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Research Article

Identification, quantification and comparative assessment of costunolide in wild, cultivated and *in vitro* callus culture of *Saussurea lappa* by HPLC technique

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Abstract

Saussurea lappa is an endangered medicinal plant. Serious efforts are needed to conserve it by rapid in vitro technique. Plant tissue culture is one of the best way for its conservation. Its root contains a bioactive compound, costunolide which possess anti-carcinogenic, anti-ulcer, antimicrobial and anti-inflammatory etc properties. A simple high performance liquid chromatographic (HPLC) method for identification and quantification of costunulide was established. Shoot and root derived calli were harvested after seven and fifteen days. The ethanolic extracts of calli, cultivated and wild plant roots, petiole and lamina were used. Standard compound, Costunolide and plant materials were extracted into 70% ethanol and separated on a Perkin Elmer, 200 series using reverse phase column C18 (25 cm × 4.6 mm, 5 µm) with UV visible detector at 225 nm. The mobile phase was HPLC grade methanol and water (70: 30 v/v) at flow rate of 1ml minute^{-1}. The total analysis time per sample was set 20 minutes. The establish method showed a good linerity ($R^2 = 0.988$). Costunolide was identified only in roots and root derived calli extracts. Wild root (root c) produced high quantity 1.257 µgml^{-1} of Costunolide followed by fifteen days old (callus b) callus 1.119 µgml^{-1} and seven days old callus 1.118 µgml^{-1} (callus c) while the quantity from cultivated root (root b, root a) was 1.105 µgml^{-1}, 1.102 µgml^{-1} respectively.

Keywords: Calli; Costunolide; High Performance Liquid Chromatography; Plant Roots

Introduction

S. lappa C.B. Clarke, belongs to Family Asteraceae (Compositae) and commonly known as costus **[1]**. It is an endangered long, erect perennial herb and is endemic to the valley of Kashmir as well as western Himalayas between 2500 to 3000 m above the sea level of Pakistan and India **[2]**. Its root contain a bioactive compound called costunolide which is used against a lot of

diseases like headache, pain, arthritis, skin diseases, ulcers, cough, flatulence, asthma, colic, fever, general weakness [3] and diarrhea [4]. It warms the center area while regulating the stomach hormones, dyspepsia, vomiting, tenesmus and dysentery [5] as well as treat migraine [6]. Its detail studies verified its pharmacologic action used to treat cardiovascular disorders as well as its anti-ulcer, anticancer, anti-inflammatory and antimicrobial properties [7]. In addition it regulates the central nervous system [8], immune system [9] and inhibits oxidization along with angiogenesis [10]. Besides it regulates growth of the plants as well as repels insects [11, 12] and affects parasites [13, 14]. It is also reported that costunolide possess anti-carcinogenic [15, 16], anti-ulcer [17, 18, 19], antimicrobial and anti-inflammatory properties as well as extensively investigated for the protein tyrosine phosphate inhibitory behaviour [20]. The over exploitation of *S. lappa* root for diverse commercial and medicinal purposes gradually decreases the availability of this plant in wild. The plant is enlisted in Endangered plant Species of Wild Fauna and Flora (CITES) and is one of the 37 endangered Himalayan therapeutic plant [21]. Tissue culture is the most efficient technique that allowed inducing and accumulating the metabolite in a short time and small space independent on seasonal and weather changes then the mother plant did, used to obtain higher amounts of bioactive compound directly from callus without sacrificing the endangered *S. lappa* plants in the wild.

Materials and methods

Sterilization of the media

Murashige and Skoog media was dispensed in flasks and then sterilized for 20 minutes by autoclaving at 121 ˚C. Other equipment like scalpel, spatulas and forceps were also sterilized with media. After autoclaving the whole materials were directly transferred to laminar flow cabinet, where subjected for 20 minutes to ultra violet (UV) radiation. The whole culture was carried out in sterilized and aseptic environment in laminar flow cabinet [22, 23, 24]. The present research work was carried out in Plant Tissue Culture Laboratory, Institute of Biotechnology and Genetic Engineering, Agriculture University Peshawar.

Plant materials

Micropropagated plant shoot and root were surface sterilized [22, 23] and used to derive callus. The used root derived calli were 7 days and fifteens days old. The wild growing plant collected from Kashmir hills (root c) whereas cultivated plants (root (a), leaves and petiole) were collected from Botanical garden of Pakistan Forest Institute, Peshawar and root (b) from Kozagali, Abbottabad. These plants materials were used for High Performance Liquid Chromatography (HPLC) analysis.

