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IN VIVO AND IN VITRO PRODUCTION OF FLAVONOIDS FROM CALLUS CULTURES OF BUTEA MONOSPERMA

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ABSTRACT

Flavonoids are antioxidants present in many herbal plants. A protocol for the production of three important flavonoids namely Kaempferol, Quercetin and Luteolin from various plant parts and in vitro grown callus cultures of a medicinally important plant of family fabacece viz. *Butea monosperma* was developed. *Butea monosperma* is popularly called as “dhak” or “palas” and commonly known as “Flame of forest”. The crude extracts of various plant parts of this plant possess antibacterial, antifungal, hypoglycemic and anti-inflammatory activities Kaemferol, Quercetin and Luteolin are three important flavonoids which have been isolated and identified from plant parts viz. seeds, leaves, flowers and unorganized callus cultures of *Butea monosperma* raised and maintained by frequent subculturing on Murashige and Skoog’s medium (1962) supplemented with various growth regulators. Quantification data revealed that the total amount of free flavonoids and bound flavonoids was higher in callus as compared to plant parts and the amount of free flavonoids is more than bound flavonoids both in vivo plant parts and in vitro callus cultures.

KEYWORDS: Kaempferol, Quercetin, Luteolin, Tissue culture, *Butea monosperma*.

INTRODUCTION

Medicinal plants are rich source of secondary metabolites[1] which include various compounds biosynthetically derived from primary metabolites but restricted to specific taxonomic genera of plant kingdom and specific part of plant body. Flavonoids, one of the important groups of secondary metabolites, are water soluble phenolic glycosides imparting

colour to flowers and fruits of higher plants. Flavonoids are group of substances found in fruits and vegetables essential for processing vitamin- C and needed to maintain capillary wall. They may aid in protecting against infection. Deficiency can result in bruise. In chemical structure, flavonoids are polyphenolic compounds possessing 15 carbon atoms, two benzene rings joined by a linear 3 carbon chain. These flavonoids display a remarkable array of biochemical and pharmacological actions *viz*., antiinflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic activities. These compounds appear to play vital roles in defence against pathogens and predators and contribute to physiological functions such as seed maturation and dormancy. They are synthesized from phenyl propanoid and acetate derived precursors. Quercetin works as antiinflammatory, antioxidant, anticancer agents.[2] Quercetin functions like other flavonoids in enhancing the collagen network (structural integrity) of blood vessel. Antioxidant activity of quercetin[3-4] and antimicrobial activity of many flavonoids[5-8] has been discussed. Fabaceae is a large family of plants possessing medicinal properties. *Butea monosperma* is an important plant of this family and is popularly called as "dhak" or "palas" and commonly known as "Flame of forest". The crude extracts of which has been investigated in the present study for its flavonoids contents. Considerable progress has been acheived regarding the biological activity and medicinal application on this plant. It is now considered as a valuable source of unique natural products. It has possess both medicinal and nutritional properties. The objective of the present investigation was to isolate bioactive flavonoids qualitatively and quantitatively from this medicinally important plants viz. *Butea monosperma* in *vivo* and in vitro tissue cultures.

MATERIAL AND METHODS

Seeds, leaves and flowers of healthy plants of *Butea monosperma* were collected from Rajasthan university campus, Jaipur. Unorganised static cultures of *Butea monosperma* were established from its nodal segments on Murashige and Skoog's (1962) medium[9] supplemented with 3% *(w/v)* sucrose, 0.8% (*w/v*) agar and various growth hormones (BAP+NAA). Callus initiation took place after 15-24 days of inoculation of nodal segments. The callus cultures thus obtained were maintained on MS medium for 12 months by subculturing the tissue in to fresh MS-medium with same growth hormone concentration at 4-6 weeks intervals. The various plant parts (seeds, leaves and flowers) as well as calli of different time intervals (2, 4, 6 and 8 weeks) of subculturings, dried at 60° C and were subjected to extraction of flavonoids separately.

Different plant parts (Flowers, leaves, seeds) along with callus tissue of *Butea monosperma* were air dried and powdered, separately. Each of these extracted separately with 80% methanol on water bath for 24 hours. The methanol soluble fractions were filtered, concentrated *in vacuo* and aqueous fractions were fractioned by sequential extraction with petroleum ether (Fr1), diethyl ether (FrII) and ethyl acetate (FrIII) separately. Each step was repeated thrice for complete extraction, fraction 1 was discarded in each case because it contained fatty substance, whereas fraction II and fraction III were concentrated and used for determining flavonoids. Fraction III was further hydrolyzed by refluxing with 7% sulphuric acid ($10mLg^{-1}$ plant material for 2 h), filtered and filtrate was extracted thrice with ethyl acetate. All ethyl acetate layers were pooled separately, neutralized by distilled water with repeated washings and concentrated *in vacuo*. Both fraction II and III were taken up in small volume of ethanol (2-5mL) before chromatographic examination.

