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Research Article

COMPARATIVE ANALYSIS OF TOTAL FLAVONOIDS AND QUERCETIN CONTENT *IN VIVO* AND *IN VITRO* AND ENHANCEMENT OFQUERCETIN VIA PRECURSOR FEEDING IN*PLUCHEA LANCEOLATA* OLIVER & HIERN.

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ABSTRACT

Objective: The present investigation deals with the comparative analysis of total flavonoids and quercetin content *in vivo* (leaf, stem, and root) and *in vitro*(unorganised static callus) plant parts as well as the enhancement of quercetinvia precursor feeding in callus cultures of *Pluchealanceolata*.

Methods: Unorganised static callus culture was established from young leaf explants onMurashige and Skoog's (MS) basal medium supplemented with NAA (1.0 mg/l) in combination with BAP (0.5 mg/l). Isolation and identification of bioactive secondary metabolite quercetin was carried out by the colour reaction, TLC behaviour, IR spectrum, and HPLC techniques.Enhancement of quercetin in callus cultures was achieved by the addition of various concentration of precursor/s (phenylalanine and cinnamic acid) in culture media.

Result: Total flavonoids was found to be maximum (25.7 mg/g dry wt) in leaves. Maximum quercetincontent (0.23 mg/g dry wt) was obtained in 6 weeks old callus tissues. Exogenous incorporation of cinnamic acid (5 mg/100ml) increased the yield of total quercetin about 7 to 8 fold in comparison to control (control = 0.23mg/g dry wt; fed=1.86 mg/g dry wt), whereas incorporation of phenylalanine (50 mg/100ml)increased the yield of total quercetinonly 1 to 2 fold in comparison to control(control=0.23mg/g dry wt; fed=0.38mg/g dry wt). Therefore, cinnamic acid is a more effective precursor than phenylalanine.

Conclusion: Hence,the present study proves that theaccumulation of quercetin in callus cultures can be exploited for its large-scale production in a possible array of health promoting benefits as anti-oxidant, anti-inflammatory and anti-cancerous agents in future.

Keywords: *Pluchealanceolata*, Callus, Quercetin, Enhancement, Cinnamic acid

INTRODUCTION

Pluchealanceolata Oliver &Hiern.is an important xerophytic herbbelonging to family Asteraceae and commonly known as Rasna. All parts of the plant are extensively used in indigenous system of medicine. It has anti-inflammatory and analgesic activityand is greatly used in rheumatoid arthritis, neurological diseases, sciatica, edema, bronchitis, dyspepsia, cough, psoriasis and piles [1, 2, 3]. The plant contains different secondary metabolites viz.flavonoids (quercetin,isorhamnetin, daidzein), triterpenes,sitosterols, taraxosterols, pluchine etc. which gives it anti-inflammatory and analgesic properties [3, 4, 5, 6].Flavonoids are one of the major secondary metabolite in *P. lanceolata* imparting medicinal values for human beings. Flavonoids are a class of secondary metabolites with basic structure of two aromatic rings and an oxygen atom. They are water soluble phenolic glycosides imparting colour to flowers and fruits of higher plants. They are also reported for their preventative activity in circulatory diseases and post climacteric osteoporosis [7].Of these, quercetin is considered as an active ingredient which has many biological roles including anti-inflammatory, anti-cancerous, antibacterial, antiviral, anti-gonadotropic and anti-hepatotoxic activities[8].

Over the past several years, tissue culture technology has been exploited as an efficient and useful tool for production of commercially important metabolites, biotransformation of intermediates into pharmaceutically important products and genetic enhancement of medicinal plants.Phenylalanine [9, 10] and cinnamic acid [11] are reported to be the precursors of quercetin (flavonoid). Therefore, this paper describes a comparative analysis of total flavonoids and quercetin content *in vivo*(leaf, stem, root) and *in vitro* (unorganised static callus) plant parts as well as the effect of precursors viz.cinnamic acid and phenylalanineon growth and production of quercetin in callus cultures of the plant. To the best of our knowledge, this is the first report on a comparative analysis of total flavonoids and quercetin content *in vivo* and *in vitro*as well as enhancement of the bioactive secondary metabolite quercetin by precursor feeding *in vitro*.