Preparation of extracts for high performance liquid chromatography (HPLC)

The determination of costunolide was carried out at centralized laboratory Physics department, University of Peshawar. *S. lappa* roots (collected from different regions), petiole, lamina and calli derived from micropropagate plant roots (callus “a”, seven days old and callus “b” fifteen days old) were shade dried at room temperature. All materials were ground to powder (20 g each) and subjected to successive extraction using ethanol. These materials were left for a week but were shaken daily. The extracts were filtered through Buchner funnel with Watman number one filter paper. The filtrates were evaporated to dryness under reduce pressure at 40 ˚C using rotary evaporator. Ethanol extracts were dried and from each samples 1mg of sample was dissolved in 1 ml HPLC grade methanol, filtered and injected into HPLC machine [24].

Preparation of stock solution and calibration standards

Costunolide standard (97% pure) for HPLC was purchased from sigma chemicals (Korea). To make costunolide stock solution, weighed 1.0 mg of

costunolde was dissolved in 1.0 ml of HPLC grade methanol. Calibration standards were prepared to obtained three concentration levels i.e 10 ppm, 50 ppm and 100 ppm by diluting the costunolide stock solution (1000 ppm) with HPLC grade methanol. The calibration curve was created by plotting the the ratio of costunolide standard peaks area [24]. The retention time for each sample was developed 20 minutes. The standard concentrations were first injected in HPLC and then run each samples one by one.

High performance liquid chromatography (HPLC)

For HPLC analysis Perkin Elmer, 200 series was used comprising UV visible detector at 225 wave length. Chromatographic separation was carried out with C18 reverse phase column (25 cm × 4.6 mm, 5 µm). The mobile phase was composed of HPLC grade methanol and water (70: 30 v/v) at flow rate of 1ml minute^{-1} and injection volume was 20 µl the total analysis time per sample was 20 minutes [24].

Results

Identification and quantification of costunolide

High Performance Liquid Chromatography of standard compound

Data regarding identification analysis of costunolide in standard compound is shown in (Table 1). Three different known concentrations of standard were used which was 10 mg/ml, 50 mg/ml and 100 mg/ml and the total flow time was set 20 minutes. Different peaks were found at different retention time but the only high peak was obtained at 14.8 minutes in all three concentrations. This peak indicates the presence of standard costunolide compound. The peaks were observed with different peak areas but at same retention time are shown in (Table 2).

Table 1. Data regarding to HPLC analysis of standard compound with different known concentrations showed different peak, peak area, height area % and normal percentage at the same retention time

Peak No.	Sample name and conc.	Time (minutes)	Area [micro volt seconds]	Height micro volt [µV]	Area [%]	Norm. Area [%]
20	Costunolide (10 mg/ml)	14.8	40665.74	1923.47	51.79	51.79
13	Costunolide (50 mg/ml)	14.8	609618.53	14780.98	91.87	91.87
23	Costunolide (100 mg/ml)	14.8	1665458.24	38062.11	38061.11	95.98

Table 2. Data regarding HPLC analysis of different samples showed different peaks, areas, heights, area % and normal percentage at same retention time. Callus c (fifteen days old), callus b (seven days old), Root a, Root b (cultivated) and Root c (wild)

Peak No.	Sample name	Time [min]	Area [µV sec]	Height [µV]	Area [%]	Norm. Area [%]
11	Callus c	14.8	562.85	33.63	0.00	0.00
17	Callus b	14.8	587.58	38.45	0.05	0.05
15	Root a	14.8	287.15	30.73	0.00	0.00
14	Root b	14.8	326.34	34.14	0.06	0.06
13	Root c	14.8	1090.86	65.97	0.02	0.02

Three different peak areas of costunolide and their concentrations

In (Figure 1) the series 1 shows the chromatogram of the standard compound at concentration of 100 mg/ml. The high peak areas at 2.1, 2.2, 2.5, 3.4, 4.4, 6.2, 14.8 and 19.8 minutes were observed 3722.9, 4247.5, 4342.6, 29515.5, 2668.1, 19276.6,

