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RP HPLC ANALYSIS FOR COUMARIN CONTENT IN *CICHORIUM INTYBUS* – AN IMPORTANT MEDICINAL PLANT

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ABSTRACT

Cichorium intybus is a medicinal plant commonly used in traditional medicine to treat various infectious diseases. In the present investigation, coumarin was detected in various extracts. An RP- HPLC procedure suitable for the determination of the considered compounds was also developed. Studies were carried out on analysis of secondary metabolites by RP-HPLC from different parts of *C.intybus*. HPLC detected the coumarin from *C*. i*ntybus* in various part of the plant and leaf derived callus. The plant parts used for the analysis included leaf, root and leaf derived callus. Among all the plant parts used, leaf derived callus recorded the highest concentration of coumarin at 219.73µg/ml. The study indicated that almost all parts of the plant contain coumarin component.

KEY WORDS: ***Cichorium intybus*, HPLC, secondary metabolite, extraction technique, coumarin.**

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INTRODUCTION

Cichorium intybus (Chicory) is a member of Asteracae family. It is an erect, glandular and annual herb. The tuberous root of this plant contains a number of medicinally important compounds such as inulin, bitter sesquiterpene lactones,flavonoids, coumarin etc. For any medicinal plants as a medicine, standardization is very important to confirm the plant drug authenticity and its content of active principles according to the parameters which is utilized as the criteria of plant quality[1, 2]. Coumarin (1, 2 benzopyrones) are ubiquitously found in higher plants where they originate from the phenylpropanoid pathway [3]. The persistence of plants being involved in the process such as defence against phytopathogens, response to abiotic stress and regulation of oxidative stress [4,5]. It has been found that coumarin and its derivatives show a wide range of biological activities such as anti coagulant, vasodilator, antimicrobial, estrogenic, anti inflammatory, anti fungal, anti ulcer, dermal photo sensing etc[6]. In the present study, we report a simple, rapid and selective HPLC method for the separation and determination of Coumarin compound in crude plant material and callus. However, no instrumental chromatographic method for the standardization of *C. intybus* plant material has been reported.

MATERIALS AND METHODS

Collection of plant material and sample preparation

The collection of plant material in the form of seeds was done from Jamnagar, India (Imported and Marketed by spring Haven, France). The seeds were brought to our laboratory and sowed. The leaves and root were the plant material used for extraction of coumarin. Along with this, leaf derived callus was developed. The leaf derived callus was obtained from MS Media supplemented with growth regulators 1.5mg/l of NAA + 0.25mg/l of BAP in the tissue culture laboratory and was used for the extraction of coumarin compounds. The samples were surface sterilized thoroughly. The fresh and dried samples were ground using a mortar and pestle. Both these fresh and dried samples were grounded along with acetonitrile and water respectively.

Extraction

Fine tissue powder from each tissue sample (1.5g) was extracted in 10ml of acetonitrile: water (40:60) for 30 minutes. 1ml of extract from each sample was centrifuged at 10,000rpm for 5 minutes. The supernatant was passed through 0.45µm filter before injecting to HPLC column.

A) Extraction by Maceration

Powdered fresh callus and dry callus (1.5g) were macerated with ethanol: water (1:1; v/v, 10ml) and left at rest (7 days, room temp).The material was filtered and the crude extract obtained was analyzed directly by HPLC- UV. This procedure was repeated in duplicate.

B) Extraction by Maceration under sonication

Powdered dry callus (1.5g) and fresh callus with ethanol: water (1:1); v/v, 10ml) and macerated under sonication (water bath, room temp). The material was filtered and the crude extract obtained was analyzed directly by HPLC-UV (SPD-20A, Shimadzu Corporation Kyoto, Japan). The procedure was repeated in duplicate.

Extraction using Acetonitrile: water (40:60)

A) Maceration

Powdered fresh callus, dry callus, leaf, root and seed (1.5g) were macerated with acetonitrile: water (40:60; v/v, 10ml) and the material were filtered and the crude extract obtained was analyzed directly by HPLC- UV. This procedure was repeated in duplicate.

B) Infusion

Powdered fresh and dry callus (1g) was added to boiling distilled water (10ml). The recipient was covered until reaching room temp. This material was filtered and the crude extract obtained was analyzed directly by HPLC – UV. This procedure was repeated in duplicate.

