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ORIGINAL ARTICLE

Effect of Seasonal Variation on Euphadienol Content in *Euphorbia antiquorum* L. and *Euphorbia tirucalli* L. using High-Performance Thin-Layer Chromatography Method

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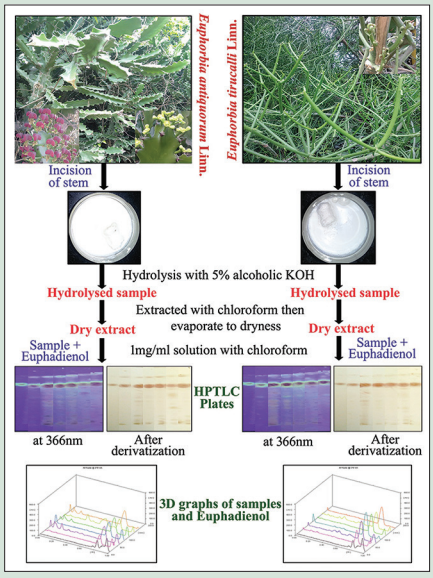
ABSTRACT

Background: Ayurveda emphasizes that different parts of plants (both organized and unorganized) should be collected on a certain season to get their better yield and therapeutic value. In general, *Kshira* (latex) should be collected in *Sharada rutu* (autumn season). However, the latex of *Snuhi* has been stated to be collected at *Shishiranta* (end of winter). The latex of *Snuhi* has an important place in different therapeutic formulations. One of the important mentions is for the preparation of *Ksharasutra*, one of the widely used paramedical procedures in Ayurveda. **Objectives:** The objective of this study was to evaluate the impact of seasonal variation on the quantity of euphadienol content (one of the major active constituents) in latex sample of *Euphorbia antiquorum* L. (EA) and *Euphorbia tirucalli* L. (ET). **Materials and Methods:** The latex was collected individually during six different seasons in a calendar year from two species i.e. EA and ET. Then, after suitable sample preparation, it was subjected to high-performance thin-layer chromatography study using euphadienol as a standard biomarker and scanned at 210 nm. **Results:** The euphadienol content was found to be present in all the six seasons across the calendar year. The content was found maximum in *Hemanta rutu* and *Sharada rutu* samples in case of EA and ET, respectively. **Conclusion:** The study proves that changes of seasons do have a certain effect on the phytoconstituents in the plants.

Key words: Euphadienol, *Euphorbia antiquorum*, *Euphorbia tirucalli*, high-performance thin-layer chromatography, *Snuhi*

SUMMARY

- Impact of seasonal variation of *Euphorbia antiquorum* L. (EA) and *Euphorbia tirucalli* L. (ET) latex samples was evaluated by high-performance thin-layer chromatography study using euphadienol as a reference standard. The results of the study highlight that the euphadienol was found to present in all six season samples and the content was maximum in *Hemanta rutu* and *Sarada rutu* samples in case of EA and ET, respectively.



Abbreviations Used: TLC: Thin-layer chromatography; HPTLC: High-performance thin-layer chromatography, HPLC: High-performance liquid chromatography; KOH: Potassium hydroxide; N_2: Nitrogen; H_2SO_4: Sulfuric acid; R_f: Retention/retardation factor.

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DOI: 10.4103/pr.pr_1_20

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INTRODUCTION

Ayurveda advocates single or multiple drugs of herbal, herbo-mineral, and animal origins for management of different disease conditions. There are many poisonous plants reported in ancient scriptures of Ayurveda that have been advocated in management of many disorders after a proper *Sodhana* (detoxification) process. Among the parts used, the *Kshira* (latex) of some enlisted poisonous plants, namely *Arka* and *Snuhi,* has been delineated for its therapeutic value. The latex of *Snuhi* is stated to be collected at the end of *Shishira rutu* in particular. *Snuhi*, one of the plants of Upavisha, has been quoted in management of different diseases such as *Gulma* (tumor), *Kushtha* (leukoderma), and *Udara* (ascites).[1] Currently, this latex finds an extensive use in preparation of *Ksharasutra* which is used clinically in treating anal fistulas. Classical texts of Ayurveda delineate different varieties of *Snuhi,* namely *Bahukantaka and Alpakantaka*[2] and *Rakta snuhi*.[3] The Ayurvedic Pharmacopoeia of India has given the botanical equivalent of *Snuhi* to be *Euphorbia neriifolia* Linn.[4]

Cite this article as: Roy S, Acharya R, Shukla VJ, Ranade A. Effect of seasonal variation on euphadienol content in *Euphorbia antiquorum* L. and *Euphorbia tirucalli* L. using high-performance thin-layer chromatography method. Phcog Res 2020;12:236-42.

