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Research Article

HPLC DETERMINATION OF COSTUNOLIDE AS A MARKER OF *Saussurea lappa* AND ITS HERBAL FORMULATIONS

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ABSTRACT

Costunolide, isolated from the plant *Saussurea lappa* is a pharmacologically active sesquiterpene lactone, having important medicinal properties and uses in the treatment of cancers, inflammations, ulcers, skin, viral and microbial infections, etc. A simple isocratic reverse phase high-performance liquid chromatographic method has been developed and validated not only for analysis of costunolide in different commercial herbal formulations but also to study the effect of different solvents on the extraction of costunolide from *Saussurea lappa* roots. The method was validated for linearity, precision, accuracy, and system suitability. The average recovery of the method at three levels was 92.16-100.84% and linearity was good for costunolide over a relatively wide range of concentration (1.0-100 μg/mL).

Keywords: Costunolide, *Saussurea lappa*, Plant extracts, HPLC and Herbal formulations.

1. INTRODUCTION

Herbal drugs have been used since ancient times as medicines for the treatment of a range of diseases. Medicinal plants have played a key role in world health. In spite of the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care. Over the past decade, interest in drugs derived from higher plants, especially the phytotherapeutic ones, has increased expressively[1]. According to the World Health Organization (WHO), because of poverty and lack of access to modern medicine, about 65-80% of the world's population living in developing countries depends essentially on plants for primary health care. It is now increasingly accepted worldwide that screening natural products is a more effective strategy for discovering new chemical entities as natural product libraries. The expanded use of herbal medicines worldwide has led to concerns relating to its safety, quality and effectiveness. The quality control of herbal crude drugs and their formulations are of paramount importance in justifying their acceptability in modern system of medicine. One of the major problems faced by user industry is non- availability of rigid quality control profiles and evaluation parameters for herbal formulations.

Saussurea lappa Clarke belongs to the family compositae. It is indigenous to India, Pakistan and China, where it grows in the Himalayas at 2500-3500 m altitude. Dried roots of this plant are known as costus roots and have reputation for their usage in traditional systems of India, China and Japan. Costunolide is one of the major bioactive constituent of *Saussurea lappa* root. This compound was previously isolated from *Saussurea lappa*[2,3]. Pharmacological studies on the activity of costunolide extracts contains anti-ulcer[4,5], anti-carcinogenesis[6,8], antimicrobial and fungicidal[9-11], anti-inflammatory[12] and protein tyrosine phosphate inhibitory activities[13] are also being widely investigated. Costunolide has been considered

as a potential candidate for various types of tumors[14-16].

Several methods have been reported for the determination of Costunolide in plants, herbal preparations and in biological matrix. Determination of costunolide in Xin-ke-shu preparation by HPLC[17]. ^{13}C-NMR spectroscope detection[18], thin-layer chromatography[19] and high-speed counter-current chromatography[20]. Rapid determination of costunolide in human plasma and Chinese patent medicine Xiang Sha Yang Wei capsule using HPLC-DAD coupled with second-order calibration was reported[21]. Determination of Costunolide in rat plasma by UPLC[22] and HPLC[23] method for costunolide in mice plasma and tissues were also reported. The present study was aimed at development of a simple and selective method for quantification of costunolide in *Saussrea lappa* extracts and polyherbal formulations. The study includes determination of costunolide in various extracts of S*aussurea lappa* roots using HPLC and the costunolide in the extracts and herbal formulations was confirmed online by LC-MS. Application of the proposed method for marker-based standardization of some commercial polyherbal 'Ayurvedic' formulation containing the roots of *Saussurea lappa.*