Analysis of the extracts for coumarin by HPLC

The standard coumarin ($C_9H_6O_2$) was purchased from Sisco research laboratories pvt. Ltd, Andheri (E) (Mumbai, India). Coumarin standard solutions within the range from 1-100µg /ml concentration were prepared for HPLC analysis. The coumarin standard was prepared by dissolving in a solution of gradient grade acetonitrile for HPLC in the ratio of 40:60 (v/v). The standard sample solution for HPLC was filtered during 0.45 µm syringe filter before injection. The analysis of extracts was done in High performance liquid chromatographic system (HPLC) equipped with L C18 a pump SPD–M10A vp photo array detector (Shimadzu). The presence of coumarin in the samples was detected by comparing with the retention time of the standard sample and the amount of coumarin in the extracts was calculated. The chromatographic conditions for the analysis were as follows: Mobile Phase: acetonitrile: water (40:60) Separations were done in the binary mode, using acetonitrile: water (40:60; v/v) at a flow rate of 1 ml min-1; with an injection volume ("loop") of 20µl; UV detection was at 274nm.

Amount of coumarin present in the fresh callus and dry callus of the sample was calculated using the formula,

$$CP(s) = \frac{AP(s) \times CP(st)}{AP(st)}$$

CP(s) - concentration of the solute in the mixture

AP(s) - area of the peak for the sample in HPLC chromatogram.

CP (st)-concentration of the standard used for injecting in HPLC.

AP (st) - area of the peak for the sample in HPLC chromatogram.

HPLC injection for each samples and standards were done in duplicates.

Standard solutions

Coumarin (100mg) was dissolved in 40ml of acetonitrile and 60ml of water in volumetric flask. The final solution contained 100µg/ml of coumarin. For the working standard, 80µl of the stock solution was taken and made up to 1ml with 40:60 acetonitile and water respectively. The same was carried for 60µg, 40µg, 20µg, 10µg, 1µg concentration.

Quantitative analysis

Determination of coumarin in plant material was performed by the external standard method, using pure coumarin as standard. Stock solutions of 1, 10, 20, 40, 60, 80,100µg/ml were utilized. Each determination was carried out in triplicate.

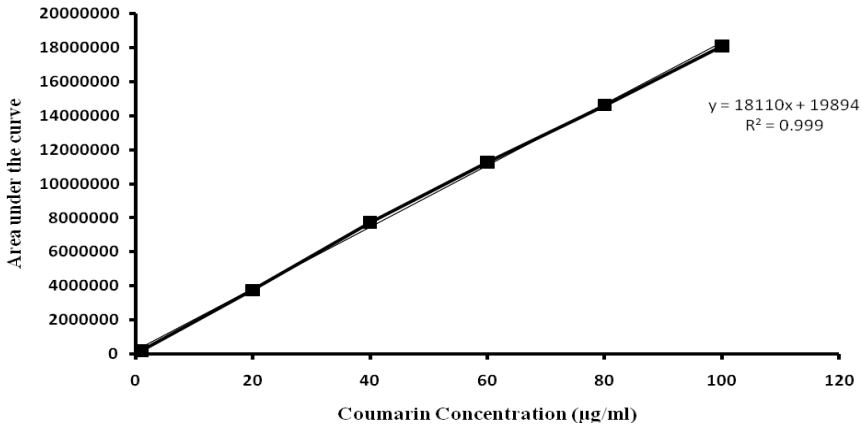

Figure 1
Calibration curve for the estimation of coumarin

Linearity

Linearity was studied by preparing standard solutions at different concentration levels. The linearity range was found to be 1µg/ml – 100µg/ml. (Figure.1)

