

Production of Flavonoids from *Terminalia arjuna* (ROXB.) *in vivo* and *in vitro* Tissue cultures

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Abstract: In the present investigation extraction, isolation, purification and identification of flavonoids were carried out *in vivo* (stem, bark, leaves, flower and fruits) and *in vitro* (callus) tissues of selected plant species *Terminalia arjuna* (family-Combretaceae). Unorganized cultures were raised and maintained by frequent subculturings on MS medium supplemented with 2,4-D, using seedling as explant. TLC, Spectrophotometry and infra-red spectral studies were used for qualitative and quantitative estimation of flavonoids. Three flavonoids namely Luteolin, Kaempferol, Quercetin have been identified.

Keywords: flavonoids, medicinal plants, MS medium, *Terminalia arjuna*.

INTRODUCTION

Flavonoids are polyphenolic glycosides, water soluble and occur almost universally in higher plants. They impart color of flowers and fruits and their correlation between flower color and attraction of insects for pollination is well known¹. Over 6000 naturally occurring flavonoids have been described and many of them are common in higher plants (Harborne and Williams, 2000). Eleven of these are found commonly, Anthocyanidins-Pelargonidin, Cyanidin, Peonidin, Delphinidin, Petunidin and Malvidin, Flavonols-Kaempferol, Quercetin and Myricetin; and Flavones-Apigenin and Luteolin. They have been found to own potent antioxidant and free radical scavenging activities and also show biological effects such as anti-anginal, antiallergic, anti-ulcer, anti-hepatotoxic, anti-viral, anti-inflammatory and anti-spasmodic. Flavonoids appear to play vital roles in defence against pathogens and predators and contribute to physiological functions such as seed maturation and dormancy²⁻³. Present study deals with the isolation, identification and quantitative estimation of flavonoids from different plant parts as well as from unorganized tissue of *Terminalia arjuna* (Roxb.) Wt. and Arn. (commonly known as 'Arjuna').

Materials and Methods

In vivo

Plant samples used in present investigation were collected from different locations of Bikaner. The plant parts-leaves and fruits were collected during the months of April-October, flowers during April- May and bark collected during March-July. Plant parts (stem, bark, leaves, flower and fruits) were separated, dried and powdered for extraction of their flavonoid contents along with *In vitro* tissue samples.

In vitro

For *in vitro* studies seedling as explants were used to initiate callusing. Explants presoaked in 0.1% liquid detergent for 30 minutes, were washed with running tap water and then surface sterilized with 0.5% (w/v) mercuric chloride for 3 minutes followed by two or three rinses of sterile distilled water. Murashige and Skoog's medium (1962)⁴ supplemented with various concentrations and combinations of growth hormones were used. Callus was maintained for six months by frequent subculturing at interval of 6 to 8 weeks at $26 \pm 1^\circ\text{C}$, 60% relative humidity and diffused light conditions (3000 lux). Growth Indices (GI) of tissue was calculated at 2, 4, 6, 8 and 10 weeks time intervals.

Extraction Procedure

The dried samples were separately soxhlet extracted by Subramanian and Nagarajan (1969)⁵ method, in 80% ethanol (100 ml/g.d.w.) on a water bath for twenty four hours to extract flavonoids from them. Each of the extract was concentrated and re-extracted in petroleum ether (40-60°C, fra I), ethyl ether (fra II) and ethyl acetate (fra III) in succession. Fraction II was analyzed for free flavonoids while the fraction III was hydrolyzed by refluxing with 7% H_2SO_4 (10 ml/gm residue) for two hours. The mixture was filtered and the filtrate was extracted with ethyl acetate in separating funnel. The ethyl acetate layer (upper layer) was washed with distilled water to neutrality, dried *in vacuo* and analyzed for bound flavonoids.

