so as to allow the diffusion of the substances and then incubated at 35-37 ºC for 18-24 hours in case of bacteria and 48-72 hours for fungi. The inhibition zones were measured and compared with the standard reference antibiotics [13].

Results and Discussions

The explants first showed swellings at cut end within 4-5 days of inoculation and after one week the swollen portion bursted out to release undifferentiated callus mass. The tissues of cultivars CINA343 and G.Cot.23 were creamish yellow and fragile while that of LRK 516 greenish yellow and compact in nature. Growth index of the tissues showed liner increasing up to a period of six weeks after which it showed a decline in eight weeks.

The antimicrobial activities of twelve extracts obtained from seeds and callus biomass in three *Gossypium* species against eight bacteria and two fungi species, measured by the diameter of the zone of inhibition by using the disc agar diffusion assay, are shown in table-1. *G. arboreum* showed very poor antimicrobial activity as compared to other two species. However its free flavonoid fraction of seeds exhibited maximum activity against *Salmonella typhimurium*, while the bound flavonoids of the callus extracts displayed antimicrobial activity against *S.aureus* and *P. aeruginosa.* Free flavonoid fraction of seeds of *G. herbaceum* and *G. hirsutum* showed activity against *B. cerus, S. epidermidis, T. viride* and *Salmonella typhimurium, E. coli, T. viride* respectively.

While growth of *P. aeruginosa* was inhibited only by bound flavonoids of CINA 343 callus extracts, *M. smegmatis* and *P. vulgaris* by bound flavonoids of G.Cot.23 callus extracts, the *E. coli* growth was reduced by only by free flavonoids of LRK516 seed extracts.

Free and bound flavonoid fraction of seed extracts of *G. herbaceum* as well as the free flavonoids of the callus extracts was active against *T. viride*, while those of *G.hirsutum* were found to be active against *S. aureus* and *S. thyphimurium.* Free flavonoid fraction of *G. arboreum* callus did not show activity against any of the microorganism tested but that of *G. herbaceum* and *G. hirsutum* showed activity against *B. cerus* and *S. thyphimurium*. Bound flavonoid fraction of callus of *G. herbaceum* showed activity against four bacterial strains, *G.arboreum* against two bacteria while *G. hirsutum* against only one bacteria. Free and bound flavonoids fraction (seeds and callus) of all the three *Gossypium* species did not show any activity against *C. albicans*.

It has been observed that antimicrobial activity of flavonoid fractions of *Gossypium* cultivars is not uniform against microorganisms used in the present investigation. Veliky and Lata [11] screened several plant extracts against bacteria and fungus *C. albicans* but none of the extract showed activity against *C. albicans* as also was observed in the present investigation. The antimicrobial activity in seeds and callus is due to flavonoids.

Table1. Antimicrobial activity of flavonoid fractions of cotton cultivars

S.No.	Organism	*G.arboreum* CINA343				*G.herbaceum* G.Cot.23				*G.hirsutum* LRK516			
		Seeds		Callus		Seeds		Callus		Seeds		Callus	
		FF	BF	FF	BF	FF	BF	FF	BF	FF	BF	FF	BF
1.	*Bacillus cerus*	-	-	-	-	0.32	-	0.32	-	-	-	0.42	-
2.	*S. aureus*	-	-	-	0.44	-	-	-	0.32	-	0.32	-	-
3.	*S. epidermidis*	-	-	-	-	0.60	-	-	-	-	-	0.40	-
4.	*M. smegmatis*	-	-	-	-	-	-	-	0.32	-	-	-	-
5.	*P. aeruginosa*	-	-	-	0.30	-	-	-	-	-	-	-	-
6.	*Proteus vulgaris*	-	-	-	-	-	-	-	0.32	-	-	-	-
7.	*S. thyphimurium*	0.75	0.40	-	-	-	-	0.64	0.40	0.50	0.40	0.60	0.45
8.	*E. coli*	-	-	-	-	-	-	-	-	0.35	-	-	-
9.	*T. viridie*	-	-	-	-	0.35	0.28	0.28	-	0.43	-	-	-
10.	*C. albicans*	-	-	-	-	-	-	-	-	-	-	-	-

Activity index [Ratio of diameters (mm) of inhibition zone of the plant part extracts and the inhibition zone of reference antibiotic discs]

FF= free flavonoids (fraction II), FB= bound flavonoids (fraction III).

Average inhibition zone with reference disc: Chloramphenicol (25µg) 25mm zone were produced against (Gram positive bacteria) B. cerus, S. aureus, S. epidermidis and M. smegmatis. Ampicillin (10µg) against P. aeruginusa 30mm, Proteus vulgaris 25mm, S. typhimurium 20mm, and E. coli 28mm; Fluconazole (10µg) for (fungus) Trichoderma viridie 23mm and Candida albicans 22mm.

Contini et al [22] reported that flavonoids of Chromolaena species found to be active against gram positive, gram negative bacteria and the fungi C. albicans and C. tropicalis, where as flavonoids in the current work exhibited activity only against bacterial strains. The study also favours that the antimicrobial activity in seeds and callus tissues of cotton cultivars is due to flavonoids.

The results presented here contribute to the scientific validation for the use of these cotton cultivars in traditional medicine and serve as guide for selection of plants with antimicrobial activity for further phytochemical work on the isolation and the identification of the active compounds. Further more our results showed the potential of some of these cultivars for the development of standardized culturally acceptable herbal medicines for local use as broad spectrum antimicrobial agents.

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