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Research Article

Simultaneous Estimation of Taraxerol and Taraxasterol in Root Callus Cultures of *Taraxacum officinale* Weber.

*Kiran Sharma, Rasheeduz Zafar

Deptt.of Pharmacognosy and phytochemistry, Faculty of pharmacy Jamia Hamdard, New Delhi-110062.

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ABSTRACT

A simple, selective, rapid, precise and economical reverse phase high-pressure liquid chromatographic method has been developed for the first time for the simultaneous estimation of Taraxerol and Taraxasterol, in natural root and root callus cultures of Taraxacum officinale (TO), maintained on MS media using hormone combinations, MS+IAA+BA (2ppm+1ppm) and MS+IAA+BA+2,4 D (0.5ppm+1ppm+0.5ppm). Column of ODS, C8-3 (250 mm x 4.6 mm i.d, partical size 5 µ) was used for the separation with a mobile phase consisting of acetonitrile: 0.1% phosphoric acid in methanol (70:30: v/v) at a flow rate of 0.6 ml/min, detection was carried out at 210 nm. The retention time of taraxerol and taraxasterol were 4.975, 3.5 min. respectively. The developed method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantification. The proposed method can be used for estimation of these compounds in herbal formulations as well in crude drug extracts.

Key Words: *Taraxacum officinale* (TO), Callus culture, Triterpenes, Taraxerol, Taraxasterol, *RP-HPLC.*

INTRODUCTION

Taraxacum officinale (TO) belonging to family Asteraceae, is a traditional medicinal herb known as common dandelions have been used in herbal medicines for their chloretic, anti- rheumatic, anti-inflammatory, diuretic and anti-carcinogenic properties. TO roots are known to possess antiulcer, antiviral, antioxidant, hypolipidemic, anticarcinogenic and prebiotic activities. Also the roots are used as herbal tea and substitute for caffeine free coffee. Its blanched leaves are eaten as salad and aerial parts possess anti-rheumatic activities. Whole plants possess anti-hyperglycemic, anticoagulatory, analgesic and anti-allergic properties and flowers are used for making wine.[1]Dandelion whole plant contains a wide variety of active compounds but our main focus is on the roots, which contain various types of pentacyclic triterpenoids (e.g. oleanane, ursane and taraxastane types). Among them taraxerol and taraxasterol are the important compounds possessing important pharmacological actions.[2] Also it has been known to benefit in Alzhiemer's and Parkinsonism.[3-4] Taraxerol is known to induce apoptosis,[5] COX inhibitor,[6] acetyl cholinesterase inhibitor[7] and possess antimicrobial potential.[8-9] Taraxasterol is anticarcinogenic,[10] anti-allergic,[11] anti-oxidant and anti-inflammatory.[12] Both these compounds are known for their potential in Alzhiemer's.[13]

Biotechnology represents a convenient experimental system for plant science research and a promising technology for the production of medicinal plants and their active constituents. Both these components acquire high therapeutic efficiency and root tissue culture proves to be a promising technology for improving the yield of these secondary metabolites. Although there are several reports available which suggest the presence of these active components in *T. officinale* root callus cultures but this is the first time where we have explored taraxerol and taraxasterol from the natural and root callus culture of *T. officinale* simultaneously and quantified by RP-HPLC. The primary objective of the current study was to establish a stable root callus and quantify the presence of Taraxerol and Taraxasterol in it.

MATERIAL AND METHODS

Plant material*: Taraxacum officinale* was brought from Kashmir and grown in herbal garden Jamia Hamdard, New Delhi and collected from there and authentification of plant and seeds were done at Pusa institute, NISCAIR (Raw Materials Herbarium and Museum, its voucher specimen number is NISCAIR/RHMD/Consult/2013/2273/53.