2. EXPERIMENTAL

2.1 Materials and chemicals

S*aussurea lappa* whole plant was obtained from Kashmir forest area, India. The plant authenticity was confirmed by Prof K. Madhava Shetty professor, Department of Botany, Sri Venkateswara University, Tirupati, India. Costunolide was obtained from our laboratory which was isolated from *Saussurea lappa* by column chromatography. Costunolide (Figure 1) was identified and confirmed by NMR and Mass spectral data[16] supported by previous reports and purity of costunolide was approximately > 99.1%. HPLC grade acetonitrile and methanol was purchased from M/s Merck India Pvt Ltd (Mumbai, India). All the solvents used for extraction of plant material were of analytical reagent grade purchased from M/s S.D. Fine chemicals (Mumbai, India). Ultrapure water was obtained from Synergy water purification system (Millipore, USA). Microtips and centrifuge tubes (2 mL) were purchased from M/s Thermo fisher scientific Ltd (Mumbai, India). 0.45µm syringe filters obtained from M/s PAL Millipore (India). Eppendorf micropipettes were purchased from M/s Perala agencies (Hyderabad, India).

2.2 Chromatographic conditions

Agilent HPLC system (1100 series, Germany) was used for chromatographic separations. The separation of analyte was achieved by using Phenomenex Luna C8 (250 x 4.6mm, 5µ). The mobile phase consisted of acetonitrile and water in 60:40, % v/v ratio, both solvents containing 0.1% formic acid, pumped at a flow rate of 1.0 mL/min and detection wavelength was 210 nm. The injection volume was 20 µL and the total analysis time per sample was 20.0 min. LC-MS analysis was performed on Agilent LC-MSD Trap SL mass spectrometer (Waldron, Germany), equipped with electrospray ion interface, operating in positive ion polarity. Nitrogen gas was used as nebulizer and curtain gas. Collision induced dissociation was achieved using helium gas.

2.4 Preparation of stock solution and calibration standards

Accurately weighed amount of 10 mg of costunolide was dissolved in 10 mL methanol in volumetric flask. Calibration standards were prepared by diluting the appropriate volume of stock solution with methanol to obtained concentration levels of 1, 2, 5, 10, 20, 50 and 100 µg/mL.

2.5 Study of extraction solvent

Powdered roots of *S. lappa* were extracted with different organic solvents like hexane, ethyl acetate, chloroform, dichloromethane, acetonitrile, ethanol, methanol and acetone by sonication for 30 min. The obtained extracts were centrifuged at 4000 rpm for 20 min and the supernatant liquid was concentrated under vacuum at 40^{o}C. The individual concentrated extracts were diluted with methanol and injected onto HPLC system for the estimation of Costunolide.

2.6 Preparation of sample solution from extracts

The dried roots of *Saussurea lappa* (approx. 500 mg) was accurately weighed and extracted with hexane, ethyl acetate, chloroform and methanol separately in centrifuge tubes. The extraction was carried out with each solvent separately by taking 500 mg of powered plant material, to which 5 mL of extraction solvent followed by sonication for 30 min and followed by centrifuge at 4000 rpm for 20 min. The supernatant liquid was decanted; the same extraction cycle was repeated three times. The resultant solution was concentrated to dryness and 33.3 mg/mL solutions of these extracts were prepared. The sample solutions were injected directly on to HPLC system.

2.7 Preparation of sample from herbal formulations

Powder, tablets and oil formulations were extracted using different procedures. Powder was weighed accurately 1.0 g transferred to centrifuge tubes separately. For tablets average weight of 10 tablets was taken and finely powdered from which 1.0 g weighed accurately. For oil directly 1.0 g was taken for extraction. For all the above materials 5 ml of extraction solvent was added, followed by sonication for 30 min and centrifuge at 4000 rpm for 20 min. The supernatant liquid was decanted; the same extraction cycle was repeated three times. The resultant solution was concentrated to dryness and 100 mg/mL solution was prepared from the above samples. The prepared samples were injected directly on to HPLC system.

2.8 Method validation

Stock solution of the costunolide was prepared diluted to appropriate concentration for the construction of calibration curves. Seven concentration of costunolide solution were analyzed in triplicate and then the calibration curves were constructed by plotting the peak area versus concentration of costunolide. The calibration curves were analyzed using linear regression equation and coefficient of determination.