Qualitative Analysis

Thin layer chromatography

Thin glass plates (20 X 20 cm) coated (wet thickness, 0.2-0.3 mm) with silica gel 'G' (30 gm /60 ml) were dried at room temperature. The dried plates were activated at 100°C for 30 minutes in an oven and cooled at room temperature. Ethyl ether and ethyl acetate fractions from each of the test sample of *T. arjuna* were separately applied 1 cm above the edge of the plates along with the standard reference compounds - apigenin, isorhamnetin, scutellarein, kaempferol, luteolin, quercetin, myricetin, scopoletin and negretin. These glass plates were developed in an air tight chromatography chamber containing about 200 ml of solvent mixture of n-butanol, acetic acid and water (4:1:5, upper layer). Some other solvent mixtures such as ethyl acetate saturated with water, acetic acid, (6:4); acetic acid, concentrated hydrochloric acid and water (10:3:30) were also tried, but solvent mixture of n-butanol, acetic acid and water (4:1:5, upper layer) gave best results in all the cases examined. The developed plates were air dried and visualized under UV light (254 nm) which showed one fluorescent spot in ethyl ether fraction (II) and two spot in ethyl acetate fraction (III) in all the instances, coinciding with those of the standard samples of luteolin (yellow, Rf 0.78), quercetin (yellow, Rf 0.82) and kaempferol (deep yellow, Rf 0.93) in both *in vivo* and *in vitro*. The plates were also placed in a chamber saturated with ammonia vapours to observe the colours of the spots (luteolin-yellow, quercetin-yellow, kaempferol-deep yellow). On spraying the developed plates with 5% ethanolic FeCl_3 solution one spot was seen in ethyl ether fraction (II) and two spots in ethyl acetate fraction (III). Fraction II spots were coinciding with those of reference quercetin (yellowish brown) and that of fraction III with luteolin (yellow) and kaempferol (deep yellow) in all samples. The Rf values were calculated as an average of the five replicates.

Preparative thin layer chromatography (PTLC)

Glass plates (20 x 20 cm), thickly coated (wet thickness, 0.4-0.5 mm) with silica gel 'G' (45 gm/80 ml water), activated at 100°C for 30 minutes and cooled at room temperature, were used for preparative thin layer chromatography (PTLC). The extract of both the fractions (II and III) of *T. arjuna* were applied on separate

plates and developed plates were air dried and visualized under UV light (254 nm). Each of the fluorescent spot coinciding with those of the standard reference compounds of luteolin, quercetin and kaempferol were marked.

Quantitative Analysis

Spectrophotometry

Quantitative estimation of the identified flavonoids was carried out colorimetrically following the method of Kariyon *et al.* (1953)⁶ and Naghski *et al.* (1975)⁷ in case of quercetin and of Mabry *et al.* (1970)⁸ in case of luteolin and kaempferol. Stock solutions of luteolin, quercetin and kaempferol were separately prepared by dissolving the authentic samples in methanol. Six concentrations (25 mg/ml to 150 mg/ml) of each of the standard samples were spotted on silica gel 'G' coated and activated plates. Separate plates for each of the conc. of luteolin, quercetin and kaempferol were used and these chromatograms were developed in the same solvent system as used for qualitative method (n-butanol:acetic acid:water, 4:1:5; upper layer). Such developed chromatograms were air dried and visualized under UV light (254 nm). The fluorescent spots were marked and collected along with the absorbent in separate test tubes in methanol. The mixture was shaken vigorously, centrifuged and supernatants collected separately. The volume of elutes was made up to 10 ml by adding spectroscopic methanol. To each of these samples 3 ml of 0.1 M AlCl₃ was added stoppered tightly and the mixture shaken vigorously. Such tubes were kept at room temperature for 20 minutes. Five such replicates were prepared in each case and optical densities (O.D.) measured, using spectronic 20 colorimeter (Bausch and Lomb), set at 440 nm for quercetin and at 423 nm for luteolin and kaempferol against a blank (10 ml spectroscopic methanol + 3 ml of 0.1 M AlCl₃). Regression curves for luteolin, quercetin 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 luteolin, quercetin 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 luteolin, quercetin and kaempferol in the sample were then determined (mg/g.d.w.) by comparing with those of their respective standard regression curves. Five such replicates were examined and mean values calculated (SE < 0.5%).

Result and Discussion

Presence of Luteolin (Rf 0.78, m.p. 328-331°C, UV max 255, 350, yellow to green with FeCl₃), quercetin (Rf 0.82, m.p. 309-311°C, UV max 258, 373, yellowish blue with FeCl₃) and kaempferol (Rf 0.93, m.p. 271-273°C., UV max 268, 368 deep yellow to brown with FeCl₃) have been identified and confirmed in all plant parts, unorganized and organized tissue of *T. arjuna*. The characteristic IR spectral peaks were coinciding with those of their respective standard reference compounds of luteolin, quercetin and kaempferol in particular samples (Table-1).