Establishment of root callus culture: The seeds were collected in the month of October and washed with water 2-3 times, again washed with teepol solution and rinsed thoroughly with Tap water 2-3 times and then treated with 0.1% sodium hypochlorite for 6 min. Then these sterile seeds were aseptically transferred into sterile petriplates containing absorbent cotton and filter paper. The petriplates were then covered with aluminum foil and kept in BOD incubator at conditions (24± 2ºC, 55-60% RH) for

*Author for correspondence: E-mail: kiransharmapharma@gmail.com

R1: R1-MS+IAA+BA+2, 4-D (0.5ppm+1ppm+0.5ppm)

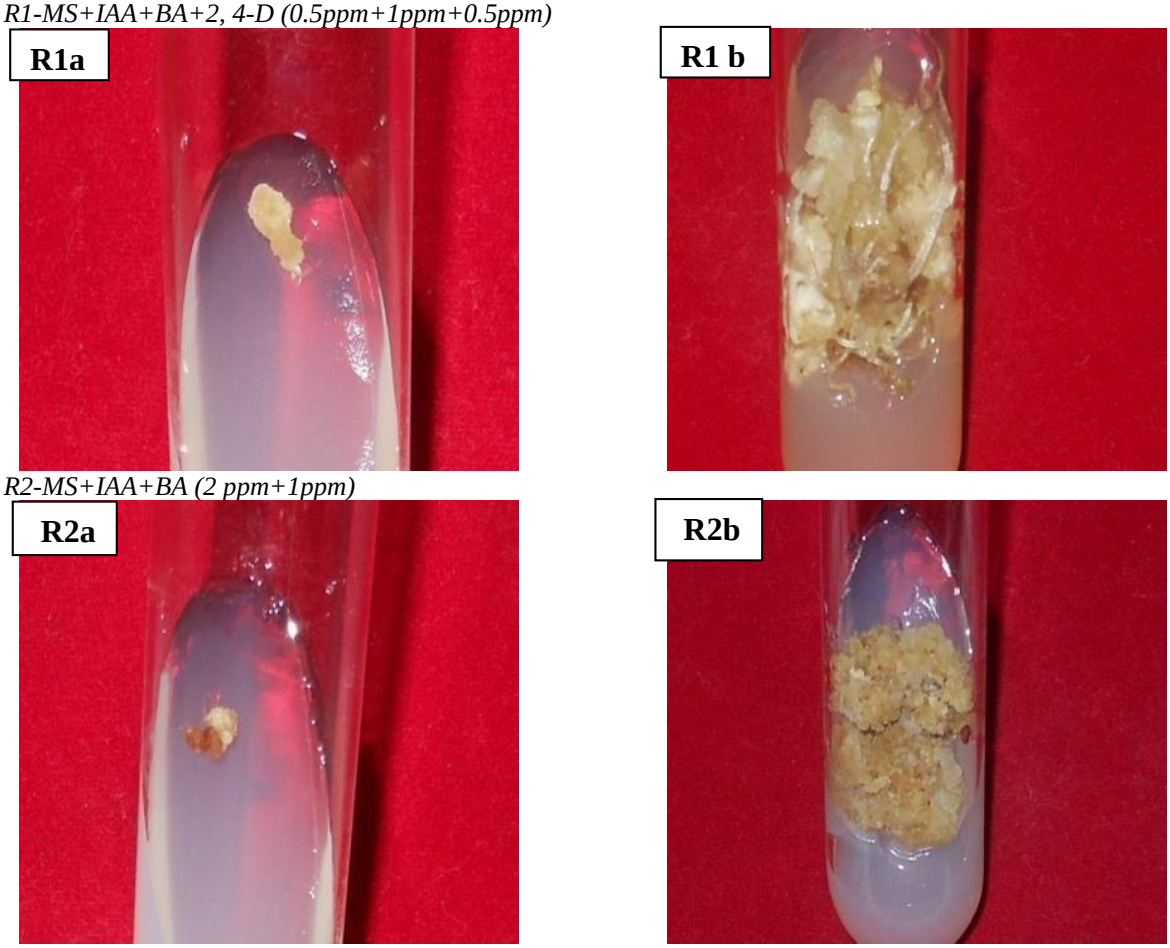


Fig 1: Initiation and maintenance of root callus using different combinations of hormones (R1 & R2). R1a & R2a: initiation of root callus; R1b & R2b: 90 days old root callus.