Submitted: 12-Jan-2020 **Revised:** 26-Feb-2020
Accepted: 29-Apr-2020 **Published:** 14-Aug-2020

Recent texts of Ayurveda consider *Euphorbia antiquorum* (EA) L. and *Euphorbia tirucalli* (ET) Linn as the botanical source of *Tridhara snuhi* and *Kanda snuhi,* respectively. Both the plants belong to the family Euphorbiaceae.[5,6]

EA, known as triangular spurge, is widely distributed over the world in tropical and temperate regions, ranging from annual weeds to trees height up to 8–10 m. The study reports that the latex of EA has inhibitory effects on several different cancer cell lines. It also has insecticidal activity. As per traditional uses, fresh milky latex or gum is an acrid irritant which applied externally to relieve warts and other cutaneous infections and also relieve pain of gout, rheumatism, and toothache. Latex mixed with the flour of *Cicer arietinum* is administered in pills in gonorrhea. The latex is also said to be an aphrodisiac.[7,8]

ET, commonly known as pencil tree, is a perennial shrub or tree up to 10–15 m tall and most widespread of all tree *Euphorbia* species. It is originated from tropical East Africa. The latex is a caustic milky white sap when damaged, like many other *Euphorbia* species. ET has been reported to treat rheumatism, stomach ailments, cough, earache, epithelioma, skin tumors, asthma, etc., among Brazilian indigenous communities. It is also used as a folk medicine for the treatment of cancer. ET latex diluted in 2 L of water was indicated to treat neoplasm. High dilution of ET latex modifies the viability and glycolytic metabolism of nontumor melanocytes and human breast cancer cells.[9,10]

Previous study reports that both the plants contain a common phytoconstituent, i.e. euphol (euphadienol), which belongs to the subclass triterpenoids with the molecular formula $C_{30}H_{50}O$ [Figure 1].[8,10] Euphol selectively induced gastric cancer cells apoptosis by modulation of ERK (extracellular signal-regulated kinases) signaling and could thus be of value for cancer therapy. It is also used in anti-inflammatory and antiviral activities on human gastric cancer cells. Euphol is used in reducing the severity of colitis and might be a potential molecule in the management of inflammatory bowel diseases.[11,12]

Phytoconstituents are the responsible factors for the drug action, and it was found that the quality and quantity of the phytoconstituents of plants can fluctuate with seasonal changes.[13,14] Since the latex of these two plants have an important place in the management of different disease conditions, as the seasonal variations, if any, occurring in these plants has not been reported still yet. Hence, the present study has been designed to evaluate the impact of seasonal variation on the quantity of euphadienol content in latex sample of EA and ET using standard euphadienol through high-performance thin-layer chromatography (HPTLC) method.

MATERIALS AND METHODS

Identification and authentication of plants

Two species of *Snuhi*, growing naturally in abundance, in the peripheral area of Jamnagar, Gujarat were botanically identified as *Euphorbia tirucalli* L., *Euphorbia antiquorum* L. by studying the morphological character as noted in flora.[15] Color photographs were taken during different seasons, and wet specimen of each sample was prepared following standard guidelines. For authentication and preservation, fresh twig of each plant species along with inflorescence was stored in AAF (70% ethyl alcohol: glacial acetic acid: formalin) solution in the ratio of 90:5:5 for further study.[16] Sample specimens were authenticated by an expert of pharmacognosy laboratory of Gujarat Ayurved University, Jamnagar, and wet specimen of each sample has been deposited to institute's pharmacognosy museum for the further referencing. The specimen numbers are PHM/2015-2016/6268 and PHM/2015-2016/6269 of EA and ET.

Collection of sample

The fresh crude latex of EA and ET was individually collected from stem by incision method in clean glass vials at morning time in all six *rutus* (seasons) [Table 1].[17] The time for collection of latex was kept constant (within 7 am–9 am) and in the 2nd week of each *rutu*.

