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Extraction Of Flavonoids From In Vivo And In Vitro Tissue Culture Of Some Important Halophyes Of Western Rajasthan

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Abstract: Kaempferol and quercetin have been isolated and identified from plant parts (root, shoot and fruit) and unorganized cultures of *Haloxylon recurvum*, *Haloxylon salicornicum* and *Salsola baryosma* raised and maintained by frequent subculturings on Murashige and Skoog's medium[1] supplemented with 3mg/l BAP + .5 mg/l 2, 4-D in *H. recurvum*, 1 mg/l BAP + 2.5 mg/l 2,4-D in *H. salicornicum* and 2mg/l BAP + 2mg/l 2,4-D in *S. baryosma*. In the present investigation, maximum content of flavonoids was observed in fruits of *H. recurvum* and minimum in roots of *S. baryosma*. Among the plant parts the fruits contained highest percentage of flavonoids as compared to shoots and roots in all the three species. The content of isolated flavonoids in various plant parts was observed in this order; Fruits > Shoots > Roots. In callus culture the maximum flavonoids content was found in 8 week old callus culture as the maximum growth indices was observed there. There was a progressive increase in flavonoids content from 2 to 8 weeks old callus culture while decrease with 10 weeks old callus culture in all the three plants.
Keywords: Flavonoids, Tissue culture, *Haloxylon recurvum*, *Haloxylon salicornicum*, *Salsola baryosma.*

INTRODUCTION

Plants of arid and semi arid zone are good sources for the production of various types of senondary metabolites which make them resistant to various environmental stress *e.g.*scarcity of water, salinity pathogens etc. These compounds include alkaloids, flavonoids, steroids, phenolics, terpenes, volatile oils etc. Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. More than 6000 flavonoids have been identified in plants[2]. These compounds appear to play vital roles in defense against pathogen and predators and contribute to physiological function such as seed maturation and dormancy[3], impart colour to flowers and fruits and attract insects for pollination provides resistance to plants[4]. Flavonoids are important for human beings due to their antioxidative and radical scavenging effects as well as their potential estrogenic and anticancer activities[5]. So far no work has been done on the extraction of flavonoids from *Haloxylon salicornicum* (Moq.) Bunge in Boiss, *Haloxylon recurvum* (Moq.) Bunge ex Boiss and *Salsola baryosma* (Roem. et Schult.) *in vivo* and *in vitro*. Hence work was carried out on production of flavonoids in these three plant species.

MATERIAL AND METHODS

In vivo

H. recurvum, H. salicornicum, and *S. baryosma* plant parts (root, shoots and fruits) as identified and authenticated by taxonomist, Central Arid Zone Research Institute (CAZRI), Bikaner (Rajasthan) India, were collected at their luxuriant growth, dried, powdered and used for the estimation of flavonoids content along with the *in vitro* tissue samples.

In vitro

Unorganized cultures of all the three plant species were established from nodal segments on Murashige and Skoog's medium supplemented with 3mg/l BAP + .5 mg/l 2, 4-D for *H. recurvum,* 1 mg/l BAP + 2.5 mg/l 2, 4-D for *H. salicornicum* and 2mg/l BAP + 2mg/l 2, 4-D for *S. baryosma*. The cultures were maintained for 12 months by subculturing at 6-8 weeks intervals at 25±2°C, 55-60% humidity and 300-400 lux light intensity under 16 hours photoperiod using a combination of cool white fluorescent and incandescent light. The tissues were harvested at the transfer age of 2, 4, 6, 8 and 10 weeks and kept at100^0c for 5 min so as to inactivate any enzymatic activity and later at 60^0c till a constant weight was achieved. Growth index was calculated using following equation:

Growth Index (GI) =

$$\frac{\text{Final fresh weight of tissue} - \text{Initial fresh weight of tissue}}{\text{fresh weight of tissue}}$$

EXTRACTION PROCEDURE

The dried, powered plant materials and callus tissue were separately soxhlet extracted in 80% ethanol for 24 hours[6]. 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.