Table 1: Qualitative chemical tests for the presence/absence of various phytoconstituents

S.No.	Class of compounds	Natural root	Root callus A	Root callus B
1	Alkaloids	+	+	+
2	Amino -acids	+	+	+
3	Flavonoids	-	-	-
4	Carbohydrates	+	+	+
5	Phenolics	+	+	+
6	Steroids	+	-	-
7	Tannins	+	+	+
8	Saponins	+	+	+
9	Mucilage	+	+	+
10	Proteins	+	+	+
11	Anthraquinone Glycosides	-	-	-
12	Terpenoids	+	+	+

+Present, - Absent

germination. After one week seeds showed germination. The aseptically grown seedlings were taken and its root tip was cut and aseptically transferred into solid Murashige and Skoog's (MS) medium as basal medium containing hormone combinations MS+IAA+BA (2ppm+1ppm) and MS+IAA+BA+2,4 D (0.5ppm+1ppm+0.5ppm). The callus initiation was seen after 15 days. After initiation of root callus, they were separated aseptically from the mother explants and the calli initiated on root in 16-21 days and were transferred to the same medium on which they were initiated in order to develop an independent calli. The growth was observed for further 3 weeks. The developed calli were chopped down and transferred aseptically in MS medium supplemented with the similar hormonal combinations which showed excellent results in the previous experiment. During the maintenance, the sub-culturing was routinely done after every three weeks. The callus cultures were further subculture into the fresh media as independent callus cultures and maintained up to 90 days.

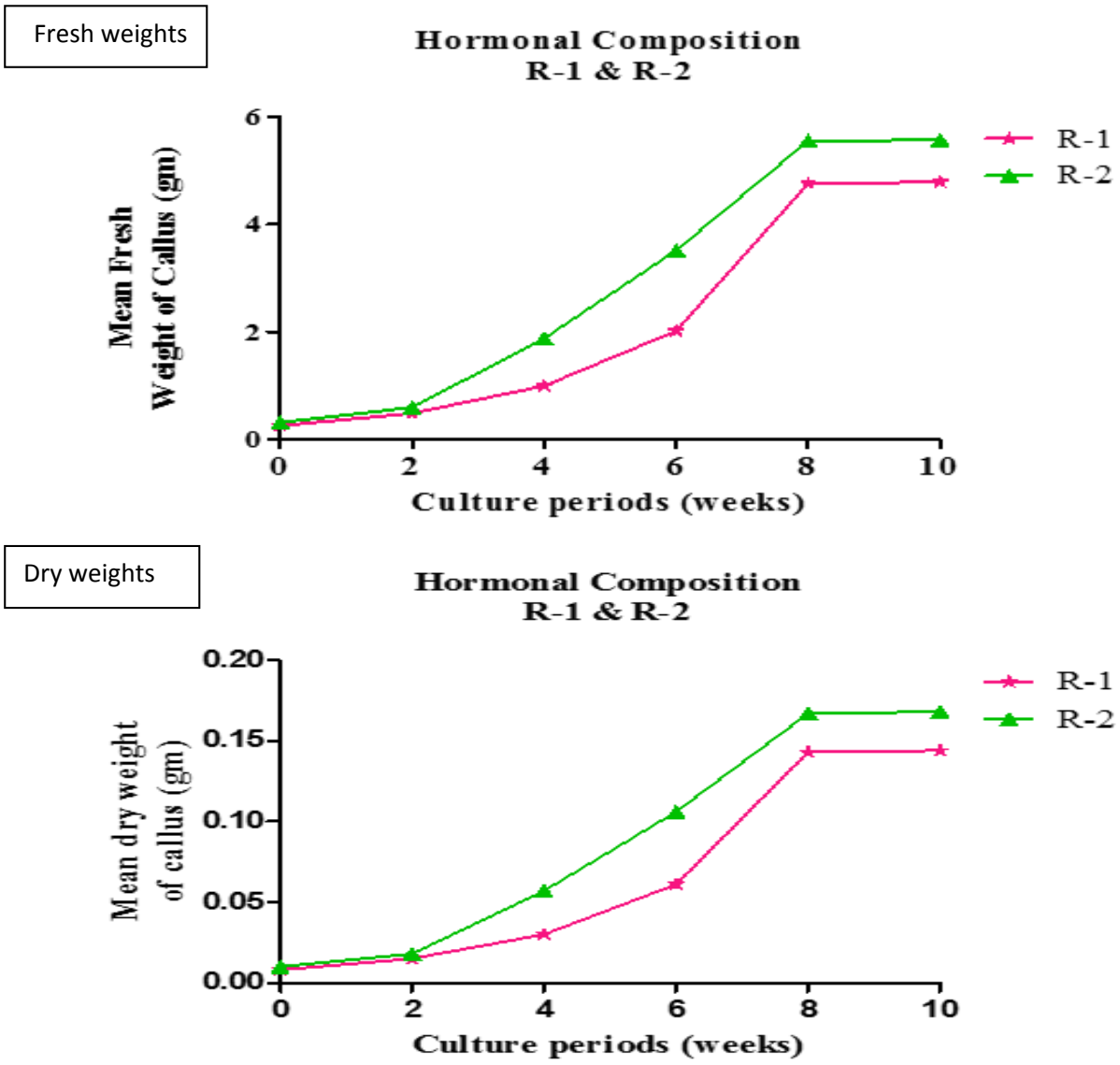


Fig 2: Increase in fresh weight (gm) and dry weights (gm) of R1 and R2.