1665458.2 and 3513.6 µVsec respectively. The (Figure 1) shows that highest peak area, 1665458.2 µV was observed at 14.8 minute. Series 2 indicates the chromatograme of the same standard compound but with 50 mg/ml concentration. The high peak areas at 2.0, 2.1, 2.3, 3.1, 3.4, 4.4, 6.2,14.8 and 19.8 minute were found 6995.8, 11902.6, 4399.3, 1124.9, 13744.3, 4539.1, 8709.0, 609618.5 and 1017.4 µVsec respectively. The maximum peak area 609618.5 was observed at retention time 14.8 minute. Series 3 shows the chromatogram of the standard compound with 10 mg/ml concentration. The peak areas at 10.061, 14.843, 16.657, 17.694, 17.694 and 19.580 minutes were recorded 46.36, 40665.74, 22.29, 16.09 and 1.40 µVsec respectively. The highest peak area was 40665.74 µVsec among these peak areas recorded at 14.8 minute. All the three concentrations showed peaks on the same retention time and were taken as standard peaks areas to calculate the unknown concentrations of the tested samples.

Calibration curve for different concentrations of costunolide

ln order to observe the unknown concentrations of the calli and root samples calibration curve for costunolide was obtained under optimum condition by plotting costunolide peaks areas verses its three different concentrations (10, 50 and 100 mg/ml) as shown in (Figure 2). The calibration curve showed good linearity in relatively wide concentration range. The regression equation used for standards was $y = 18179x - 19762$ ($R^2 = 0.988$).

Data regarded quantitative analysis of costunolide through HPLC is presented in (Table 2) showing different peaks at different time for each samples but costunolide showed the same retention time. Root collected from Kashmir forest (root c) covered high area is 1090.86 µVsec and also large height 65.97 µV of the peak followed by fifteen days old root callus (callus b) 587.58 µV sec and 38.45 µV sec and seven days old root callus (callus c) 562.85 µV sec and 33.63 µV while root collected from Koza gali, Abbottabad (Root b) showed 326.34 µV sec area and 34.14 µV height of the peak. However, the root collected from Pakistan Forest Institute Peshawar (root a) showed less peak area 287.15 and height 30.73 compared to all other samples.

Shoot callus (callus 'a'), lamina and petiole excised from natural plant, extracts were also prepared properly and run through HPLC. Many unknown peaks were found at different time but no peak was observed at 14.8 minute to determine costunolide. The peak area of each sample was used to quantify costunolide in each sample and presented with its chromatographs.

HPLC chromatogram of ethanol extract callus "b"

The chromatogram of the ethanolic extract of callus 'b' is shown in (Figure 3) with the time shown in minutes. It is clear from the (Figure 3) that at 14.2, 14.8, 17.8 and 17.9 minutes the peaks areas 43.7, 587.5, 34.5 and 26.8 µV sec were found respectively. The peak area 587.58 µV sec of costunolide was observed at 14.8 minute with the calculated concentration of 1.119 µg/ml.

HPLC chromatogram of ethanol extract callus "c"

The chromatogram of the ethanolic extract of callus 'c' is shown in (Figure 4). It is evident from the (Figure 4) that at 14.6, 14.8 and 17.641 minutes the peaks areas were found 26.43, 562.85 and 983.02 µVsec respectively. The costunolide peak area 587.58 µVsec at retention time 14.8 minute was observed as shown in (Figure 4). In callus 'c' the concentration of costunolide was recorded 1.118 µg/ml.

HPLC chromatogram of ethanol extract of root "a"

The chromatogram of the ethanolic extract of root 'a' is presented in (Figure 5). It is obvious from the (Figure 5) that at retention time 14.3 and 14.8 minutes the peaks areas were recorded 97.51 and 287.15 µVsec respectively. While no peak was appeared on 15 and 16 minutes as

cleared from the (Figure 5). The costunolide concentration was observed about 1.1028 µg/ml.

HPLC chromatogram of ethanol extract of root "b"

The chromatogram of the ethanolic extract of root 'b' is shown in (Figure 6). It is clear from the (Figure 6) that at retention time 14.8 minute the peak area found was 326.34 µVsec. However, no other peak was observed at 14, 15 and 16 minutes. In root 'b' the concentration of costunolide was about 1.105 µg/ml.

HPLC chromatogram of ethanol extract of root "c"

The chromatogram of the ethanolic extract of root 'c' is shown in (Figure 7). It is observed from the (Figure 7) that at retention time 14.8 minute the costunolide peak area was appeared 1090 µVsec. While no peak was observed on 15 to 16 minutes as clear from (Figure 7). In root 'c' the concentration of costunolide was about 1.257 µg/ml. Non significant results were observed in all the tested samples.