MATERIALS AND METHODS

Plant material and culture establishments

The plant parts of *Pluchealanceolata* were collected during the month of July from the forest regions of Jaipur and adjacent areas. The plant material was authenticated by herbarium ofthe Department of Botany, University of Rajasthan, Jaipur. A voucher specimen was deposited in the herbarium of the Department.The plant materials (leaves root and stem) were air dried at room temperature and under shade, and then powdered to a fine grade by using a laboratory scale mill.These shade dried parts of the plant were powdered and kept in air tight plastic bag until use. Unorganised callus cultures (eighteen months old) were grown on MS medium[12]consisting of basal salts and vitamins with 3% (w/v) sucrose and 0.8% agar with NAA (1.0 mg/l) and BAP (0.5 mg/l) using leaf explants[13].These cultures were allowed to grow upto their maximum growth age (6 weeks) [14].

To examine the effect of incorporation of precursor/s on biosynthesis of quercetin and growth of the tissues, callus tissue was transferred to fresh MS medium with above mentioned additives and singly supplemented with different concentrations of cinnamic acid (2.5-10 mg/100ml) and phenylalanine (25-100 mg/100ml) separately. These cultures were allowed to grow upto their maximum growth age (6 weeks). The callus tissues were dried, till a constant weight was achieved, powdered, weighed and subjected to quercetin analysis. Five replicates were used in each case and their mean values were taken into consideration.

Extraction and quantitative estimation of total flavonoids in plant extract

1 gm of each plant samples *in vivo* (leaf, stem and root) and *in vitro* (unorganised callus tissues) was extracted with 25 ml of 90% ethanol at 37°C for 24 hours. After filtration, extract was adjusted to 25 ml with 85% ethanol. Now the extract was subjected to quantitative analysis of total flavonoids. The total flavonoid was measured using a colorimetric assay [15].

Briefly, 1ml of appropriately diluted samples was added to a 10 ml flask containing 4 ml of double distilled water. At time zero, 0.3 ml of 5% $NaNo_2$ was added to each volumetric flask; after5 min., 0.3 ml of 10% $Alcl_3$ was added; after 6 min., 2 ml of 0.1M NAOH was added. Each reaction flask was then immediately diluted with 2-4 ml of double distilled water and mixed. Absorbance of the mixture upon the development of pink colour was determined at 510nm relative to a prepared blank.The total flavonoid of the samples was expressed as mg/g of quercetin equivalents. All samples were prepared in 5 replicates.

Extraction, isolation and identification of Quercetin

The dried samples were separately soxhlet extracted in 80% methanol (100 ml/gm dry weight) on a water bath for 24 hrs.[16].Each of the extracts was concentrated and reconcentrated in petroleum ether (40°-60°C) (fraction-I), ethyl ether (fraction-II) and ethyl acetate (fraction-III) in succession. Each of the steps was repeated three times to ensure complete extraction in each case. Fraction I was rejected since it was rich in fatty substances whereas fraction II and fraction III of each of the test samples was hydrolysed by refluxing with 7% H_2SO_4 (10 ml/gm residue) for 5 hours. The mixture was filtered and the filtrate extracted with ethyl acetate in a separating funnel. The ethyl acetate layer was washed with distilled water till neutrality and dried. The residues were taken up in small volumes of ethanol separately and then subjected to various tests for quercetin.

The extracts were applied on TLC (silica gel 'G' coated plates) along with standard reference compound of quercetin) developed in a closed chromatography chamber containing n-butanol, acetic acid and water (4:1:5). Identification of compound was carried out by spraying with 5% ethanolic $FeCl_3$ and also by exposing the plates in I_2vapours, NH_3vapours and UV light chambers. Rf values were calculated for isolated samples and compared with coinciding standard.

Further analysis of compound was confirmed by high performance liquid chromatography (HPLC) and Infra-red spectral (Perkin-Elmer 337 Grating, Infra-redspectrophotometer using nujol or potassium bromide pellets) methods in comparison with the authentic samples of quercetin. HPLC separation of quercetin ineach plant sample was performed on a ODS (18) column by usingacetonitrile: methanol (20:80 v/v) as mobile phase [20]. The flow ratewas1ml/min and the elution was monitored at 254 nm. Peakidentification was carried out on the basis of an authentic sample ofquercetin [17].