Reagents and Chemicals: Acetonitrile (HPLC grade) and Methanol (HPLC grade) was purchased from Merck specialties pvt. Ltd. (Worli, Mumbai, India) and Water (HPLC grade) was purchased from Loba Chemie (Mumbai, India). Phosphoric acid AR was purchased from Merck and S. D. Fine Chemicals, Mumbai respectively. All other reagents used were of HPLC grade. Working standards of Taraxerol and Taraxasterol were provided by eBiotech Inc., Devali, New Delhi.

Preparation of standard solutions: Standard stock solution of Taraxasterol and Taraxerol 1mg/ml was prepared by dissolving in methanol followed by ultra - sonication.

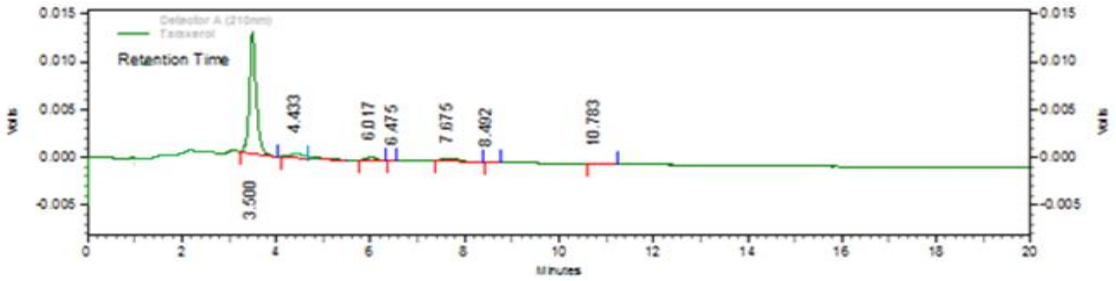
Extraction of cultured root callus and preparation of sample solutions: 1 gm each of powdered natural root and calli (Root callus 1 and root callus 2) were dried at 60^0 was extracted with 25 ml of methanol separately, filtered and filtrates were concentrated. The final volume was made upto 10 ml. 1 ml solution was taken out from this and filtered through 0.2 µm membrane filter (Gelman Science, India). The samples were analyzed by HPLC for the quantitative estimation of taraxerol and taraxasterol by a Shimadzu model HPLC equipped with quaternary LC-10A VP pump, ODS, C8-3 column (250 mm x 4.6 mm i.d, partical size 5 µ) was used for the separation, variable wavelength programmable UV/VIS detector, SPD-10AVP column oven (Shimadzu), SCL 10AVP system controller (Shimadzu), Rheodyne injector fitted with a 20 µL loop. Class-VP 5.032 software was used for the routine drug analysis.

Determination of content of Taraxerol and Taraxasterol

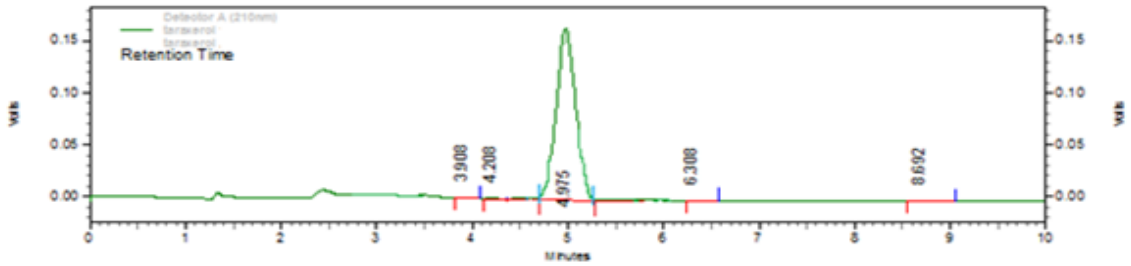
Method Development: Different mobile phases containing methanol, water, acetonitrile, and different buffers in different proportion were tried and finally of acetonitrile: (0.1% phosphoric acid in methanol) (70:30 v/v) was selected as mobile phase was delivered at a flow rate of 0.6 ml/min with detection at 210 nm. The mobile phase was filtered through a 0.22µ membrane filter and degassed. The injection volume was 20 µl analysis was performed at ambient temperature which gave good resolution and acceptable peak parameters for both Taraxasterol and Taraxerol.