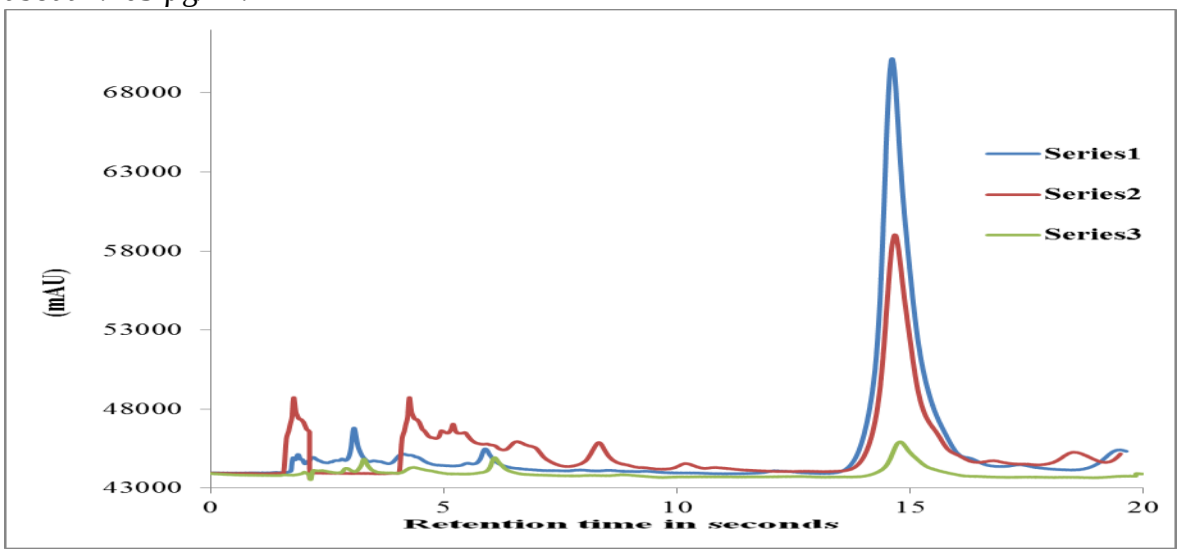

Figure 1. Three different peaks of Costusnolide and its concentrations
Series 1: The chromatogram of Costunolide 100 mg/ml concentration
Series-2: The chromatogram of Costunolide 50 mg/ml concentration
Series-3: The chromatogram of Costunolide 10 mg/ml concentration

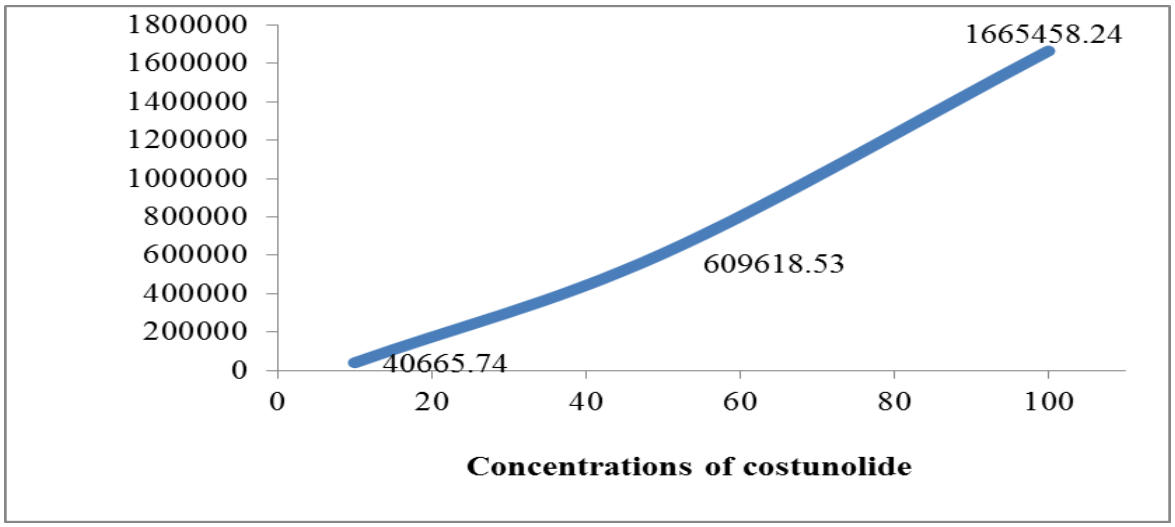

Figure 2. Calibration curve plotted for three different concentrations of costunolide High Performance Liquid Chromatography analysis of root calli and root samples