Quantitative estimation of Quercetin

Quantification of the isolated and identified quercetin in all plant samples was carried out colorimetrically[18, 19].The amount of quercetin in various test samples was then determined (mg/g dry weight) by comparing with those of their respective standard regressive curve.Five such replicates were examined and mean values were calculated.

RESULTS AND DISCUSSION

During the present set of investigation,total flavonoidwas found to be maximum in leaves (25.7 mg/g dry weight) as compared to the other *in vivo* and *in vitro* plant parts (Fig.1).Unorganised callus tissues showed 24.5 mg/g dry weight of total flavonoids as compared to root (21 mg/g dry weight) and stem (14.1 mg/g dry weight).Realising the importance of flavonoids, some researchers have also quantified total flavonoid contents in some plant species e.g. *Melothriamaderaspatana* [20] and *Ecliptaalba* [21].

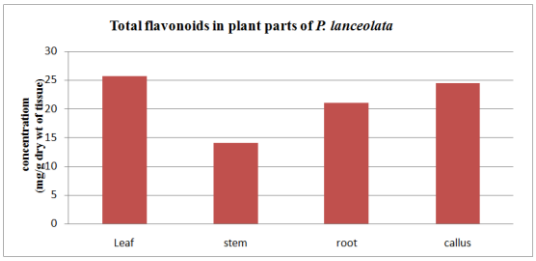

Fig. 1: Quantification of total flavonoids in*P.lanceolata in vivo* and *in vitro*

The bioactive compound, quercetin was isolated and identified from all *in vivo* and *in vitro* plant parts on the basis of colour reaction of TLC, HPLC and IR spectra. When the developed plates of TLC were sprayed with 5% ethanolic $FeCl_3$ solution it showed spots which coincided with that of the standard reference quercetin,bluish grey in colour(Fig. 2). When plates were placed in a chamber saturated with ammonia vapours and iodine vapours, it also showed deep yellow and yellowcolour of quercetinrespectively.Rf value (0.82) of quercetin isolated from the plant samples coincided with the Rf value of standard quercetin (0.82). The plates developed under UV light showed fluorescent spots coinciding with the standard sample of quercetin (Blue). Presence of quercetin was also confirmed by superimposed IR spectra of standard and isolated quercetin from *in vivo* and*in vitro* plant parts.

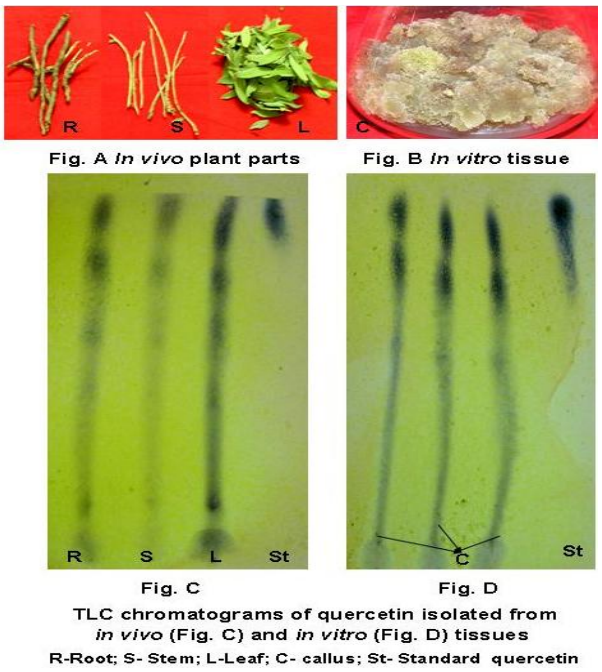

Fig. 2: Thin Layer Chromatograms of*P. lanceolata*plant parts against quercetin reference

HPLC peak of isolated quercetin was identified on the basis of retention time (t_R ~ 2.9 min)of authentic sample of quercetin (Fig. 3-6). Some unknown peaks were also observed in plant extracts.