Intra-day and inter-day variations were utilized to determine precision of the method. The calibration samples of costunolide was analyzed during a single day (n = 3) and in triplicate on three consecutive days. The coefficient of variation (CV) was taken as a measure of precision. Accuracy was determined by the recovery test. Known amounts of standard costunolide was added to the dried costus roots, and then determined as described above. Each sample was analyzed in triplicate. The limits of detection and quantification for each compound were determined by the signal- to-noise (S/N) ratio for costunolide. LOD was calculated as the amount of the injected sample gave a signal-to-noise ratio of 3, and LOQ was determined when the S/N ratio was 10.

3. RESULTS AND DISCUSSION

3.1 HPLC method development

The primary aim of the present study was to separate costunolide from various compounds of plant extracts and herbal formulations. This included mobile phase selection, flow rate and column type. Different volume ratios of water–methanol and water–acetonitrile combinations were tried as mobile phase, along with formic acid, ammonium formate and ammonium acetate buffers in varying strength on Waters Atlantis dC_{18} (150 mm × 4.6 mm, 5 µm), Agilent Zorbex XDB C_{18} (150 mm × 4.6 mm, 5µm), Phenomenex Luna C8 (250 mm × 4.6mm, 5 µm), Oyster ODS 3 (50 mm x 4.6mm, 3 µm) and Oyster C8 (50 mm x 4.6mm, 3 µm). In addition, the effect of flow rate was also studied from 0.4 to 1.0 mL/min, which was also responsible for acceptable chromatographic peak shapes. Phenomenex Luna C8 (250 mm × 4.6mm, 5 µm) was selected for its effective separation of costunolide from other constituents in extracts and formulations (Figures 2 & 3). The mobile phase consisting of acetonitrile: water (60:40, v/v) both solvents containing 0.1% formic acid was found most appropriate for faster elution, improved efficiency and peak shape. The retention time for costunolide was 12.1 min at a flow rate of 1.0 mL/min. the mobile phase used in this method was very much compatible for LC-MS analysis. The costunolide presence in extracts were confirmed by molecular ion peak $[M+H]^+$ in mass spectrum (Figure 4).

The effect of different solvents on extraction was studied by comparing the peak area of costunolide obtained in extract samples. The results given in Figure 5 indicate that methanol is the best solvent for the extraction of costunolide from dried costus root.

3.2 Validation

Under the optimum conditions described above, calibration curves for costunolide was obtained by plotting peak areas versus seven different concentrations of standard solutions. The calibration curves showed good linearity in a relatively wide concentration range with regression equation y = 46.75x-26.62 and correlation coefficient >0.9996. The limits of detection (LOD) and quantification (LOQ) of costunolide were 0.15 and 0. 48 µg/mL respectively.

The results of precision were included in Table 1. The intra-day and inter-day variation of costunolide was 2.7-4.2 and 2.6-3.9% (CV), respectively. All of the values were lower than 5%, which revealed good precision of the analytical method. The results of recovery test are indicated in Table 2, which demonstrated that the recoveries of costunolide was in the range of 92.16-100.84%, with variation coefficients ranging from 1.3 to 4.8%, which were less than 5%. The corresponding results indicated that the analytical method for the quantification of the costunolide in the samples exhibited quite good accuracy.

3.3 Sample analysis

The optimum extraction procedure and chromatographic conditions described above were applied to the extraction and determination of costunolide in four herbal preparations. The chromatographic separation of costunolide from other constituents present in commercial herbal formulations was shown in Figure 4. Each sample was determined in triplicate. The contents of costunolide analyzed are listed in Table 3.

4. CONCLUSIONS

A simple and an efficient method was developed for the quantitative analysis of the costunolide in various solvent extracts and four commercial herbal formulations. Sample pretreatment using sonication extraction was simple and the proposed HPLC method was validated as rapid, precise and accurate. The validation procedure and assay results, suggested the proposed HPLC method suitable for the quality assessment of costunolide preparations.