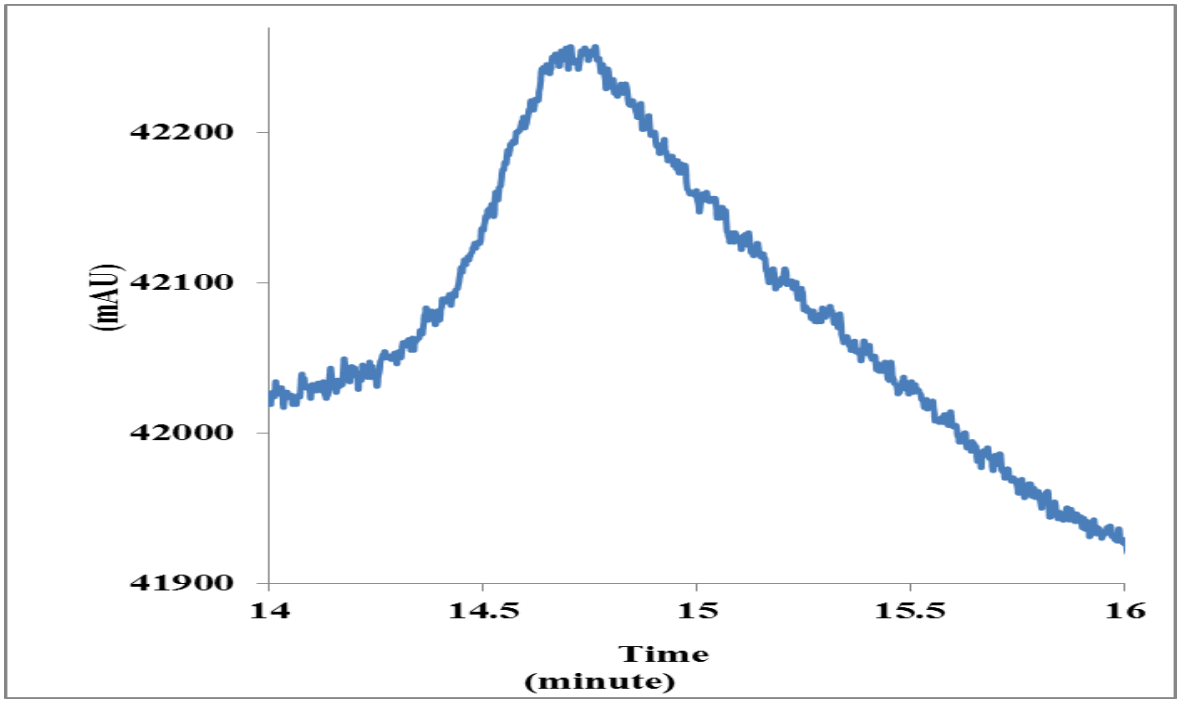

Figure 3. HPLC chromatogram of ethanol extract callus "b"