Reference standard and chemicals

Reference standard Euphadienol (purity 98.0% of HPLC grade), precoated silica gel 60 F_{254}, HPTLC aluminium plates (20 cm x 10 cm) were procured from E. Merck Ltd. (Mumbai, India). All the chemicals, including solvents such as n-hexane, ethyl acetate, chloroform, methanol, and sulfuric acid, were of analytical grade of Merck Ltd.

Preparation of standard (euphadienol) solution

A common stock solution (1 mg/ml) of euphadienol was prepared by dissolving 1 mg of euphadienol in 1 ml of HPLC grade methanol, and different amounts, i.e. 4 µl, 5 µl, and 6 µl standard solution, were loaded onto HPTLC plate to get the calibration curve and linearity.

Calibration curve for standard euphadienol

The calibration was performed by analysis of working standard solutions of euphadienol and was spotted on precoated HPTLC plate. The HPTLC plate was developed, dried by hot air, and analyzed as per the chromatographic condition mention below. Then, the calibration curve was prepared by plotting peak area versus concentration, corresponding to each spot.

Sample preparation for high-performance thin-layer chromatography

One milliliter of each sample of latex was individually subjected to basic hydrolysis using 1 ml of 5% alcoholic potassium hydroxide.[4] After hydrolysis, the solution was cooled and transferred to a separating funnel. An approximately equal amount of chloroform was added, and the separating funnel was shaken thoroughly. Then, the chloroform layer was separated and the remaining part was again treated with an equal amount of chloroform. Then, the chloroform layer so separated was pooled with the earlier obtained chloroform fraction. The pooled chloroform fraction was evaporated on a water bath. Then, the residue obtained was reconstructed with chloroform to make the solution of 1 mg/ml and used directly for HPTLC analysis. Then, the samples were named such as:

- Sample 1: *Vasanta rutu* sample
- Sample 2: *Grishma rutu* sample
- Sample 3: *Varsha rutu* sample
- Sample 4: *Sharada rutu* sample
- Sample 5: *Hemanta rutu* sample
- Sample 6: *Shishira rutu* sample.

Table 1: The six *rutu* and their corresponding Hindu lunar months along with Gregorian month

Rutu	Season	Hindu lunar month	Gregorian month
Vasanta	Spring	*Chaitra* and *Vaishakha*	March-April
Grishma	Summer	*Jyeshtha* and *Ashadha*	May-June
Varsha	Rainy	*Shravana* and *Bhadrapada*	July-August
Sharada	Autumn	*Ashvina* and *Kartika*	September-October
Hemanta	Prewinter	*Margashira* and *Paushya*	November-December
Shishira	Winter	*Magha* and *Phalguna*	January-February

Method specifications and chromatographic condition

A CAMAG Linomat V HPTLC system equipped with an automatic TLC sampler, TLC scanner III, and integrated software winCATS was used for the analysis. The plate consisted of 20 cm × 10 cm, precoated with silica gel 60 F_{254} TLC plates with aluminum sheet support. The sample was spotted using Hamilton syringe. The samples and standard were applied on the plate as a 2-mm wide band with a constant application rate under a flow of N_2 gas. The linear ascending development was carried out in a CAMNG twin through chamber (20 cm × 10 cm), which was presaturated with a 36-ml mobile phase, i.e., n-hexane: ethyl acetate (9:3 v/v) for 30 min, at room temperature. The length of the chromatogram run was up to 7.5 cm. Then, the plate was dried and scanned at 210 nm. Then, the dried plate was derivatized with the use of 5% methanol H_2SO_4 reagent. To prepare the reagent, 5 ml of methanol was mixed with a 95-ml concentration of sulfuric acid. The plate was immersed in the methanol H_2SO_4 reagent for 1 sec and then heated at 100°C for 10 min. After the development, bands of the samples were identified by matching their R_f values with those obtained for standard. The amount of euphadienol in the latex samples was quantified through peak area of the developed spot.

Analysis

The plotting of calibration curve and the quantification of euphadienol in latex sample was performed using Microsoft Excel 2013.

