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ORIGINAL PAPER

Antimicrobial principles from tissue cultures of *Balanites aegyptiaca*

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Abstract

Flavonoid extracts of callus tissue of Balanites aegyptiaca (fam. Balanitaceae) were screened against Escherichia coli, Proteus vulgaris, Pseudomonas aureginosa, Citrobacter amalonaticus, Staphylococcus aureus, Micrococcus lylae, Bacillus subtilis and Sporolactobacillus adopting disc diffusion method. Results were compared with the zone of inhibition produced by commercially available standard antibiotics. Maximum activity was observed in free flavonoid fraction of callus tissue.

Key words: *Balanites,* tissue culture, flavonoids, antimicrobial activity.

Introduction

Balanites aegyptiaca is a multipurpose plant used as fodder, medicines, charcoal and pesticides. The plant has antidiabetic, antihelmintic, antibacterial and antiviral activities mainly attributed due to the production of various types of secondary metabolites like flavonoids, steroids and alkaloids. Antimicrobial activity of the various plants has been reviewed by many workers [1, 2]. Callus tissues maintained as static cultures and /or suspension cultures have also been screened for their antimicrobial substances [3 - 12]. However, there have been only a few attempts [13, 14], to isolate the active antimicrobial principles from tissue cultures and even less has been done on the identification of these substances. The present work deals with the screening of *B. aegyptiaca* cultures for their antimicrobial activity against eight microorganisms and isolating, identifying and testing of the possible antimicrobial substances produced by it.

Materials and Methods

Plant tissue culture

Unorganized tissue culture of *Balanites aegyptiaca* was established from cotyledons and maintained on Murashige and Skoog medium [15] supplemented with 5mg/l indole-3-acetic acid and 1mg/l kinetin (Kn) for twelve months by frequent sub culturing at interval of 4 to 6 weeks at 26 ± 1ºC, 55% relative humidity under diffused light conditions (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 121ºC and 15lb psi for 20 minutes. Callus tissues at the transfer ages of maximum growth index (eight weeks) were used in antimicrobial screening.

Isolation of active principles

The dried tissues were extracted in a soxhlet with hot ethanol [16] (100 ml/gm dry weight of tissue) and filtered. The filtrate was dried *in vacuo* and the residue extracted with petroleum ether, ethyl ether and ethyl acetate in succession. 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 (30 ml 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.

Identification of active principles

The isolates were examined by thin layer chromatography (silica gel G coated plates) along with standard reference compounds quercetin and kaempferol. The plates developed in n-butanol, acetic acid and water (4: 1: 5, upper layer) were seen under uv light, placed in a chamber saturated with ammonia and were sprayed separately with 5% ethanolic ferric chloride solution. Each of the isolates was purified by preparative tlc using the same solvent system as for tlc. Isolates (each spot separately) were eluted with ethyl acetate and crystallized from chloroform. The purified isolates were subjected to mp, mmp, uv and ir spectral studies for identification.

Quantitative Estimation of active principles

The quantitative estimation of the active principles identified as flavonoids was carried out colorimetrically [17 -19].

Test microorganisms

The microorganisms used for antimicrobial screening were gram negative bacteria - *Escherichia coli, Proteus vulgaris, Pseudomonas aureginosa, Citrobacter amalonaticus* and gram positive bacteria - *Staphylococcus aureus, Micrococcus lylae, Bacillus subtilis and Sporolactobacillus.*

The bacterial cultures of gram +ve and gram –ve bacteria were maintained on nutrient broth (10% peptone, 0.5% lablamco and 0.5% NaCl, pH adjusted to 7.5). These microorganisms were allowed to grow at 35ºC - 37ºC temperature. The inoculum was prepared by adjusting the concentration of microorganisms at 40% transmittance for gram +ve and gram -ve bacteria using UV-VLS spectrophotometer – 119 set at 630 nm.

Antimicrobial activity of the test samples was compared with the antibiotics known to be effective against each of test microorganisms in their established doses. These antibiotics were used as reference for comparison. These were streptomycin (10µg) for gram –ve bacteria and penicillin (10 units) for gram +ve bacteria.