Preparation of Calibration Curve: Standards of Taraxerol and Taraxasterol were used to construct a calibrated graph by plotting peak areas versus the amount of Taraxerol and

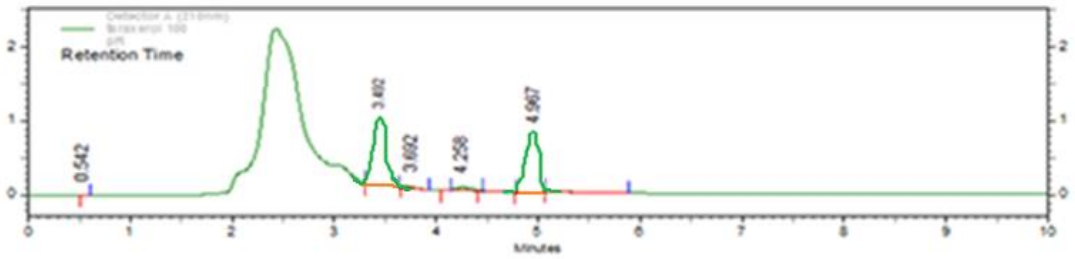
2a: HPLC Chromatogram of Standard compound (Taraxasterol) at 210 nm



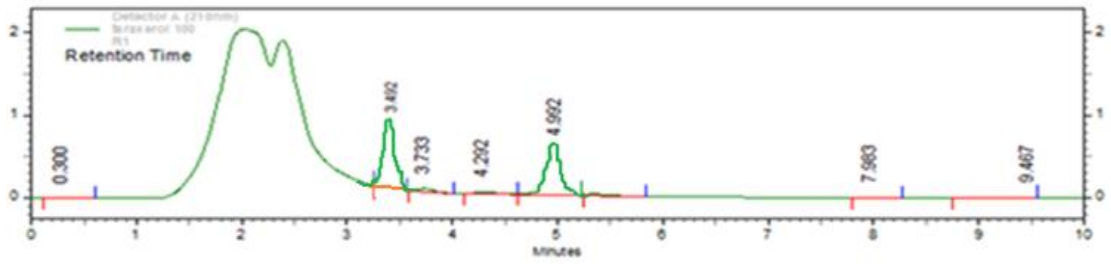
2b: HPLC Chromatogram of Standard compound (Taraxerol) at 210 nm.



2c: HPLC Chromatogram of root extract



2d: HPLC Chromatogram of root callus (R1-MS+IAA+BA+2,4-D (0.5ppm+1ppm+0.5ppm)



2e: HPLC Chromatogram of root callus (R2-MS+IAA+BA (2 ppm+1ppm)

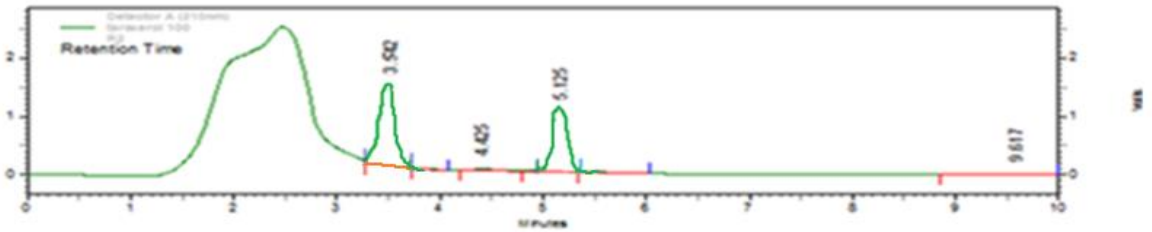


Fig 3: RP-HPLC HPLC Chromatograms of Taraxerol and Taraxasterol in methanol extracts of callus, original root and regenerated roots of T.officinale.