Coumarin standard Histogram

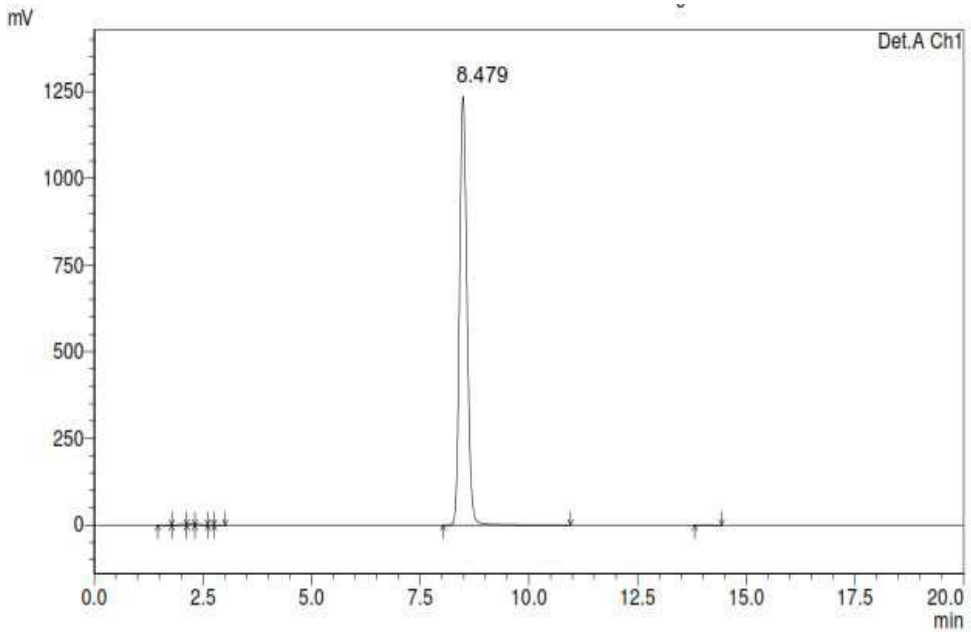

Figure 2
HPLC chromatogram for coumarin standard concentration

Fresh callus Histogram

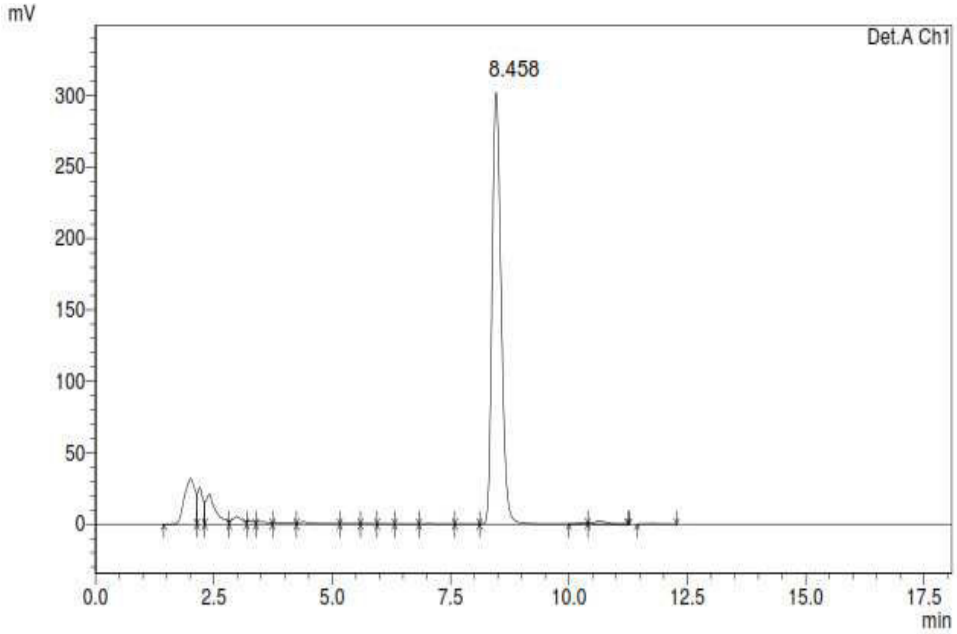

Figure 3
HPLC chromatogram showing presence of coumarin in fresh callus of C. intybus (Acetonitrile: water 40:60)