QUALITITATIVE ANALYSIS

Thin layer chromatography

The glass plates (20 x 20 cm) coated (0.2 – 0.3 mm thick) with silica gel G (30 gm/60 ml) were dried at room temperature. The dried activated at 100^0 C for thirty min. in an oven and cooled at room temperature. Ethyl ether and ethyl acetate fraction from each of the test sample were separately applied 1 cm above the edge of the plates along with the standard reference compound (Apigenin, Kaempferol, Luteolin, Quercetin and Vitexin). Several other solvent mixture such as ethyl acetate saturated with water, acetic acid (6:4); forestall system (acetic acid, conc. HCl and water; 10:3:30) were also tried. The solvent mixture of n-butanol, acetic acid, water (4:1:5) gave the best results in all the cases examined. The developed plates were air dried and visualized under UV light which showed two fluorescent spot in both the fractions II and III in all the instances co-including with those of the standard sample of quercetin (blue, Rf 0.82) and kaempferol (bright yellowish blue, Rf 0.93). The plates were placed in a chamber saturated with ammonia vapors to observe the color of the spots (quercetin-yellow; kaempferol-deep yellow). On spraying the developed plates with 5% ethnolic FeCl3 solution which also showed only one spot (in both the fraction II and III) in which fraction II was considered with that of the reference of quercetin (yellowish brown) and fraction III with kaempferol (deep yellow). The Rf values were calculated an average of the 5 replicate.

Preparative thin layer chromatography

The glass plates (20 x 20 cm) thickly coated (0.4 – 0.5 mm) with silica gel (45 gm/ 80 ml water) and activated at 100^0c for 30 min and cooled at room temp. were used for prepative thin layer chromatography (PLC). The extract of both the fraction (II and III) were applied on separate plates and developed plates were air-dried and visualized under UV light. Each of the florescent spot coinciding with those of the standard reference compounds of quercetin and kaempferol were marked.

QUANTITATIVE ANALYSIS

Quantitative estimation of the identified flavonoids was carried out calorimetrically following the method of Kariyone *et al*.[7] and Naghhski *et al*.[8] in case of quercetin; and Mabry *et al*.[9] in case of kempferol. Stock solution (25 micro gm/ ml) of querceting and kempferol were separately prepared by dissolving the authentic sample in methanol. Six conc., (25 micro gm/ml – 150 micro gm/ml) of each of the standard samples were spotted on silica gel coated and activated plates. Separate plates of each of the conc. of quercetin and kaempfrol were used and these chromatograms were developed in the

same solvent system as used for the quantitative method (n-butanol; acetic acid; water, 4:1:5, upper layer). Such developed chromatograms were air dried and visualized under UV light. The fluorescent spots were marked and collected along with the adsorbent in separate test tubes the mixture shaken vigorously, centrifuged and supernatants collected separately. The volume of the replicates was made up to 10 ml by adding spectroscopic methanol. To each of these sample 3 ml of 0.1 mole AlCl3 was added, stopper tightly and the mixture shaken vigorously. Such tubes were kept at room temp. for 20 min. Five such replicates were prepared in each case and optical density is measured using spectronoic – 20 colorimeter set at 440 nm for quercetin and 423 nm for kaempferol against a blank (10 ml spectroscopic methanol + 3 ml 0.1 mole AlCl3). Regression curves for quercetine and kaempferol were separately plotted in between their respective concentrations and optical densities, which followed Beer's law.

Each of the ethyl ether and ethyl acetate extracts was dissolved in 1 ml of spectroscopic methanol and applied (0.1 ml) on silica gel 'G' coated plates along with authentic quercetine and kaempferol markers and developed as above. Fluorescent spots coinciding with those of the reference compounds were marked, scrapped, eluted with methanol separately and the samples were prepared as detailed above. The optical density in each case was colorimetrically recorded as above. The amount of quercetine and kaempferol in the sample were then determine (mg/g.d.w.) by comparing with those of their respective standard curves. Five such replicates were examined and mean value calculated (SE< 0.5%).

Table: 1 Growth indices of static cultures of *H. recurvum, H. salicornicum* and *S. baryosma.*

Plant Name	Growth Indices				
	2 Weeks	4 Weeks	6 Weeks	8 Weeks	10 Weeks
H. recurvum	2.67	3.81	4.42	6.38	6.02
H. salicornicum	2.88	4.09	5.27	7.69	6.34
S. baryosma	3.01	4.62	5.87	8.03	7.84

Table No: 2 Flavonoids isolated (mg/g.dw) from various plant parts of *H.recurvum*

Plant Parts	Kaempferol	Quercetin	Total (mg/g.dw)
Root	1.08	0.97	2.05
Shoot	1.20	1.12	2.32
Fruit	1.89	1.30	3.19

Table No: 3 Flavonoids isolated (mg/g.dw) from *in vitro* tissue culture of *H.recurvum*

