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SHORT COMMUNICATION

FLAVONOIDS OF MOTH BEAN CULTIVARS AS ANTIMICROBIAL AGENT

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Quercetin and Kaempferol have been isolated and identified from seeds, leaves and unorganized cultures of *Vigna aconitifolia* (moth bean) cultivars (RMO-257, RMO-40, IPCMO-880 and Jwala) raised and maintained by frequent subculturing on Murashige and Skoog's medium supplemented with 0.5 mg/l kinetin and 1.5 mg/l of 2,4-D and screened for their antimicrobial activity against gram positive, gram negative and fungal pathogen.

Key words* : Kaempferol, quercetin, tissue culture, *Vigna aconitifolia.

Flavonoids are secondary metabolites that are present at high levels in most plant seeds and grains. These compounds appear to play vital role in defence against pathogens and predators and contribute to physiological functions such as seed maturation and dormancy (Brenda 1998).

The wide distribution of antibiotic principles has comprehensively been discussed by Skinner (1955). Nickell (1959) surveyed 174 papers which covered the distribution of antibiotics in 147 plant families. Various plant parts have also been tested for their antimicrobial activity against gram positive and gram negative bacteria and fungal pathogen (Harsh *et al.*1983, Jit *et al.* 1986). Attempts were also made to isolate active antimicrobial principles from tissue cultures (Khanna *et al.* 1971, Khanna and Nag 1973). The present work deals with the isolation, identification, quantitative estimation of quercetin and kaempferol from seeds, leaves and unorganized cultures of moth bean cultivars (RMO-257, RMO-40, IPCMO-880 and Jwala) and their screening for antimicrobial activity.

Seeds and leaves of four cultivars of Moth bean collected afresh from the experimental field, Agriculture Research Station Beechwal, Rajasthan Agriculture University, Bikaner. Unorganized cultures of *V.aconitifolia* (moth bean) cultivars were established from young leaf explant of 7 days old *in vitro* seedling on Murashige and Skoog's (1962) medium supplemented with 0.5 mg/l kinetin and 1.5 mg/l 2,4-D for 12 months by frequent subculturing at interval of 6-8 weeks at 26 ± 1^0C, 55% relative humidity and diffused light conditions (300 lux). The growth indices were calculated at different time interval of 2, 4, 6, 8 and 10 weeks.

The unorganized tissues were harvested at the transfer age of maximum growth index (8 weeks) for present investigation. The test organisms *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* were procured from the Department of Microbiology and Immunology, S.P. Medical College, Bikaner.

The growth medium used for *S.aureus* and *E.coli* was nutrient broth and for *C.albicans* Sabourd's liquid medium. The inoculum was prepared by adjusting the concentration of microorganisms at 40% transmittance for bacteria and 65% for *C.albicans* (Khanna & Staba 1968) using spectronic-20 colorimeter (Bausch and Lomb) set at 630 nm.

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***Isolation of flavonoids* :** Dried, weighed and powdered seeds, leaves and 8 weeks old tissue of moth bean cultivars were soxhlet extracted with 80% hot ethanol on a water bath for 24 hours and filtered (Subramanian & Nagrajan 1969). The filterate was concentrated and then re-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 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.

Identification of flavonoids : The isolates were examined by TLC (silica gel G coated plates) along with standard reference compounds, apigenen, isorhamnetin, isovitexin, kaempferol, luteolin, myricetin, quercetin, vitexin, and esculetin. 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 NH_3 and were sprayed separately with 5% ethanolic $FeCl_3$ solution. Each of the isolates were purified by preparative TLC (in a similar solvent system as for TLC). Isolates (each spot separately) were eluted with ethyl acetate and crystallized from $CHCl_3$. The purified isolates were subjected to mp, mmp, and IR spectral studies for identification.

Quantitative estimation of flavonoids : Quantitative estimation of the identified flavonoids was carried out colorimetrically following the method of Kariyone *et al.* (1953) and Naghski *et al. (*1975) in case of quercetin and Mabry *et al*. (1970) in case of kaempferol.

Testing of isolated flavonoids for antimicrobial activity : Sterilized petri-plates were preseeded with 10 ml of growth agar medium and 4 ml of inoculum in the case *of S.aureus* and *E.coli* and 6.5 ml of inoculum in the case of *C.albicans* (Khanna and Staba 1968, Khanna *et al.* 1971). Paper discs measuring 6 mm diameter, that absorbs about 0.1 ml of the test sample (isolate) and a known quantity of standard reference antibiotics (table 2 - footnote) were used. The inoculated plates were kept at 5^0C for 45-55 min and then incubated at 35-37^0C for 18 hours. The inhibition zones were measured and compared with those of the standard reference antibiotics.