Test for antimicrobial activity

Petri plates were pre-seeded with 10 ml. of growth medium and 4 ml of inoculum. Paper discs measuring 6 mm diameter, which absorb about 0.1 ml of the test samples, isolated substances (10µg) and known quantity of standard reference antibiotic, were used. The inoculated plates were kept at 5ºC for 40-45 minutes so as to allow the diffusion of the substance and then incubated at 35ºC - 37ºC for 18–24 hours. The inhibition zones were examined and compared with those of the standard reference antibiotics [20-22].

Results and Discussions

The best growth response for induction of unorganized cultures was shown by green, thick cotyledons taken as explants (Fig.1). The unorganized tissues of *B. aegyptiaca* were greenish yellow in color, soft in texture and fragile in nature. Growth indices of tissues showed a linear increase up to a period of 8 weeks, with a maximum of 10.1 after which it showed a decline to 9.2 in ten week old tissue. It was observed that the tissues became brown after a period of eight weeks, which may be due to the accumulation of secondary products in the tissues.

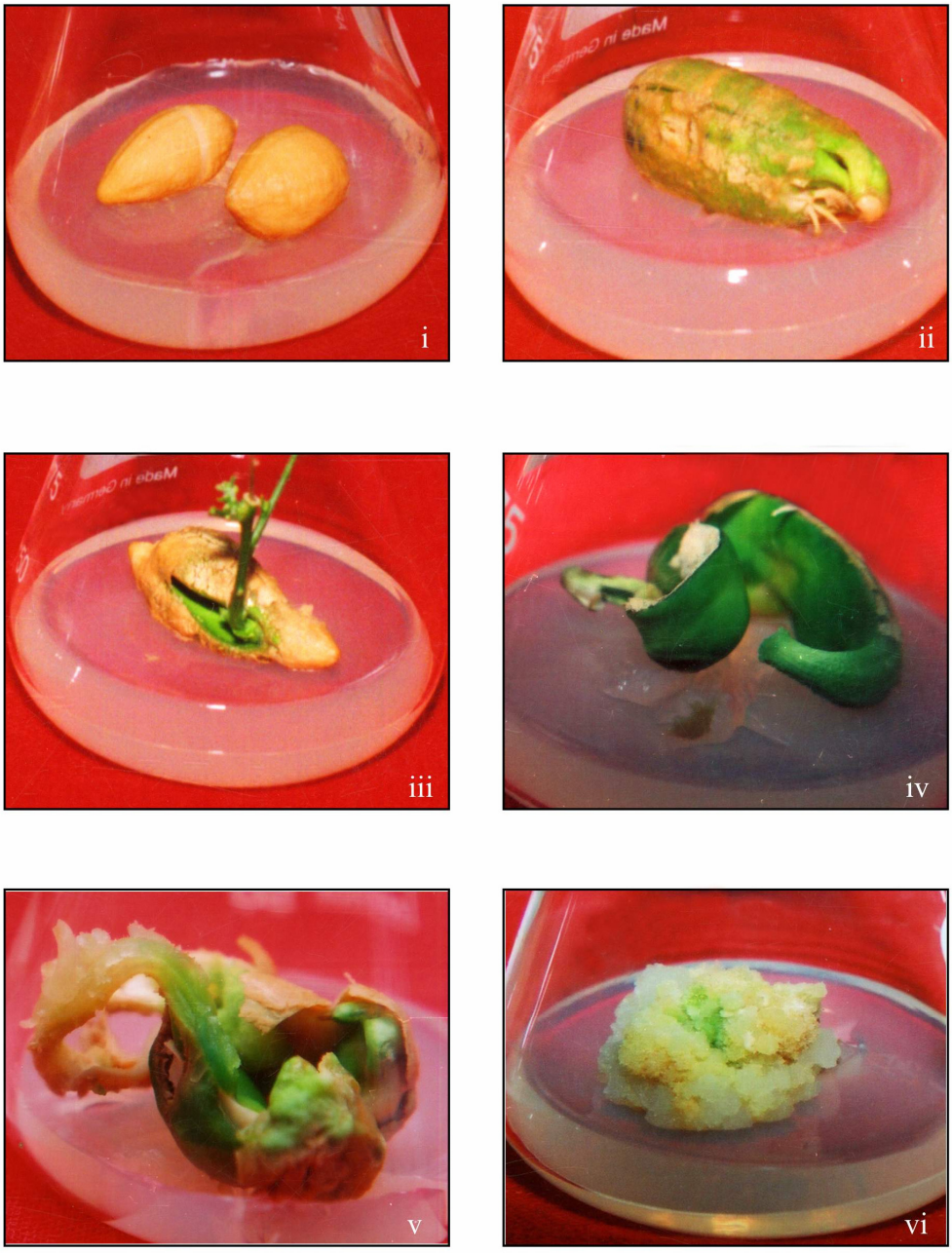

Fig. 1 . (i) Seeds inoculated on hormone free MS medium (ii) Initiation of germination, (iii) Germinating seeds (iv) Green thick cotyledons (taken as explants) (v) Cotyledons showing callogenesis (vi) Establishment of callus cultures

Tissue extracts of *B. aegyptiaca* prepared in ethyl ether and ethyl acetate showed activity against microorganisms tested. However free flavonoid extracts were found to be more active against gram +ve bacteria while the bound flavonoid extracts were moderately active against both the gram +ve bacteria as well as gram –ve bacteria. The antimicrobial activity of the tissue cultures of both the fractions containing free and bound flavonoids is depicted in the table 1. Amongst the gram –ve bacteria free flavonoids were more active against *E.coli* and *C.amalonaticus.* Among the gram +ve bacteria the free flavonoids showed high to moderate activity against all the four microorganisms in all samples, while the bound flavonoids showed moderate to high activity against *Sporolactobacillus.*

Table 1. Antibacterial activity of flavonoid extracts of *Balanites aegyptiaca* in *vitro*