Taraxasterol over a range of 100-500µg/ml. Linearity of the system was investigated by serially diluting the stock solutions to give concentrations in the range of 100-500 µg/ml for taraxasterol and 100-500µg/ml taraxerol. An aliquot (20µl) was injected using mixture acetonitrile: (0.1% phosphoric acid) methanol (70:30) v/v as mobile phase. Calibration curves were obtained by plotting the Peak area vs. concentration. The equations of the

regression lines are For Taraxasterol, y = 20061X+33915 (R^2= 0.989), For Taraxerol y = 19832X+38282 (R^2= 0.990). The response was linear over the tested concentration range.

Statistical analysis: A randomized block design was used for all experiments. Each experiment had triplicates with each replicate consisting of at least 20 explants. Data were subjected to analysis of variance to evaluate the significant differences between means. Data variability was expressed as the mean ± SE. The results were analyzed statistically.

RESULTS AND DISCUSSION

Establishment of root callus culture: In the present investigation, a callus culture of *T. officinale* root was developed from seedling in order to study the content of Taraxerol and Taraxasterol. The seeds of *T. officinale* were germinated aseptically by using various sterilants; sodium hypochlorite and mercuric chloride of different concentrations at different contact times. In contrast to mercuric chloride exposure to sodium hypochlorite of concentration 0.1% for 6 min showed better rate of germination. The roots of the sterile plantlets showed slow or no growth when cultured in MS liquid media without growth hormones. Good results have been found when these sterile rootlets were transferred to the sterilized culture tubes containing MS medium supplemented with different hormonal combinations, but combinations which showed good initiation and good growth rate were MS+IAA+BA (2ppm+1ppm) and MS+IAA+BA+2,4 D (0.5ppm+1ppm+0.5ppm) and shown in fig. 1 .

Earlier reports that showed good root callus growth resulted when sterilized root material was grown on a modified white's medium supplemented with, indole acetic acid (IAA) 2 mg l^{-1} and10% coconut milk.[14] The later as liquid medium supported the growth of callus aggregates showing root organogenesis. Again using root explants, developed and maintained callus growth on White's basal medium supplemented with 0.4 mg/l IAA and 15% coconut milk or 1mg/l kinetin.[15] So, in the present study we developed root callus, from seedling's root tip and maintained it on MS medium using hormone combinations, MS+IAA+BA (2ppm+1ppm) and MS+IAA+BA+2,4-D (0.5ppm+1ppm+0.5ppm) and obtained fairly good callus growth rate. Growth kinetics of callus culture was studied, in terms of increase in fresh weight and dry weights of root callus after every subculture and results are shown graphically in fig. 2.

Qualitative estimation*:* The qualitative chemical tests were performed for the presence/absence of various plant constituents in natural root and root callus of different hormonal combinations. It was found that the natural root, root callus culture contains alkaloids, amino-acids, carbohydrates, phenolic compounds, terpenoids, tannins, saponins, mucilage and proteins.[16]

Quantitative estimation: Pentacyclic-triterpenes, taraxasterol & taraxerol are therapeutically active biomarker compounds that are widely present in various plants. Extraordinarily high taraxerol content was reported in the leaves of *Rhizophora mangle*, especially *Rhizophora racemosa*.[17] whereas taraxasterol, quite frequently but not exclusively present in compositae family. Taraxasterol was initially explored by Furuno et al, suggesting the organ mediated accumulation of Taraxasterol in *T. officinale*. Later on in 1994 again Taraxasterol was estimated in *T. officinale*,[18] suggesting its organ mediated accumulation especially in latex.[19] According to his work biosynthesis of taraxastane appears to be associated with organ differentiation, (pseudo)-laticifer cells being the probable site of their biosynthesis.

These potential triterpenoids are both present in very lesser quantity. We are here introducing a plant model system to trigger the concentration of both these components for the first time from the root culture of *T. officinale*. RP-HPLC method is one of the most fast and reliable method for identification of phytochemicals. Quantitative analysis of Taraxerol and Taraxasterol carried out by RP-HPLC method. The method was validated in accordance to ICH guidelines by determining several parameters of

Table 2: Quantification of Taraxerol and Taraxasterol in the root cultures of *T. officinale* (R1& R2)

S.No.	Tissue/ Cell line	Constituent	Retention time (min)	Area	Conc. in injected sample (µg/ml)	Conc. (%dry weight)
1.	PR	TX	3.492	93321	2.96	0.0299
		TA	4.967	71727	1.686	0.0169
2.	RC1	Tx	3.492	94357	3.013	0.0304
		TA	4.992	73123	1.757	0.018
3.	RC2	Tx	3.542	94216	3.006	0.0303
		TA	5.125	73019	1.751	0.0177

R1: Root culture 1; R2: Root culture 2; Tx: Taraxerol; TA: Taraxasterol

Table 3: Recovery studies of Taraxasterol.