5. ACKNOWLEDGEMENTS

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Table 1: Precision data

Concentration (µg/mL)	Intra-day		Inter-day	
	Mean ± S.D (µg/mL)	% CV	Mean ± S.D (µg/mL)	% CV
2.0	2.11 ± 0.07	3.2	2.07 ± 0.06	3.1
10.0	9.38 ± 0.26	2.7	9.27 ± 0.28	3.1
50.0	47.87 ± 2.05	4.3	47.70 ± 1.25	2.6

Table 2: Recovery data

Amount added (µg/mL)	Recovery (%) (n = 3)	Mean ± S.D	%CV
2.0	98.24 97.19 95.82	97.08 ± 1.21	1.3
5.0	99.25 92.32 97.11	96.22 ± 3.55	3.7
10.0	100.84 92.15 94.01	95.67 ± 4.57	4.8

Table 3: Content of costunolide in plant extracts and herbal formulations

Sample	Dosage form	Costunolide (mg/100g)
Saussurea lappa roots	Methanolic extract	11.29 ± 0.34
Saraswatha choornam	Powder	1.28 ± 0.26
Kottamchukkadi thailam	Oil	0.31 ± 0.01
Yograj guggulu	Tablet	ND
Purim	Tablet	0.66 ± 0.01

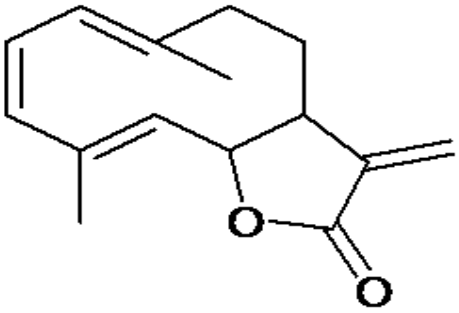

Fig. 1: Chemical Structure of Costunolide

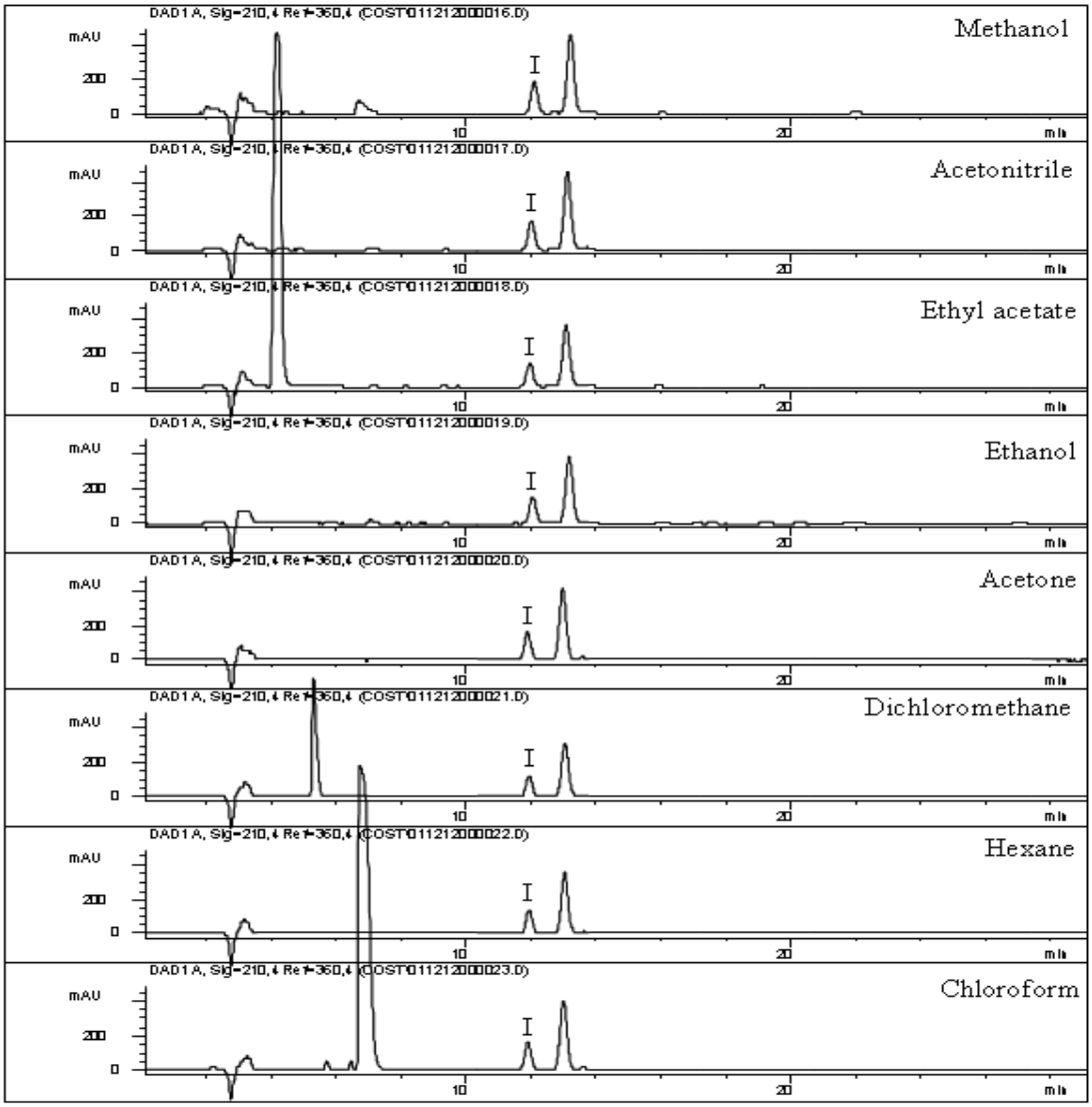

Fig. 2: Chromatograms of different extracts of *Saussurea lappa,* Costunolide (I)