Thin glass plates (20x20 cm) were coated with Silica gel G (250mμ thick). The freshly prepared plates were air dried at room temperature; thereafter these were kept at 100^0C in an oven for 30 minutes to activate and then cooled at room temperature. The freshly prepared and activated plates were used for analysis. Each of the extract was co- chromatographed with authentic flavonoid as a marker. (quercetin, luteolin, kaempferol). These plates were developed in an air tight chromatographic chamber saturated with solvent mixture (Benzene: Acetic Acid: Water: 125:72:3).[11] The developed plates were air dried and visualized under UV light by exposure to ammonia fumes. The mouth of a 100 mL containing concentrated NH_4OH was held in contact with each spot for about 5-10 seconds and fluorescent spots corresponding to that of standard markers were marked. The developed plates were also sprayed with 5% $FeCl_3$, 0.1% alcoholic $AlCl_3$ and kept in I_2 chamber separately. The coloured spots thus developed were noted and the Rf value of each spot was calculated. Several other solvent systems such as n- butanol, acetic acid, water (4:1:5), tertiary butanol, acetic acid, water (3:1:1) were also tested, but the solvent system containing benzene, acetic acid, water (125:72:3) gave better results. PTLC of aforementioned flavonoid extracts was carried out using silica gel G coated plates (BDH ; 500mμ in thickness) by spotting the extract as well as standard markers (luteolin, kaempferol, quercetin). These plates were developed in the solvent mixture of benzene, acetic acid, and water (125:72:3), air dried and examined under UV light. Each of spots corresponding with the standard markers were marked, scraped from 200 plates, and eluted with 50% methanol. The eluted fractions were filtered, air dried and again co-chromatographed along with standard markers to test their purity. The eluted

fractions were subjected to crystallization separately and melting point (mp), mixed melting point (mmp) was determined. The isolates were also subjected to ultraviolet and infrared spectral studies. The identity of the isolated flavonoids were confirmed by mp, mmp performed in capillaries (Toshniwal Melting Point Apparatus), IR (Infra-red spectrophotometer; Perkin, Elmer 337, Grating Infra-red spectrophotometer), UV (Ultraviolet and visible spectrophotometer; Carl Zeiss, Jena, DDR, VSU-2P spectrophotometer) analysis along with their respective authentic samples.

The isolated flavonoids were also estimated by spectrophotometer. Stock solution ($1mgL^{-1}$) of Kaempferol, luteolin and quercetin were prepared separately by dissolving authentic compounds in methanol. Different concentrations ranging from 20µg to 160µg of each of the compounds spotted separately on silica gel G plates. For each concentration of reference authentic standards separate plates were used and developed in the same manner as described earlier. These developed plates were air dried and visualized under UV light. The fluorescent spots were marked and collected along with the absorbance in separate test tubes. Spectroscopy methanol grade (5mL) was added to each test tube, shaken vigorously, centrifuged and supernatants were collected separately. The volume of each of the eluate was made up to 10mL by adding methanol. To each of these samples, 3mL of 0.1 M $AlCl_3$ solution was added again shaken vigorously and kept at room temperature for 20 min. Five such replicates were run in each case and their optical densities were measured using spectrophotometer at 426nm for kaempferol and luteolin and at 440nm for quercetin against blank (10mL of spectroscopic grade methanol and 3mL of 0.1 M $AlCl_3$). The standard curves were plotted between concentration and their respective average optical density of each of the compound. The regression curve so achieved followed Beer's law.

RESULTS AND DISCUSSION

Presence of three flavonoids (Kaempferol, Quercetin and Luteolin) was confirmed by thin layer chromatography (TLC). Developed silica gel plates showed three fluorescent spots under UV lamp coinciding with authentic standard samples of Kaempferol, Quercetin and Luteolin. These three spots turned to yellowish brown when the plates were kept in iodine chambers. Further identification was done by Rf values (Kaempferol 0.86, Quercetin 0.78 and Luteolin 0.56), melting point (Kaempferol 276-278°C, Quercetin 315-320°C and Luteolin 326-329^{0}C) and the colour reaction tests, when sprayed with 5% ethanolic $FeCl_3$ (spots turned to greenish grey when sprayed with 5% ethanolic ferric chloride solution). In

vivo analysis of plant parts showed highest content of total flavonoids in seeds (1.06mg/gdw) and minimum in leaves (0.6%mg/gdw). In vitro analysis of callus cultures showed maximum flavonoids in six weeks old callus cultures (1.20mg/gdw) which declined by eighth week (1.14mg/gdw) cultures (0.86mg/gdw).

CONCLUSION

Quantification data revealed that the total amount of free and bound flavonoids was found to be higher in callus cultures as compared to plant parts. Flavonoids content in its free form was more as compared to the bound form in vivo plant parts as well as in vitro callus cultures.

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