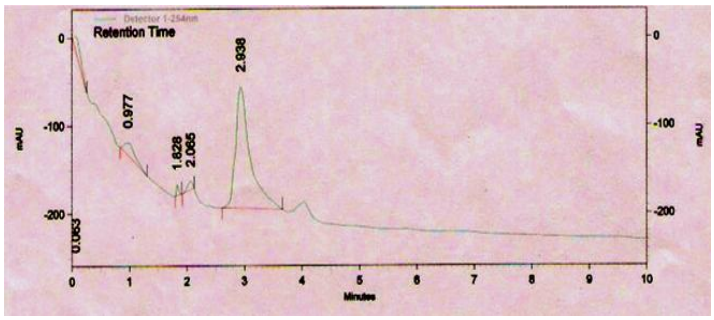

Fig. 3: HPLC Chromatogram of Standard Quercetin

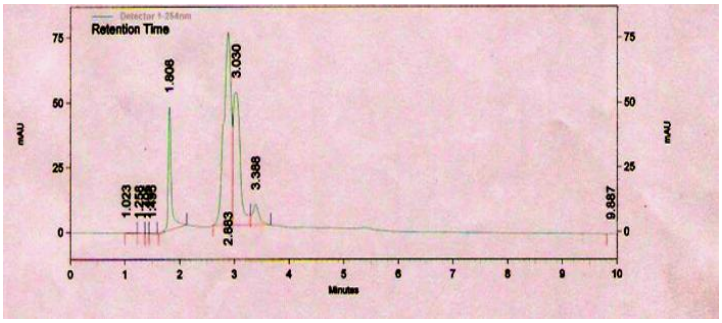

Fig. 4: HPLC Chromatogram of Quercetin isolated from leaves of *P.lanceolata*

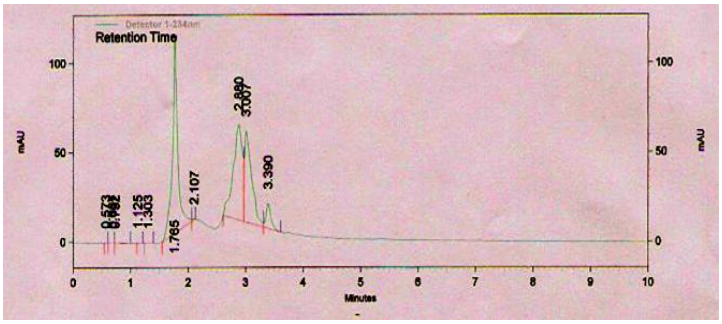

Fig. 5: HPLC Chromatogram of Quercetin isolated from stem of *P.lanceolata*

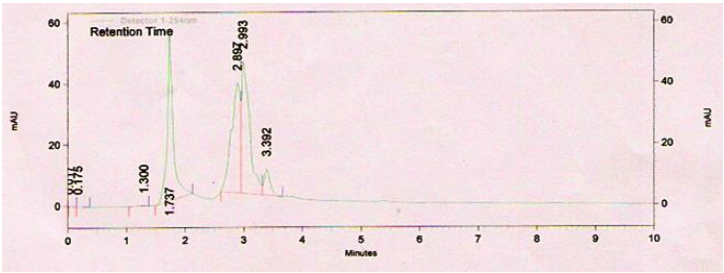

Fig. 6: HPLC Chromatogram of Quercetin isolated from root of *P.lanceolata*

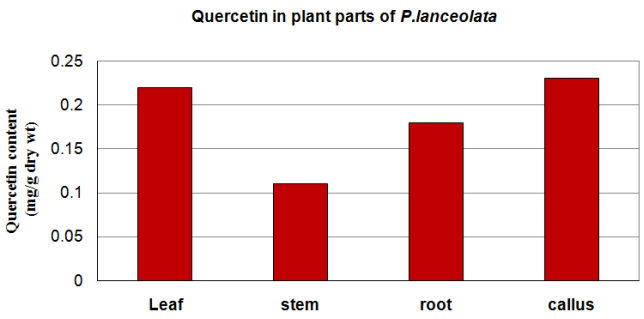

Fig. 7: Quercetin content in *P. Lanceolata in vivo* and *in vitro*

Quantitative estimation of total quercetin contents showed maximum amount in six weeks old callus tissues (0.23 mg/g dry wt) as compared to other tested plant samples (Fig. 7).In intactplant parts, total quercetin content was found to be maximum in leaf (0.22 mg/g dry wt) followed by root (0.18 mg/g dry wt) and stem (0.11mg/g dry wt). However, slight difference was observed in the amount of total quercetin contents of six weeks old callus tissue (0.23 mg/g dry wt) and leaf (0.22 mg/g dry wt).