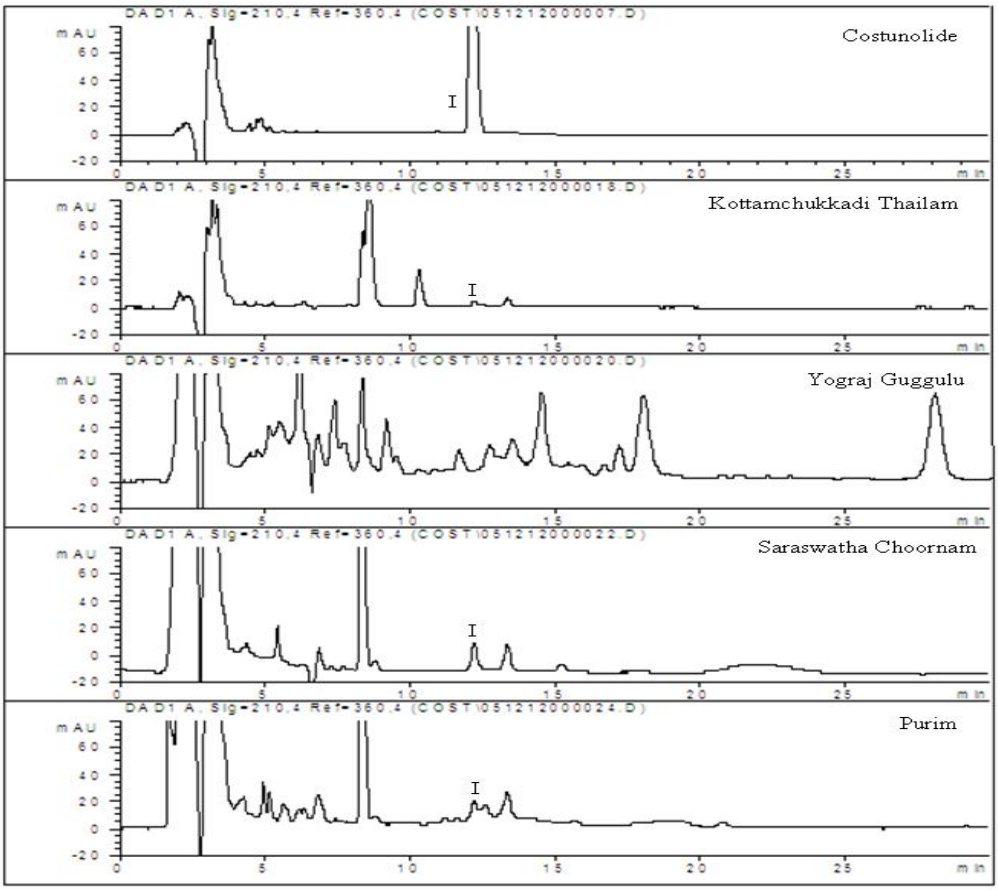

Fig. 3: Chromatograms of different herbal formulations containing *Saussurea lappa*, Costunolide (I)