Unorganized callus of moth bean cultivars was fragile and creamy with light green tinge. Growth indices of tissue showed a linear increase up to eighth week but declined in tenth week. However, among all the four cultivars, maximum GI was observed in IPCMO-880 (11.5) and minimum in RMO-40 (9.0).

Active principles isolated were identified as flavonoids and confirmed as quercetin (Rf 0.82, UV fluorescent yellow bluish; NH_3 deep yellow, $FeCl_3$ – bluish grey; mp 309^0 – 311^0, UV max 258, 373 nm in ethanol) and kaempferol (Rf 0.93; UV fluorescent bright yellowish blue; NH_3 – light yellow ; $FeCl_3$ – brownish; mp 271^0 – 273^0, UV max 268, 368 nm in ethanol) in free and bound form respectively. The characteristic IR peaks of isolated and authentic samples were identical. Of these isolated antimicrobial principles, quercetin and kaempferol were active against all the three microorganisms tested (table 2). Maximum free quercetin (1.35 mg/g dw) was found in leaves of RMO-257 and minimum (0.35 mg/g dw) in seed of IPCMO-880 whereas maximum bound kaempferol (5.5 mg/g dw) was found in leaves of IPCMO-880 and minimum (1.30 mg/g dw) in seed of RMO-40 (Table 1).

Table 1. Flavonoids (mg/g dw) from moth bean cultivars *in vivo* and in tissue culture

Cultivars	Flavonoid	Seeds	Leaves	Callus
RMO-257	Kaempferol	1.95	4.5	4.35
	Quercetin	0.67	1.35	1.20
	Total	2.62	5.85	5.55
RMO-40	Kaempferol	1.30	4.27	3.80
	Quercetin	0.62	1.10	0.99
	Total	1.92	5.37	4.79
IPCMO-880	Kaempferol	2.45	5.50	5.20
	Quercetin	0.35	0.90	0.87
	Total	2.80	6.40	6.07
Jwala	Kaempferol	2.75	5.20	4.90
	Quercetin	0.54	0.85	0.84
	Total	2.79	6.05	5.74

The quantity of isolated quercetin and kaemferol in tissues of various moth bean cultivars at maximum growth index is shown in table 1. Maximum free quercetin (1.20

Table 2. Antimicrobial screening of isolated flavonoids

Isolates	Test microorganisms				
	Staphylococcus aureus		*E.coli*		*C.albicans*
	I/C[a]	I/P[a]	I/C[a]	I/S[a]	I/M[a]
Quercetin	0.36	0.21	0.66	0.56	0.30
Kaempferol	0.43	0.33	0.55	0.35	0.23

a = Ratio of the diameter of the inhibition zone of the isolated substances (10 µg) under observation (I) to the inhibition zone of the reference disc.

Average inhibition zone :

(C) = Chloramphenicol (30 µg) against *S.aureus*=17 mm and E.coli=15 mm; (P) = Penicillin (10 units) against *S.aureus* = 22 mm; (S) = Streptomycin (10 µg) against *E.coli* = 20 mm; (M) = Mycostatin (100 units) against *C.albicans* = 21 mm.

mg/g dw) was found in RMO-257 whereas bound kaempferol (5.2 mg/g dw) was found in IPCMO-880 cultivars. The data presented in table 1 reveals that amount of flavonoid in callus culture was found to be more than the seeds but little less than the leaves of various cultivars studied. Quercetin has been reported in static cultures of *Crotolaria juncia* (Jain and Khanna 1974), *Calendula officinalis, Crotolaria burhia* and *Papaver rhoeas* (Khanna *et al*. 1980) and kaempferol from callus cultures of *Dolichos lablab, Glycin max, Pisum sativum* (Rao and Raju 1999) while both quercetin and kaempferol have been isolated from tissue culture of *Peganum harmala* (Harsh and Nag 1984) and *Tribulus alatus* (Jit and Nag 1985) and tested for anti microbial activity.

Thus it can be concluded that antimicrobial activity in plants is neither a generic character nor a family one (Harsh *et al*. 1983), but it is the feature of active principles (flavonoids) present in the plant. Moth bean cultivars investigated, are good and potential source of antimicrobials and due to presence of these biologically active substances they are more resistant to bacterial and fungal attack.

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