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ANTIMICROBIAL PRINCIPLES FROM IN VITRO TISSUE CULTURE OF *PEGANUM HARMALA*

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Antimicrobial activity of the various plants has been reviewed by many workers (1-4). Callus tissues maintained as static cultures and/or suspension cultures have also been screened for their antimicrobial substances (5-7). However, there have been only a few attempts (8-10) 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 *Peganum harmala* L. for its antimicrobial activity against three microorganisms and the isolating, identifying, and testing of the possible antimicrobial substances produced by it.

EXPERIMENTAL

PLANT TISSUE CULTURE.—Unorganized tissue culture of *P. harmala* was established from seedlings and maintained on Murashige and Skoog's medium (11) supplemented with 5 ppm of kinetin for 12 months by frequent subculturing at interval of 6-8 weeks. The growth indices (GI) were calculated at different time intervals of 2, 4, 6, and 8 weeks (GI=final dry weight of tissue − initial dry weight of tissue/initial dry weight of tissue). Callus tissues at the transfer ages of maximum growth index were used in antimicrobial screening. The test microorganisms used were *Staphylococcus aureus, Escherichia coli,* and *Candida albicans*. The growth medium used for *S. aureus* and *E. coli* was nutrient broth, and for *C. albicans* Sabouraud's liquid medium. The microorganisms were allowed to grow, and the concentration of each microorganisms was adjusted (12).

PREPARATION OF EXTRACTS.—Callus tissue of *P. harmala* and agar medium on which the tissue was grown were extracted separately. Both of the tissue samples and agar medium (45 g) were homogenized with a mixture of Et_2O and 95% EtOH (1:1; 1 g test material in 2 ml solvent mixture) for 5-10 min in a Waring Blender at low speed. An Et_2O and a 50% EtOH extract were prepared in accordance with the method reported earlier (12, 13).

ISOLATION OF ACTIVE PRINCIPLES.—All the dried tissues of 2, 4, 6, and 8 weeks were separately extracted in a Soxhlet with hot EtOH (14) (100 ml/g dry weight of tissue) and filtered. The filtrate was dried *in vacuo* and the residue extracted with petroleum ether, Et_2O, and EtOAc in succession. The Et_2O fraction was analyzed for free flavonoids while the EtOAc fraction was hydrolzed to cleave glycosides by refluxing with 7% H_2SO_4 (30 ml) for 2 h. The mixture was filtered, the filtrate extracted with EtOAc, neutralized with 5% NaOH, then dried *in vacuo* and analyzed for bound flavonoids.

IDENTIFICATION OF ACTIVE PRINCIPLES.—The isolates were examined by tlc (silica gel G coated plates) along with standard reference compounds, apigenin, isorhamnetin, isovitexin, kaempferol, luteolin, myricetin, quercetin, vitexin, and esculetin. The plates developed in *n*-BuOH-HOAc-H_2O (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 EtOAc and crystallized from $CHCl_3$. 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 (15-17).

PREPARATION OF SAMPLES AND ANTIMICROBIAL TESTING.—Petri-plates were preseeded with 10 ml of growth agar medium and 4 ml of inoculum in the case of *S. albus* and *E. coli* and 6.5 ml of inoculum in the case of C. albicans (12, 13). Paper discs of 6 mm diameter, which absorb about 0.1 ml of the extract (EtOH/Et_2O), isolated substances (10 μg), and a known quantity of standard reference antibiotics were used. Blank paper discs of 50% EtOH and Et_2O (6 mm diameter) were used as controls for screening. The inoculated plates were kept at 5° for 45-55 min and then incubated at 35-37° for 18 h. The inhibition zones were measured and compared with the standard antibiotics.

RESULTS AND DISCUSSION

Undifferentiated callus of *P. harmala* was yellowish green and fragile. Growth indices of tissue showed a linear increase up to a period of 6 weeks, with a maximum of 9.2 after which it showed a decline to 8.6 in eight-week-old tissue.

Plain Et_2O and 50% EtOH discs (control) did not show any activity, whereas tissue extracts of *P. harmala* prepared in Et_2O and 50% EtOH showed antimicrobial activity against *S. albus*, *E. coli*, and *C. albicans*. The EtOH (50%) extract of spent agar medium was active against all the microorganisms tested, but its Et_2O extract was active only against Gram-positive bacteria.

Active principles isolated were identified as flavonoids. Quercetin and kaempferol were confirmed using various parameters of tlc, mp, mmp, and uv maxima. The characteristic ir peaks of isolated and authentic samples were identical. Presence of quercetin and kaempferol were confirmed in all the tissue samples of *P. harmala* in free and bound form, respectively. Of these isolated principles, quercetin and kaempferol were active against all the microorganisms tested, except that quercetin showed no activity against *C. albicans*.

The quantity of isolated quercetin and kaempferol in various samples at different time intervals is reported in Table 1. Maximum free quercetin (4.43 mg/g dw) was found in six-week-old tissues, whereas maximum bound kaempferol (2.86 mg/g dw) was found in eight-week-old tissue.

Quercetin has been reported in static culture of *Crotolaria juncia* (18), *Calendula officinalis*, *Crotolaria burhia*, and *Papaver rhoeas* (19), while both quercetin and kaempferol have been reported in tissue culture of *Agave wightii* and *Lycopersicon esculantum* (19). Our studies also showed the presence of quercetin and kaempferol in static tissue culture of *P. Harmala*, which indicates that tissue retains the potential to synthesize these compounds efficiently.

Thus, it can be concluded that antimicrobial activity of Et_2O and 50% EtOH extracts of tissue culture of *P. harmala* may be due to the presence of quercetin and/or kaempferol in sufficient amount. The antimicrobial activity of agar medium may be due to the diffusion of some active substances into the medium.

TABLE 1. Antimicrobial Principles-Flavonoids (mg/g dry weight) from *Peganum harmala* Tissue Culture

Age of Culture (weeks)	GI[a]	Free Quercetin	Bound Kaempferol	Total Flavonoid Contents
2	6.3	2.12±0.02	1.26±0.06	3.38±0.04
4	7.9	2.90±0.01	2.23±0.05	5.13±0.03
6	9.2	4.43±0.09	2.55±0.02	6.98±0.05
8	8.6	3.98±0.07	2.86±0.01	6.83±0.04

[a]GI = growth index (final dry weight of tissue − initial dry weight of tissue/initial dry weight of tissue).

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