RESULTS

Calibration curve

Spots were applied on the plate for each concentration starting with the lowest concentration to avoid the carryover effect. The calibration curve was prepared by plotting peak area versus concentration (microgram/spot) corresponding to each spot. From the data, a linear correlation was not possible, so a polynomial equation of second-degree trend line was extrapolated on graph using the concentration range of 4–6 µl from the stock solution of 1 mg/ml. The regression equation of polynomial function for euphadienol was regression through area $y = -1026.4x^2 + 10981x - 23328$, $R^2 = 1$, and regression height $y = -17x^2 + 188.6x - 325.8$, $R^2 = 1$ [Figure 2].

Chromatographic separation of latex samples after derivatization

After derivatization with 5% methanol H_2SO_4 reagent, latex samples of EA and ET showed the similar color reaction, i.e., brown color spots as that of standard euphadienol at R_f 0.79 [Tables 2, 3 and Figures 3b, 4b].

Table 2 shows that a maximum number (10 in number) of spots were found in *Grishma rutu* and *Shishira rutu* samples where both the samples showed five similar bands at R_f 0.98, 0.79, 0.64, 0.48, and 0.17. A minimum number (3 in number) of spots were found in *Hemanta rutu* samples. All samples showed a similar band at R_f 0.98 and 0.79. Among all the R_f, 0.79 ± 0.02 is similar to that of euphadienol R_f, so 0.79 ± 0.02 R_f shows the band to represent Euphadienol after derivatization in all the samples [Table 2].

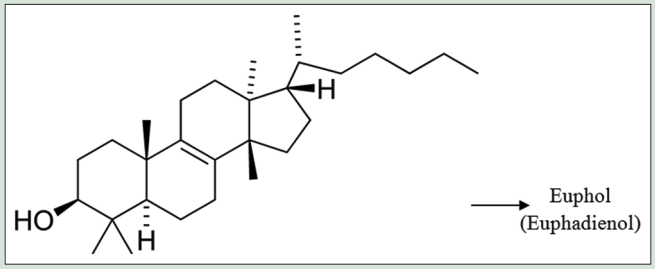

Figure 1: Structure of Euphol (Euphadienol)

Table 2: Chromatographic separation of latex sample of *Euphorbia antiquorum*

Samples	Number of spots	R_f
Euphadienol	1	0.81
Vasanta	6	0.98, 0.79, 0.72, 0.56, 0.48, 0.41
Grishma	10	0.98, 0.79, 0.72, 0.64, 0.6, 0.48, 0.35, 0.17, 0.1, 0.06
Varsha	9	0.98, 0.79, 0.72, 0.62, 0.48, 0.43, 0.35, 0.09, 0.04
Sharada	8	0.98, 0.79, 0.72, 0.62, 0.56, 0.48, 0.17, 0.09
Hemanta	3	0.98, 0.79, 0.74
Shishira	10	0.98, 0.79, 0.64, 0.56, 0.48, 0.41, 0.33, 0.27, 0.17, 0.08

Table 3: Chromatographic separation of latex sample of *Euphorbia tirucalli*

Samples	Number of spots	R_f
Euphadienol	1	0.81
Vasanta	8	0.98, 0.79, 0.72, 0.56, 0.48, 0.41, 0.33, 0.27
Grishma	6	0.79, 0.72, 0.48, 0.41, 0.33, 0.09
Varsha	9	0.98, 0.79, 0.72, 0.62, 0.48, 0.41, 0.33, 0.28, 0.1
Sharada	7	0.98, 0.79, 0.72, 0.63, 0.56, 0.48, 0.4
Hemanta	2	0.98, 0.79
Shishira	5	0.79, 0.63, 0.56, 0.41, 0.35

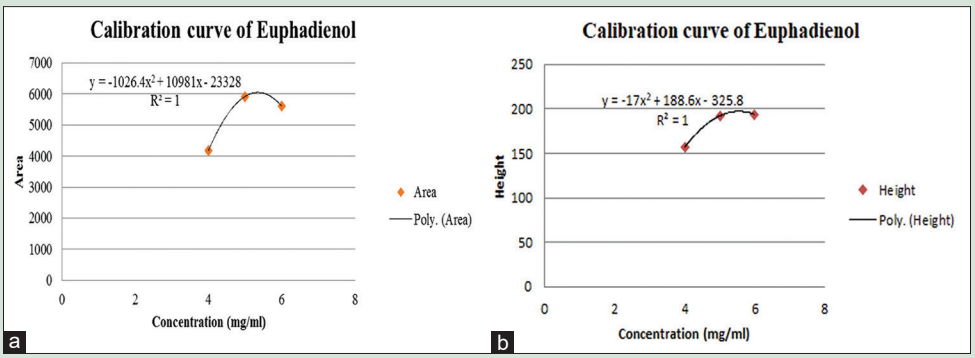

Figure 2: Calibration curve of euphadienol; (a) Polynomial function through area, (b) Polynomial function through height