Age of tissue	Kaempferol	Quercetin	Total (mg/g.dw)
2 Weeks	0.76	0.54	1.3
4 Weeks	1.45	1.27	2.72
6 Weeks	1.99	1.78	3.77
8 Weeks	2.57	2.44	5.01
10 Weeks	2.21	1.98	4.19

Table No: 4 Flavonoids isolated (mg/g.dw) from various plant parts of *H.salicornicum*

Plant Parts	Kaempferol	Quercetin	Total (mg/g.dw)
Root	1.15	1.04	2.19
Shoot	1.26	1.19	2.45
Fruit	1.37	1.21	2.58

Table No: 5 Flavonoids isolated (mg/g.dw) from *in vitro* tissue culture of *H.salicornicum*

Age of tissue	Kaempferol	Quercetin	Total (mg/g.dw)
2 Weeks	0.53	0.37	0.90
4 Weeks	0.71	0.56	1.27
6 Weeks	1.21	1.42	2.63
8 Weeks	2.38	2.12	4.50
10 Weeks	1.96	1.92	3.88

Table No: 6 Flavonoids isolated (mg/g.dw) from various plant parts of *S.baryosma*

Plant Parts	Kaempferol	Quercetin	Total (mg/g.dw)
Root	0.99	0.87	1.86
Shoot	1.21	1.09	2.30
Fruit	1.75	1.26	3.01

Table No: 7 Flavonoids isolated (mg/g.dw) from *in vitro* tissue culture of *S.baryosma*

Age of tissue	Kaempferol	Quercetin	Total (mg/g.dw)
2 Weeks	0.68	0.52	1.20
4 Weeks	0.91	0.87	1.78
6 Weeks	1.28	1.06	2.34
8 Weeks	2.51	1.96	4.47
10 Weeks	1.54	1.37	2.91

RESULT AND DISCUSSION

In the present investigation, two flavonoids *viz.*, quercetin (Rf 0.82, m.p. 309-311°c, UV max 258, 373, yellowish brown with $FeCl_3$) and kaempferol (Rf 0.93, m.p. 271-273°c, UV max 268, 368 deep yellow to brown with $FeCl_3$) and were isolated and identified from various plant parts (roots, shoot, fruit) and tissue culture of *H.salicornicum*, *H.recurvum* and *S.baryosma* and were confirmed on the basis of their Rf value, TLC behavior, color, melting point, UV studies. The characteristic IR spectral peaks were coinciding with those of their respective standard reference compounds of quercetine and kaempferol in particular samples.

In the present investigation, maximum content of flavonoids was observed in fruits of *H. recurvum* and minimum in roots of *S. baryosma*. Among the plant parts the fruits contained highest percentage of flavonoids as compared to shoots and roots in all the three species. The content of isolated flavonoids in various plant parts was observed in this order; Fruits > Shoots > Roots. In callus culture the maximum flavonoids content was found in 8 week old callus culture as the maximum growth indices was observed there. There was a progressive increase in flavonoids content from 2 to 8 weeks old callus culture while decrease with 10 weeks old callus culture in all the three plants.

In *H. recurvum* maximum amount of total flavonoids (kaempferol and quercetin) was observed in the fruits (3.19 mg/g.dw), followed by shoot (2.32 mg/g.dw) and roots (2.15 mg/g.dw; Table 2). In callus culture, the total amount of flavonoids was found to be higher (5.01 mg/gdw) in 8 weeks old tissue while minimum (1.30 mg/gdw) was found in 2 weeks old callus culture (Table 3).

Among plant parts, maximum amount of total flavonoids (kaempferol and quercetin) was observed in the fruits (2.58 mg/g.dw), followed by shoot (2.45 mg/gdw) and roots (2.19 mg/gdw) in *H. salicornicum* (Table 4). In callus culture, the total amount of flavonoids was found to be higher (4.50 mg/g.dw) in 8 weeks old tissue while minimum (0.9 mg/g.dw) was found in 2 weeks old callus culture (Table 5).

In *S. baryosma* maximum amount of total flavonoids (kaempferol and quercetin) was found to be higher in fruits (3.01 mg/g.dw), followed by shoot (2.30 mg/g.dw) and roots (1.86 mg/g.dw; Table 6). In callus culture, the total amount of flavonoids was observed higher (4.57 mg/g.dw) in 8 week old tissue while minimum (1.2 mg/g.dw) in 2 weeks old tissue (Table 7).

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