Fresh Leaf Histogram

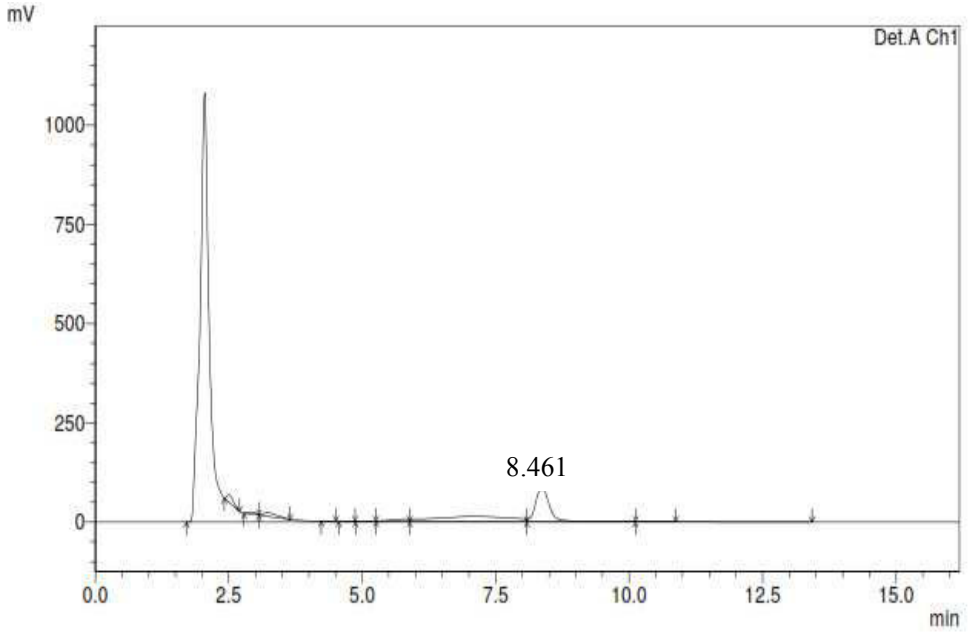

Figure 4
HPLC chromatogram showing presence of coumarin in fresh leaf of C. intybus (Acetonitrile: water 40:60)

Fresh Root Histogram

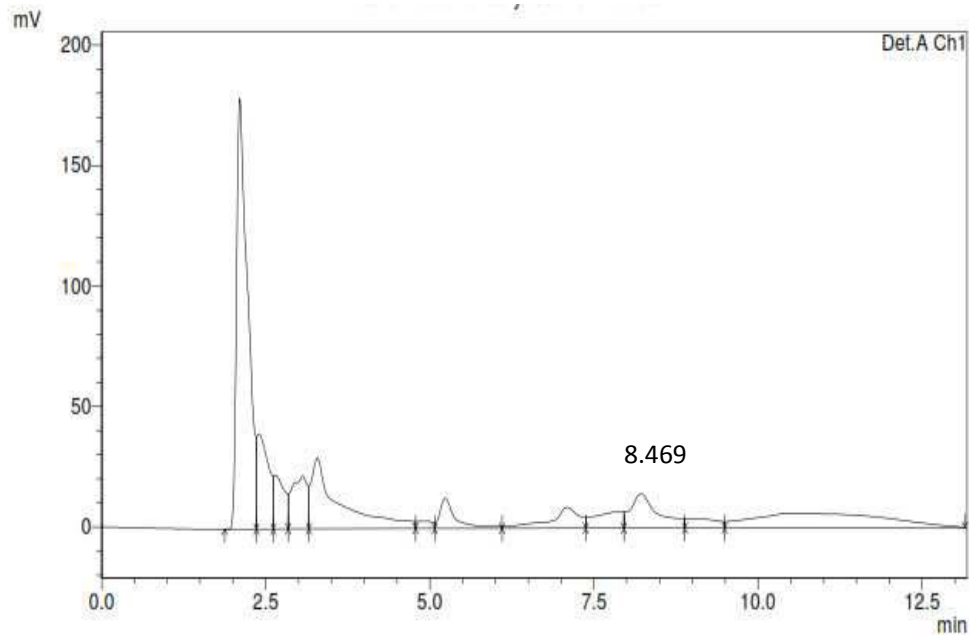

Figure 5
HPLC chromatogram showing presence of coumarin in fresh root of C. intybus (Acetonitrile: water 40:60)