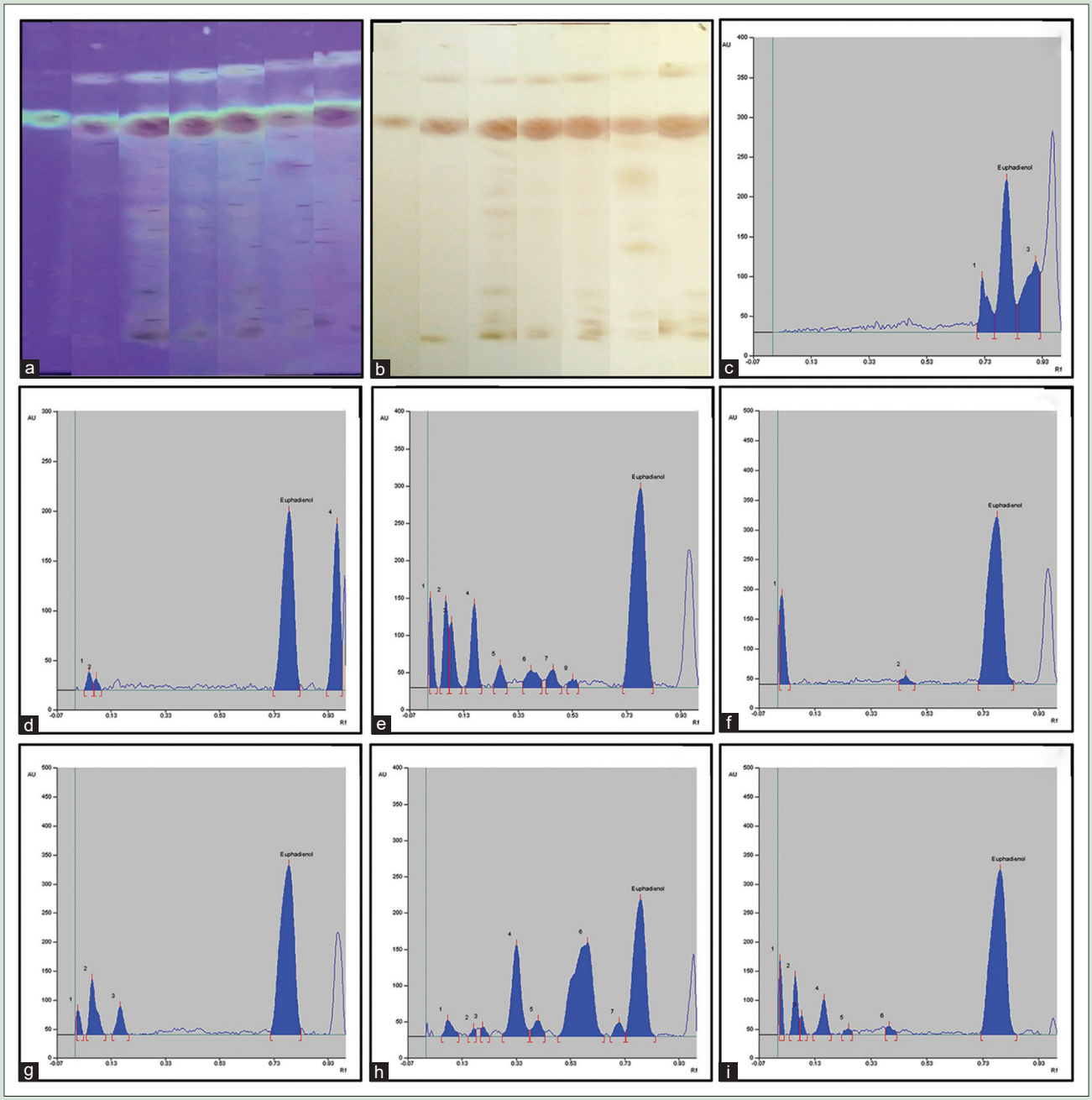

Figure 3: High-performance thin-layer chromatography profile of *Euphorbia antiquorum* L.; (a) High-performance thin-layer chromatography plate at 366 nm, (b) High-performance thin-layer chromatography plate after derivatization, (c) Densitogram of euphadienol at 210 nm, (d) Densitogram of *Hemanta* sample at 210 nm, (e) Densitogram of *Shishira* sample at 210 nm, (f) Densitogram of *Vasanta* sample at 210 nm, (g) Densitogram of *Grishma* sample at 210 nm, (h) Densitogram of *Varsha* sample at 210 nm, (i) Densitogram of *Sharada* sample at 210 nm

Table 3 shows that a maximum number (9 in number) of spots were found in *Varsha rutu* sample, whereas a minimum number (2 in number) of spots were found in *Hemanta rutu* samples. All samples, except *Shishira rutu* sample, showed a similar band at R_f 0.98 and 0.79. Among all, the R_f 0.79 ± 0.02 is similar to that of euphadienol R_f, so 0.79 ± 0.02 R_f shows the band to represent euphadienol after derivatization in all the samples [Table 3].

High-performance thin-layer chromatography densitometric quantification of euphadienol in latex samples

The present study was designed to develop a simple method for quantification of the euphadienol marker compounds in EA and ET. Euphadienol was resolved well at R_f 0.8 [Tables 4 and 5] from processed latex sample when the plate was developed in the solvent system n-hexane: ethyl acetate (9:3 v/v) and derivatized with 5% methanol H_2SO_4 reagent. The concentration of euphadienol along with Rf and peak area of EA and ET is described in Tables 4 and 5. The concentration of euphadienol in latex sample of EA was analyzed through peak area of the developed spot.

Table 4 highlights the chromatographic profile of EA latex. Euphadienol showed the band at R_f 0.8, and all samples showed the band at R_f 0.79 at 210 nm. It was found that the highest quantity of euphadienol was