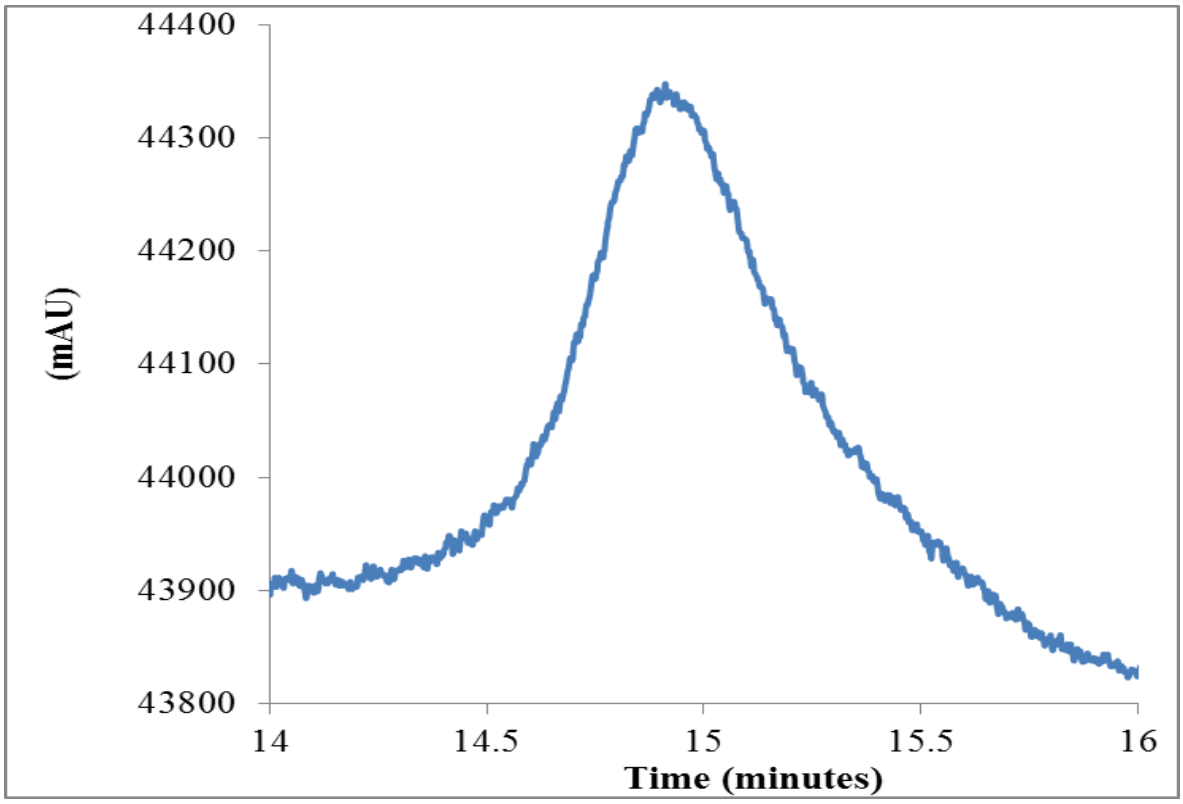

Figure 4. HPLC chromatogram of ethanol extract of callus "c"

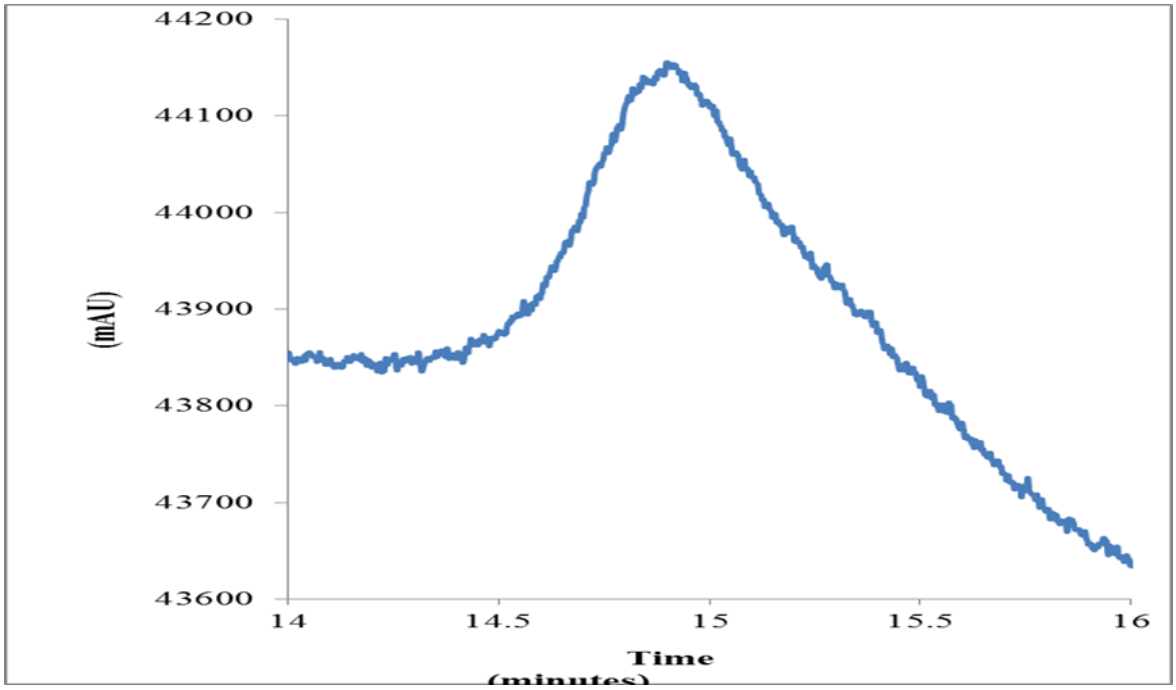

Figure 5. HPLC chromatogram of ethanol extract of root "a"