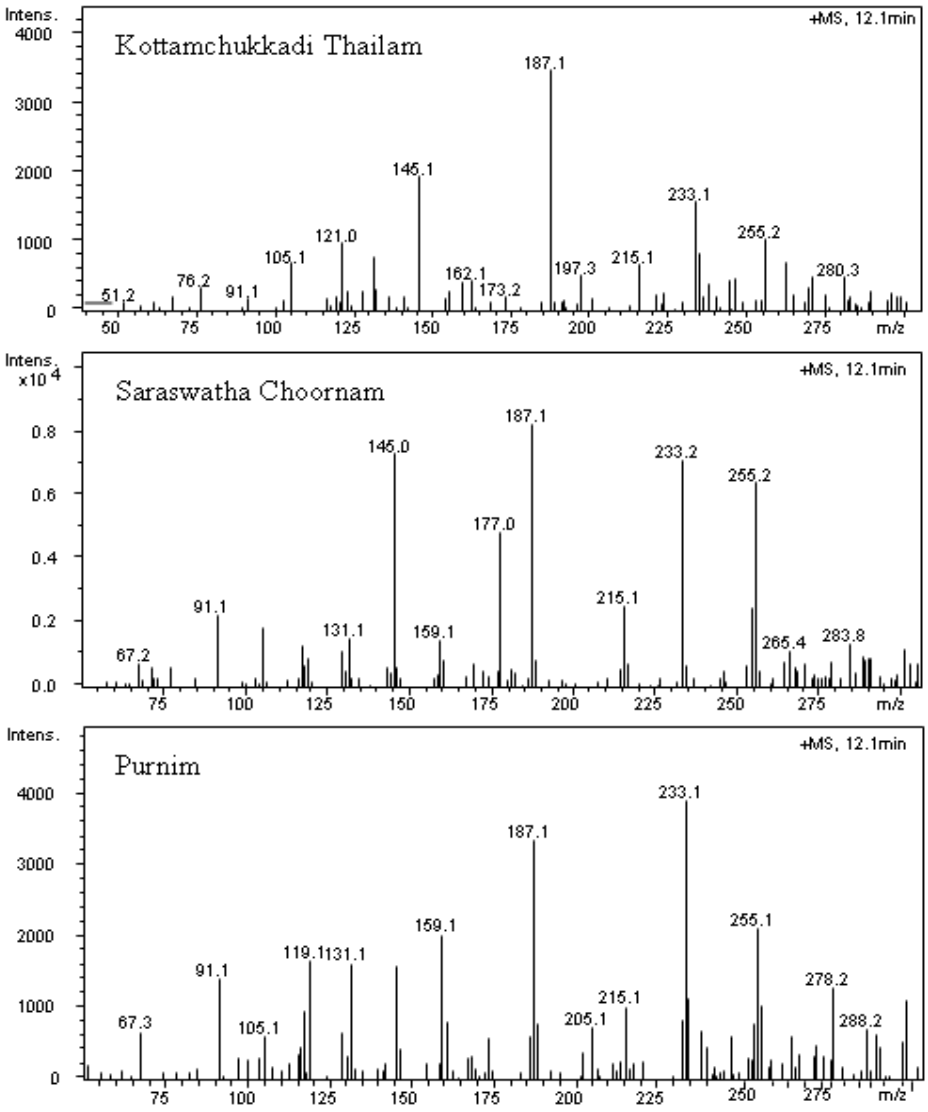

Fig. 4: Mass spectra of Costunolide (*m/z* 233) in different herbal formulations

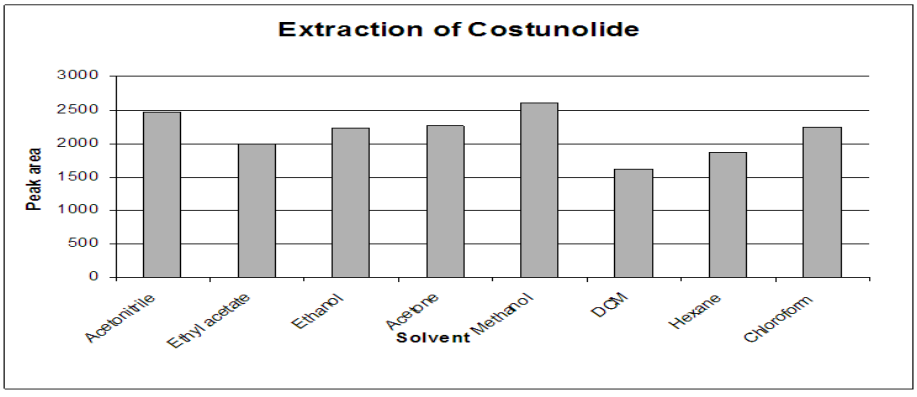

Fig. 5: Effect of different solvents in extraction of Costunolide from *Saussurea lappa*