Gram Negative	**Callus Tissues**	
	II fraction (Free Flavonoids)	**III fraction (Bound Flavonoids)**
Proteus vulgaris	++	++
Pseudomonas aureginosa	++	+
Escherichia coli	+++	+
Citrobacter amalonaticus	+++	++
Gram Positive		
Staphyllococcus aureus	++	+
Micrococcus lylae	++	+
Bacillus subtilis	+++	+
Sporolactobacillus	++	+++

+++ High activity ++ Moderate activity + Low activity

Active principles isolated were identified as flavonoids – Quercetin (Rf. 0.82, uv fluorescent-bluish yellow; NH_3 – yellow; $FeCl_3$ – bluish grey; mp = 309 º - 311 ºC, uv max – 258, 303, 375 nm in ethanol) and Kaempferol (Rf. 0.93, uv fluorescent-bright yellowish blue; NH_3 – deep yellow; $FeCl_3$ – brownish; mp = 271 º - 272 ºC, uv max – 263, 324, 368 nm in ethanol). The characteristic ir peaks of isolated and authentic samples were identical. Presence of quercetin and kaempferol were confirmed in all the tissue samples of *B. aegyptiaca* in free and bound form respectively. Of these isolated principles, quercetin and kaempferol were active against all the microorganisms. The total flavonoid content was 5.34 mg/gdw in eight weeks old tissues of *B. aegyptiaca.* The amount of quercetin was higher (4.12 mg/gdw) than that of kaempferol (1.22 mg/gdw).

Quercetin has been reported in static cultures of *Crotolaria juncia* [23], *Calendula officinalis*, *Crotolaria burhia* and *Papaver rhoeas* [24] and kaempferol from callus cultures of *Dolichos lablab*, *Glycine nax*, *Pisum sativum*[25] while both quercetin and kaempferol have been isolated from tissue cultures of *Peganum harmala* [26] and *Tribulus alatus* [27].

Contini *et al* [28] reported that flavonoids of *Chromolaena* species found to be active against gram +ve bacteria, gram –ve bacteria and the fungi *C. albicans* and *C. tropicalis*. Our studies also showed the presence of quercetin and kaempferol in static tissue cultures of *B. aegyptiaca* which indicates that tissue retains the potential to synthesize these compounds efficiently. Thus, it can be concluded that antimicrobial activity of tissue culture extracts of *B. aegyptiaca* may be due to the presence of quercetin and/or kaempferol in sufficient amount .

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