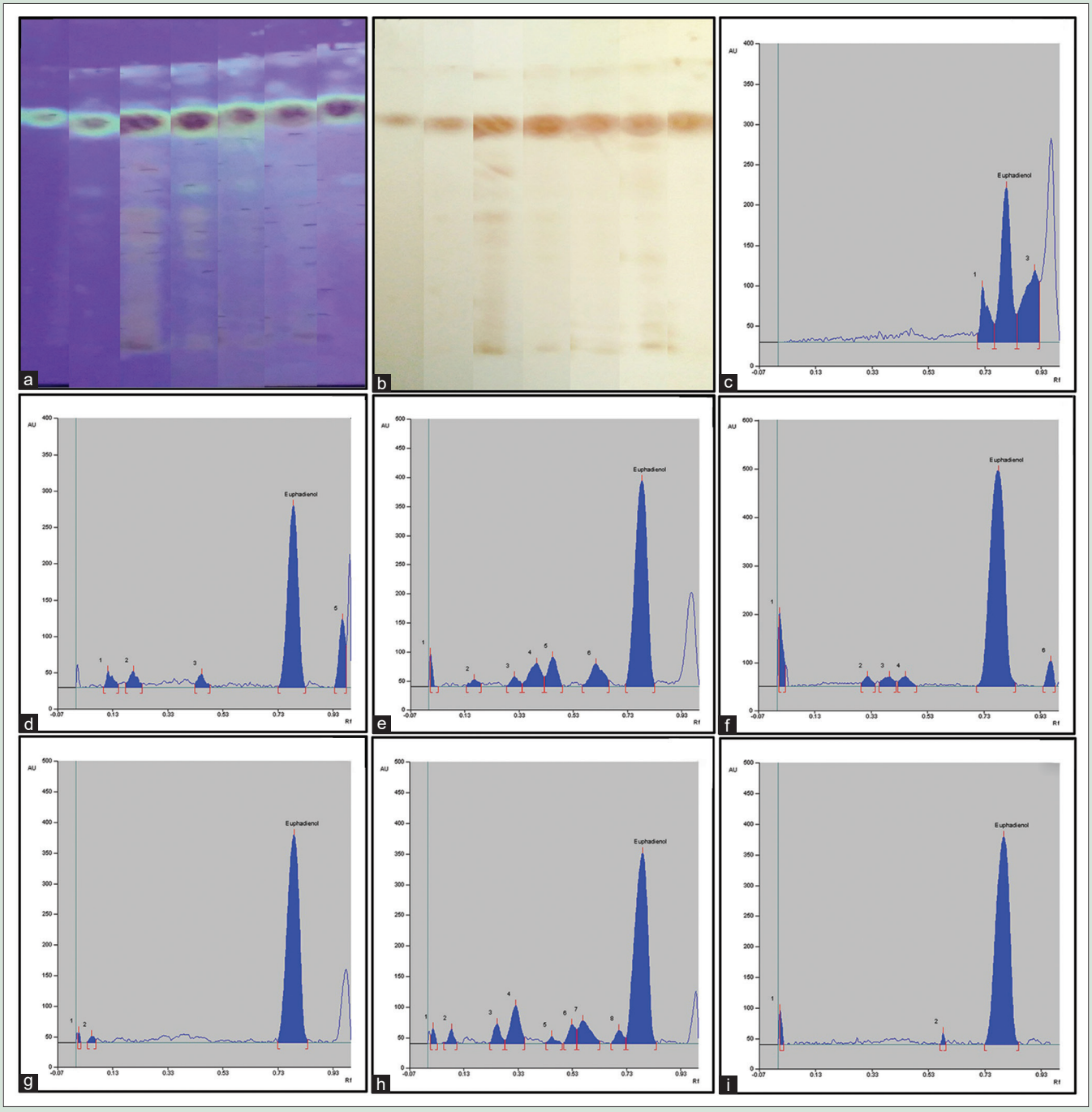

Figure 4: High-performance thin-layer chromatography profile of *Euphorbia tirucalli* L.; (a) High-performance thin-layer chromatography plate at 366 nm, (b) High-performance thin-layer chromatography plate after derivatization, (c) Densitogram of euphadienol at 210 nm, (d) Densitogram of *Hemanta* sample at 210 nm, (e) Densitogram of *Shishira* sample at 210 nm, (f) Densitogram of *Vasanta* sample at 210 nm, (g) Densitogram of *Grishma* sample at 210 nm, (h) Densitogram of *Varsha* sample at 210 nm, (i) Densitogram of *Sharada* sample at 210 nm

found in *Hemanta rutu*, followed by *Vasanta rutu*, and the lowest quantity was found in *Varsha rutu* [Figures 3 and 5a].

Table 5 highlights the chromatographic profile of ET latex. Euphadienol showed the spot at R_f 0.8, and all samples showed the band at R_f 0.79 at 210 nm. It was found that the highest quantity of euphadienol was found in *Sharada rutu* sample, followed by *Vasanta rutu* sample, and the lowest quantity was found in *Varsha rutu* sample [Figures 4 and 5b].

DISCUSSION

Chromatographic fingerprint analysis has shown to be a rational and feasible approach for the quality assessment and species authentication of traditional medicine. The developed fingerprint pattern of components can be used to determine not only the absence or presence of markers of interest but also the ratio of all detectable analytes as well. Thin-layer chromatography (TLC) or HPTLC is primarily used as an inexpensive method for separation, qualitative identification, or semi-quantitative visual analysis of samples. HPTLC has a few limitations, such as the limited developing distance and lower plate efficiency by comparison with HPLC and gas chromatography; it is still an effective tool for quality evaluation of herbal drugs due to its simplicity, low cost, and