Among all plant parts as well as callus showed maximum amount of luteolin while minimum of quercetin. Amount of kaempferol was slightly lower than luteolin but higher than quercetin in all cases. Comparing the amount of flavonoid in all plant parts, maximum amount of luteolin, kaempferol and quercetin and total flavonoid content has been estimated in bark (0.59 mg/g.d.w., 0.54 mg/g.d.w., 0.43 mg/g.d.w. and 1.56 mg/g.d.w. respectively) and minimum in leaves (0.41 mg/g.d.w., 0.35 mg/g.d.w., 0.22 mg/g.d.w. and 0.98 mg/g.d.w. respectively) (Table-2). Among all plant parts of *T. arjuna* individual flavonoid and total flavonoid content was observed in increasing order of leaves <fruits <flowers <stem <bark (Figure- 1).

Dried and powdered eight weeks old unorganized and tissue of *T. arjuna* were analyzed for qualitative and quantitative estimation of flavonoids. Amount of individual and total flavonoid content estimated was less in unorganized tissue (0.48 mg/g.d.w., 0.43 mg/g.d.w., 0.28 mg/g.d.w. and 1.29 mg/g.d.w. respectively) than bark (1.56 mg/g.d.w.) and stem (1.47 mg/g.d.w.) but higher than all remaining *in vivo* plant parts (Table-2). Higher concentrations of flavonoids in Bark of *T. arjuna* shows the medicinal value of plants as antioxidant, antiallergic and antimicrobial. Sufficient amount of flavonoids in unorganized tissue can be considered as better achievement for large scale production of flavonoids being medicinally valuable for society.

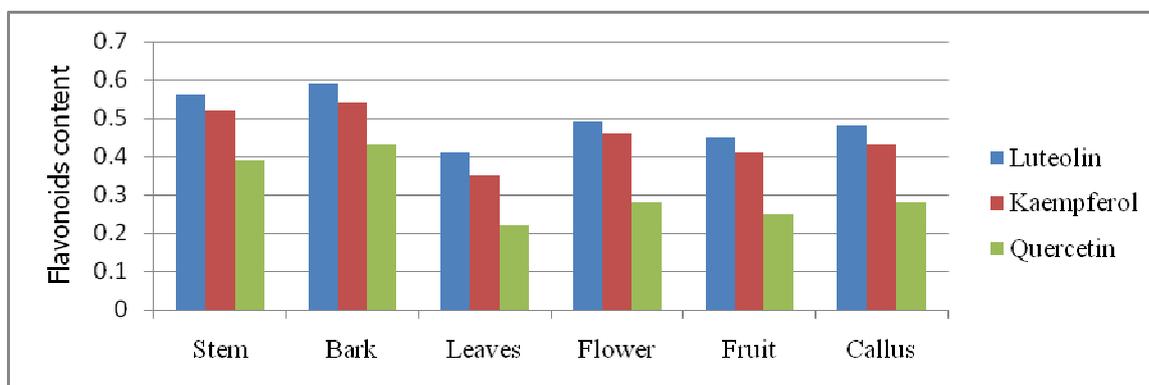
Table 1: Chromatographic behavior and chemical characteristics of isolated flavonoids

Flavonoids	Rf	Colour with spray reagent	Mp°C	UV maxima (nm)
Luteolin	0.78	Yellow to green	328-331	255-350
Kaempferol	0.93	Deep yellow to brownish	271-273	268-369
Quercetin	0.82	Yellow to bluish	309-311	258-373

Table 2: Flavonoids content (mg/100g.d.w.) in *T. arjuna* in vivo and in vitro

Flavonoids	In vivo					In vitro Unorganized tissue
	Stem	Bark	Leaves	Flower	Fruit	
Luteolin	0.56 ± .05	0.59 ± .04	0.41 ±.04	0.49 ± .04	0.45 ± .05	0.48 ± .02
Kaempferol	0.52 ± .04	0.54 ± .03	0.35 ±.05	0.46 ± .02	0.41 ± .03	0.43 ± .03
Quercetin	0.39 ± .02	0.43 ± .06	0.22 ±.04	0.28 ±.04	0.25 ± .04	0.28 ± .04
Total content	1.47 ± .05	1.56 ± .06	0.98 ±.05	1.23 ± .04	1.11 ± .04	1.29 ± .04

Values are mean of five replicates ± SD

Figure 1: Flavonoids content (mg/100g.d.w.) in *T. arjuna* in vivo and in vitro

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