S.NO	Standard Taraxasterol Conc.((µg/ml)	Root extract Taraxasterol Conc ((µg/ml)	Taraxasterol Conc. after spiking (µg/ml)	Taraxasterol Conc. Detected after spiking (µg/ml)	Recovery (%)	Average recovery of Taraxasterol
1.	10	2.96	11.48	10.247	89.2	92.523
2.	15	2.96	13.98	13.216	94.53	
3.	20	2.96	16.48	15.47	93.84	

Table 4: Recovery studies of Taraxerol.

Standard Taraxerol Conc.((µg/ml)	Root extract Taraxerol Conc. ((µg/ml)	Taraxerol Conc. after spiking(µg/ml)	Taraxerol Conc. Detected after spiking (µg/ml)	Recovery (%)	Average recovery of Taraxerol
10	1.686	10.45	10.01	95.7	94.7
15	1.686	12.91	12.23	94.7	
20	1.686	15.50	14.51	93.6	

performance quality. Linearity and detector response were determined over a range of 1.2 – 720 µgmL-1. The detector response was linear over the tested concentration range. The equations of the regression lines are: Taraxasterol, y = 20061X+33915 (R^2= 0.989), and for Taraxerol, y = 19832X+38282 (R^2= 0.990). Limit of detection for Taraxasterol and Taraxerol were found to be 10µg/ml and 20 µg/ml respectively. Limit of Quantification was found to be 30µg/ml for Taraxasterol and 40µg/ml for Taraxerol respectively. Peak purity and identity were verified by studying the PDA-data, as well as spiking sample with standards of Taraxerol and Taraxasterol. The accuracy of the method was confirmed by recovery experiments. One of the samples was spiked with a known amount of the standards of Taraxerol and Taraxasterol. The spiked samples were extracted and analyzed under the optimized conditions. Recovery of 92.52 ± 1.12% taraxasterol and 94.7 ± 1.21% of Taraxerol were obtained (Table 3 & 4). Inter-day variation of the assay was determined at 100µg/ml and 200µg/ml and showed to be lower than 1 % for Taraxasterol and 1.2 % for Taraxerol. The chromatogram showed the retention time of 3.5 mins. (A: 2140500) with standard Taraxasterol and 4.975 (a: 2181000) with standard Taraxerol in methanol (Fig. 3, 2a and 2b). The chromatogram of standard and the different extracts showed sharp and uniform peak with non significant variations in retention time. By HPLC analysis, the quantity of Taraxasterol in natural root extract was found to be 2.96µg/ml and 1.686µg/ml for Taraxerol (Fig. 3, 2c). In contrast root callus cultures, R1 (MS+IAA+BA+2, 4 D-0.5ppm+1ppm+0.5ppm) showed 3.013µg/ml of Taraxasterol and 1.757µg/ml of Taraxerol (Fig. 3, 2d), similarly R2 (MS+IAA+BA-2ppm+1ppm) showed 3.006 µg/ml of taraxasterol and 1.751 µg/ml of taraxerol (fig. 3, 2e) were found in the root culture of *T. officinale*. Previous reports suggest the presence of Taraxasterol in cultures of Taraxacum officinale and its natural root,[19] but both these compounds are quantified for the first time from root callus.

CONCLUSION

The HPLC analysis showed enhancement in the yield of Taraxerol and Taraxasterol in callus culture of root as compared to the natural root extract. The present work is significant as the two biologically active triterpenols have been quantified simultaneously for the first time in root callus cultures. Hence, tissue culture will prove to be a valuable strategy to increase the yield of these important secondary metabolites. Further, by using advanced plant tissue culture techniques, such as addition of precursors and biotic and abiotic elicitors or genetic transformation by *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens species*, will definitely result in increase in the yield of these phytoconstituents to a considerable level.

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