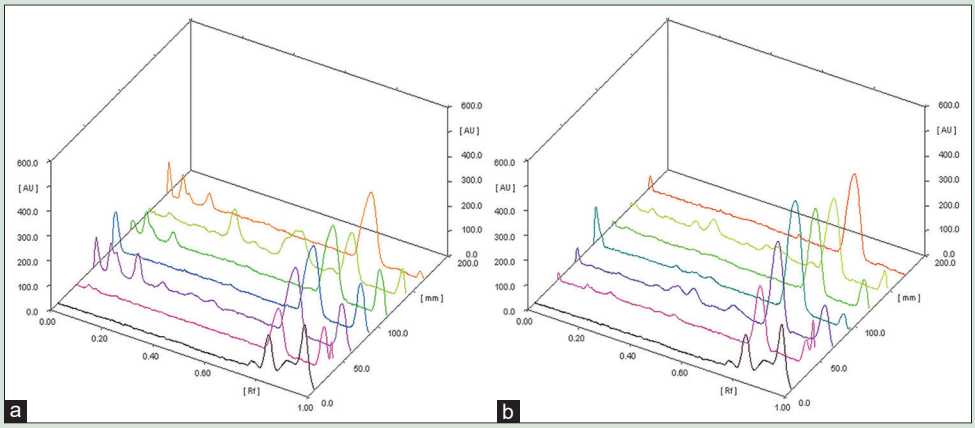

Figure 5: Three-dimensional graphs of high-performance thin-layer chromatography profile; (a) Euphadienol with all samples of *Euphorbia antiquorum* L., (b) Euphadienol with all samples of *Euphorbia tirucalli* L.

Table 4: Quantification of euphadienol in all samples of *Euphorbia antiquorum* latex

Sample	Maximum R_f	Area	Concentration
Euphadienol	0.8	5917.9	5 µg
Vasanta	0.79	12,631.0	260.289 µg/mg
Grishma	0.79	12,917.2	175.179 µg/mg
Varsha	0.79	7150.1	129.359 µg/mg
Sharada	0.79	12,879.0	193.619 µg/mg
Hemanta	0.79	6403.3	270.505 µg/mg
Shishira	0.79	11,660.8	163.3855 µg/mg

Table 5: Quantification of euphadienol in all samples of *Euphorbia tirucalli* latex

Sample	Maximum R_f	Area	Concentration
Euphadienol	0.8	5917.9	5 µg
Vasanta	0.79	19,790.2	383.500 µg/mg
Grishma	0.79	12,875.9	359.035 µg/mg
Varsha	0.79	12,784.7	173.661 µg/mg
Sharada	0.79	14,110.9	551.954 µg/mg
Hemanta	0.79	8061.3	370.160 µg/mg
Shishira	0.79	13,758.3	175.593 µg/mg

requirement, and at the same time, it has been successfully utilized to develop the chromatographic fingerprint for drug samples. TLC or HPTLC is often described as a pilot method for HPLC. However, recent reviews show that the TLC and HPTLC techniques can be used to solve many qualitative and quantitative analytical problems in a wide range of fields including medicine, pharmaceuticals, chemistry, biochemistry, food analysis, toxicology, and environmental analysis.

The therapeutic value of the plant lies in the bioactive phytochemical constituents of the plant which is also the responsible factor of showing various physiological effects on the human body.[14] One of the important bioactive phytoconstituents of both the study plants is euphol (euphadienol). Hence, the estimation and evaluation of seasonal variation of euphadienol content in both the plants is important to judge the drug action with respect to different seasons.

The simplicity of the sample preparation and the possibility of analyzing several samples of herbal products simultaneously in a short time make HPTLC the method of choice. In the present study, euphadienol was quantified from the processed latex sample through peak area of the developed spot where the highest quantity of euphadienol was found in *Hemanta rutu* sample of EA and *Sarada rutu* sample of ET. It was found that the euphadienol was present in all-season samples where they only showed the differences in the quantity, which indicates the fluctuation of biomarker content in the plant by the variation of seasons.

After derivatization with 5% methanol H_2SO_4 reagent, latex samples showed some similar and some different spots with respect to different seasons. Differences in R*f* highlight that changes of seasons have some effects on the synthesis of phytoconstituents in the same plant sample.

CONCLUSION

From the present study, it can be concluded that euphadienol is present in both the plants, i.e., EA and ET, in all seasons. The highest quantity of euphadienol is present in *Hemanta rutu* sample of EA and *Sharada rutu* sample of ET. In the present study, dissimilarity in R*f* confirms the speculation on the probable variation in phytoconstituents encouraged by the seasons. This method can be used for the estimation of euphadienol in other herbal preparations and can be utilized for standardization purpose.

Acknowledgements

The authors would like to thank the National Medicinal Plants Board, Ministry of AYUSH Govt. of India New Delhi, for the fund under the research project. The authors are also indebted to the Gujarat Ayurved University for giving the facility to conduct the whole research work.

Financial support and sponsorship

The work was supported by the National Medicinal Plants Board, Ministry of AYUSH Govt. of India, New Delhi.

Conflicts of interest

There are no conflicts of interest.

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