The present biochemical study showed that incorporation of cinnamic acid (2.5-1.0 mg/100ml) as well as phenylalanine (25-100 mg/100ml) increased the amount of total quercetin contents in callus tissues (Fig.8). However, cinnamic acid was found to be a more effective precursor than phenylalanine because exogenous incorporation of cinnamic acid (5mg/100ml) increased the yield of total quercetin about 7 to 8 fold in comparison to control (control = 0.23 mg/g dry wt; fed =1.86 mg/g dry wt), whereas phenylalanine (50mg/100ml), increased the yield of total quercetin only 1 to 2 fold in comparison to control (control = 0.23 mg/g dry wt; fed = 0.38 mg/g dry wt) (Fig.9). Similarly, the role of cinnamic acid in flavonoid biosynthesis has been describedin *Pisumsativum*seedlings[22]and *Glycyrrhizainflate*[23].Effect of phenylalanine on flavonoids has been studied in tissue cultures of *Hydrocotylebonariensis*[24]and *Silybummarianum*[25].

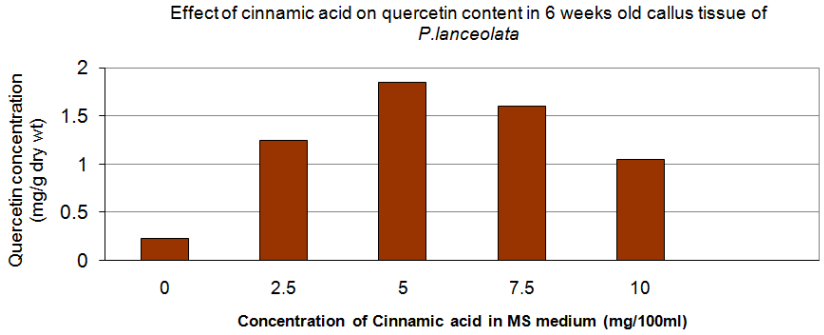

Fig. 8: Effect of cinnamic acid on growth and production of quercetin in 6 weeks old callus tissues of *P.lanceolata*

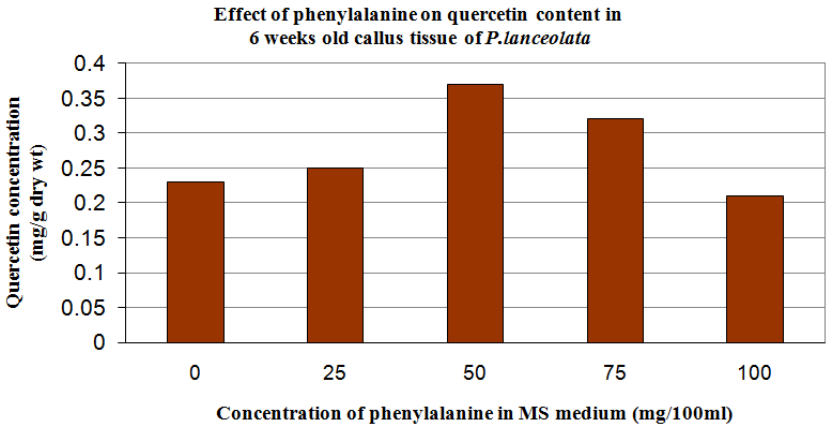

Fig. 9: Effect of phenylalanine on growth and production of quercetin in 6 weeks old callus tissues of *P.lanceolata*

CONCLUSION

Flavonoids are one of the major secondary metabolites in P. lanceolata imparting medicinal values for human beings. Of these, quercetin is considered as an active ingredient which has many biological roles. In conclusion, the present study describes a comparative analysis of total flavonoids and quercetin content in vivo and in vitroas well as enhancement of the bioactive secondary metabolite quercetin by precursor feeding in vitro. Therefore, the protocol described in this report is expected to contribute to the future studies in this plant species for large scale production of certain biologically active plant metabolites from in vitro propagated elite materials. Accumulation of quercetin in callus cultures can be exploited for its large-scale production in a possible array of health promoting benefits as anti-oxidant, anti-inflammatory and anti-cancerous agents in future.

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