Coumarin content from different parts of plants

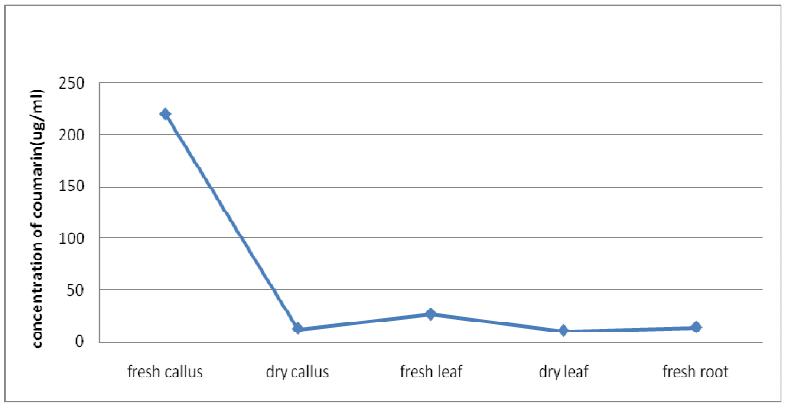

Figure 6
Coumarin content from different parts of C. intybus. Acetonitrile: water (40:60)

Coumarin content from Leaf derived callus

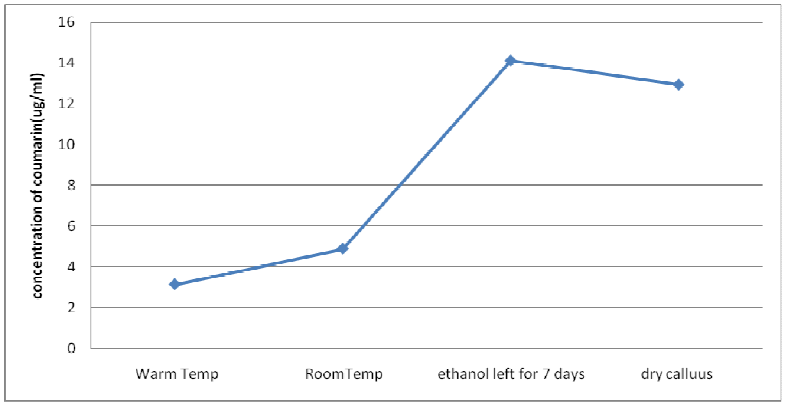

Figure 7
Coumarin content from fresh callus with variation in temperature and dry callus. Acetonitrile: water (40:60)

RESULTS AND DISCUSSION

Coumarin standards were run in HPLC and chromatogram is shown (Figure 1). The single peak coumarin was obtained at a retention time of 8.4 min. The extracts of leaf and root from *in vivo* plant of *C. intybus* showed similar peaks at retention time 8.4 min, corresponding to the coumarin standard. The callus extracts from *in vitro* derived leaf recorded the maximum content of coumarin (219.73µg/ml) in acetonitrile: water (40:60). The coumarin content recorded from leaf samples was 26.78µg/ml, then accordingly dry callus, dry leaf and fresh root recorded 12.94µg/ml, 13.02 µg/ml and 14.34µg/ml respectively in acetonitile :water(40:60)(Figure 6). The coumarin content present in fresh callus(room temp and warm temp), dry callus , fresh callus left at rest for 7 days all showed a very less amount of coumarin compound than the acetonitrile: water (40:60). The callus which was taken was about 20 days old and it was derived from the leaf inoculated with the growth regulator NAA+ BAP. Callus extracts from *in vitro* showed higher content of coumarin than the leaf extracts. This may be due to the altered physical conditions provided in the culture or by time taken by the callus, that is 20 days where the callus will be fully grown [7]. Similar results were reported by

Becker, 1970 in *Pimpinelle anisum*, *Foenicum vulgare* and *Mentha piperita*[8, 9]. No reports have been available of detection and isolation of the coumarin compounds from the callus culture of *C. intybus*. Coumarin in leaves and roots of *in vivo* grown plants at a retention time of 8.473 was identified using standard coumarin. In the present study, coumarin was detected at a flow rate of 1.0ml min^{-1} and by using the mobile phase in a binary mode. The fresh calli extracts showed higher percentage of coumarin in the calli extracts than the leaf extracts, dry calli extracts and *in vivo* plants. The calibration curve showed the linearity of the detector over the standard coumarin. 20 µl of solution was injected from solutions of standards ranging in concentrate from 1.0µg/ml- 100µg/ml and the standard curve was plotted. The amount of coumarin present in different extracts was calculated by comparing the peak area of the sample to the peak area of standard and by using the linearity equation.

CONCLUSION

These results indicated that among root, leaf and leaf derived callus of the plant used for analysis, leaf derived callus recorded a maximum coumarin.

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