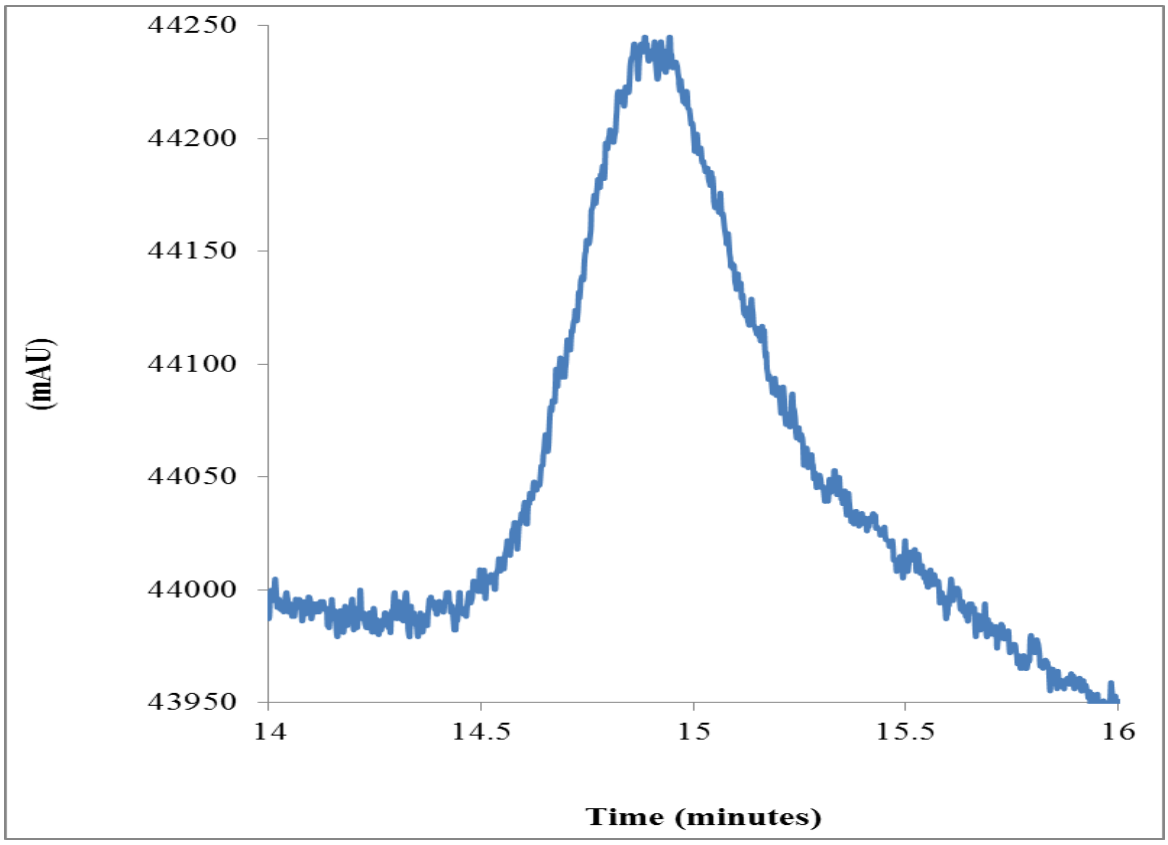

Figure 6. HPLC chromatogram of ethanol extract of root "b"

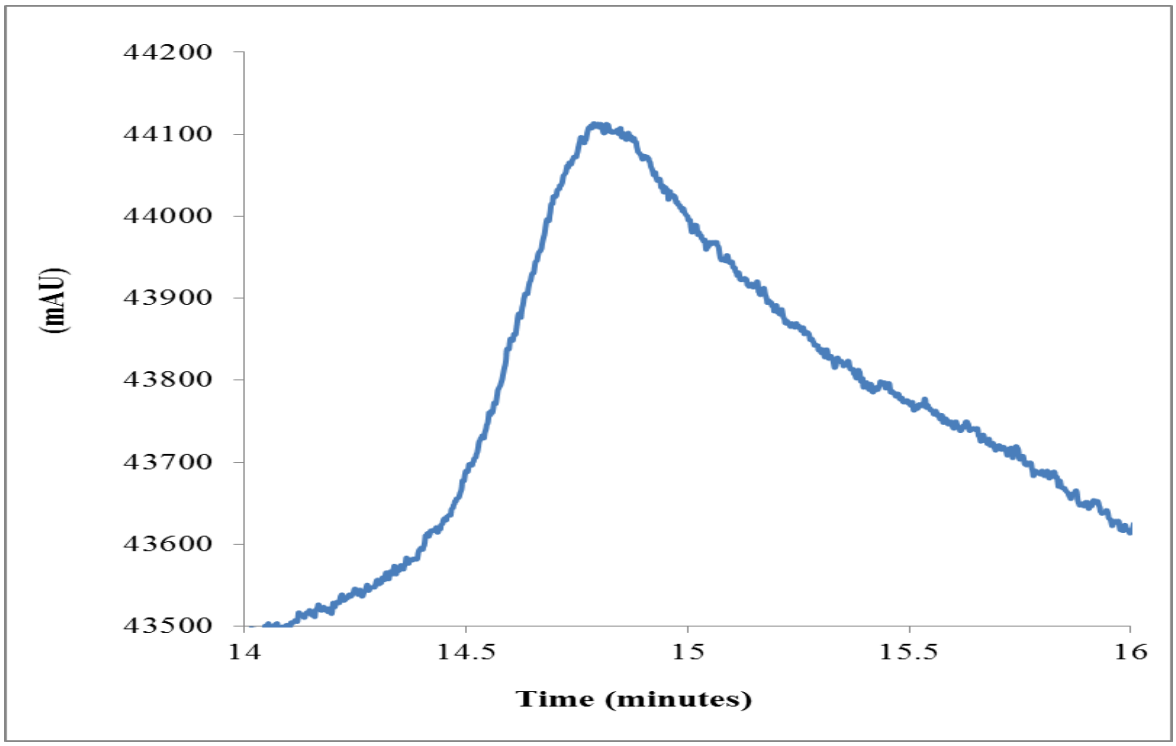

Figure 7. HPLC chromatogram of ethanol extract of root "c"

Discussion

High performance liquid chromatographic analysis

HPLC technique was employed to investigate, identify and quantify the quantity of costunolide in calli (root, petiole and lamina), wild and field grown plant's roots, petiole, lamina of *S. lappa* and compared them with each other. We developed an easy, a cheap and accurate method of HPLC for costunolide quantification in *S. lappa*. Calli cultures established from seedling and different parts of micropropagated plantlet of *S. lappa* are thought to be an important source of secondary metabolites for such studies. As naturally growing plants have been extensively exploited for medicinal and economic purposes and huge numbers of these plants have been used in folk medicines. In this case, plant tissue culture is the most efficient technique used to obtain higher amounts of bioactive compound, that allowed inducing and accumulating the metabolite in a short time than mother plant did. Our results show that 100 mg/ml costunolide has many small peaks and a single large peak was observed in 20 minutes on 14.8 seconds so the flow rate was fixed at 20 minutes. In all three concentrations many different peaks were observed but a highest peak was found at the same retention time of 14.8 seconds which identified the presence of the same bioactive compound. The other peaks may be some contamination of the solvent or extracts as these were not found in all the three series or the impurities of the compound as the standard costunolide was 97% pure. In all ethanolic extracts of natural plant petiole, lamina and callus 'a' (shoot derived callus) costunolide was not observed while in all samples of roots and root calli costunolide was observed which indicated that it is found only in roots of *S. Lappa*. Our results match with previous reports **[24, 25]**. The quantitative HPLC analysis indicated that *S. lappa* root calli cultures give slightly better results than root 'a' and root 'b'. Furthermore, our report showed that age of calli also affect the concentration of Costunolide as ffiteen days old callus (callus b) produced a bite more amount of the compound compared to

seven days old callus (callus c). In this case, it is observed that plant tissue culture is the most efficient technique used to obtain higher amounts of bioactive compound, that allowed inducing and accumulating the metabolite in a short time than mother plant did. These conclusions are in agreement with the report of Lima *et al.* **[26]**. In between root 'a' and root 'b' very little difference in content of costunolid was observed as both roots were collected from cultivated farms of different regions. However, Root 'c' (wild root) showed higher content of Costunolide than callus 'a', callus 'b', root 'a' and root 'b'(cultivated roots). These differences in concentration can be attributed to several environmental and different geographical variations. These results also match the previous reports **[24, 25, 27, 28]**. Plant material (calli) through plant tissue culture technique is free from environmental variations, therefore, it enhance the reproducibility of the experiments and products as well.

Conclusion

Based on the current research work it is concluded that quantitative HPLC analysis indicated that root *callus* culture produced the same costunolide bioactive compound and also approximately in similar amount found in the roots of intact cultivated and wild growing plant.

Authors' contributions

Conceived and designed the experiments: ZU Nisa, Performed the experiments: ZU Nisa, Analyzed the data: G Farooq, Contributed materials/ analysis/ tools: SH Shah, Wrote the paper: MA Sajad & MAS Khan.

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