6. REFERENCES

1. Calixto JB. Efficacy, Safety, Quality control, marketing and regulatory guidelines for herbal medicines. Brazillian Journal of medical and Biological research. 2000;179-189.
2. Rao SA, Kelkar GR, and Bhattacharya SC. The structure of costunolide, a new sesquiterpene lactone from costus root oil. Tetrahedron letters 1959;9:275-283.
3. Dhillon RS, Kalsi PS, Singh WP, Gautham VK and Chhabra BR. Guaianolides from Saussurea lappa. Phytochemistry. 1987;26:1209-1210.
4. Yamahara J, Kobayashi M, Miki K, Kozuka M, Sawada and Fujimura H. Chlolagogic and antitumor effect of Saussurea radix and its components. Chem.Pharm.Bull.1985;13:1285-1288.
5. Yoshikawa M, Hatakeyama S, Inoue Y and Yamahara J. Saussureamines A,B,D,E, new anti-ulcer principles from Chinese Saussurea radix. Chem. Pharm. Bull. 1993; 41:214-216.
6. Ohnishi M, Yoshimi N, Kawamori T, Ino N, Hirose Y, Tanaka T, Yamahara J, Miyata H and Mori H. Inhibitory effects of dietary protocatechuic acid and costunolide on 7,12-dimethylbenz[a]anthracene-induced hamster cheek pouch carcinogenesis. Japan Journal Cancer Research. 1997; 88:111-119.
7. Kawamori T, Tanaka T, Hara A, Yamahara J and Mori H. Modifying effects of naturally occurring products on the development of colonic aberrant crypt foci induced by azoxymethane in F344 rats. Cancer Research. 1995;55:1277-1282.
8. Gu JQ, Gills JJ, Park EJ, Mata-Greenwood E, Hawthorne ME, Axelrod F, Chavez PI, Fong HH, Mehta RG, Pezzuto JM and Kinghorn AD. Sesquiterpenoids from Tithonia diversifolia with potential cancer chemopreventive activity. Journal of Natural Products 2002;65:532-536.
9. Luna-Herrera J, Costa MC, Gonzalez HG, Rodrigues AI and Castilho PC. Synergistic antimycobacterial activities of sesquiterpene lactones from Laurus spp. Journal of Antimicrobial Chemotherapy. 2007;59:548-552.
10. Wedge DE, Galindo JCG and Macias FA. Fungicidal activity of natural and synthetic sesquiterpene lactone analogs. Phytochemistry. 2000;53:747–757.
11. Fischer NH, Lu T, Cantrell CL, Castañeda-Acosta J, Quijano L and Franzblau SG. Antimycobacterial evaluation of germacranolides. Phytochemistry. 1998;49:559–564.
12. Koch E, Klaas CA, Rüngeler P, Castro V, Mora G, Vichnewski W and Merfort I. Inhibition of inflammatory cytokine production and lymphocyte proliferation by structurally different sesquiterpene lactones correlates with their effect on activation of NF-kappaB. Biochemical Pharmacology. 2001;62:795–801.
13. Choi JY, Na MK, Hwang IH, Lee SH, Bae EY, KimBY and Ahn JS. Isolation of betulinic acid, its methyl ester and guaiane sesquiterpenoids with protein tyrosine phosphatase 1B inhibitory activity from the roots of Saussurea lappa. CB Clarke Molecules. 2009;14:266–272.
14. Lee MG, Lee KT, Chi SG and Park JH. Costunolide induces apoptosis by ROS-mediated mitochondrial permeability transition and cytochrome C release Biol Pharm. Bull. 2001;24:303-307.
15. Zhang S, Won YK, Ong CN and Shen HM. Anti-cancer potential of sesquiterpene lactones: bioactivity and molecular mechanisms. Curr Med Chem. Anti-Cancer Agents. 2005; 5: 239-243.
16. Robinson A, Vijay Kumar T, Sreedhar E, Naidu VGM, Sistla Rama Krishna, Suresh Babu K, Srinivas PV and Madhusudana Rao J. A new sesquiterpene lactone from the roots of Saussurea lappa: Structure–anticancer activity study Bioorganic & Medicinal Chemistry Letters. 2008;18:4015-4017.
17. Shu D, Yue-tao L, Jing-bo P, Hong-mei J, Zhong-mei Z and Chang-yuan Y. Simultaneous HPLC determination of costunolide and dehydrocostuslactone in Xin-Ke-shu Preparations. Pharmaceutical crops. 2011;2:74-78.
18. Ferrari B, Castilho P, Tomi F, Ana IR, Maria do Ceu C and Joseph C. Direct identification and quantitative determination of costunolide and dehydrocostuslactone in the fixed oil of Laurus novocanariensis by ^{13}C-NMR spectroscopy. Phytochem.. Anal. 2005:16:104-107.

19. Vijayakannan R. Karan M. Dutt S, Jain V and Vasist K. A rapid densitometric TLC method for simultaneous analysis of costunolide and dehydrocostus lactone in Saussurea costus. Chromatographia. 2006;63:277-281.
20. Li A, Sun A and Liu R. Preparative isolation and purification of cotunolide and dehydrocostus lactone from Aucklandia lappa Decne by high speed counter current chromatography. J Chromatogr. 2005A:1076:193-199.
21. Liu Y, Wu H, Zhu S, Kang C, Xu H, Su Z, Gu H and Yu R. Rapid determination of costunolide and dehydrocostuslactone in human plasma sample and Chinese patent medicine Xiang Sha Yang Wei capsule using HPLC-DAD coupled with second-order calibration. Chin J Chem.2012;20:1-7.
22. Fangdi H, Shila F, Yuqiong W, Yingyan B and Chunming W. Quantitative analysis of costunolide and dehydrocostuslactone in rat plasma by ultraperformance liquid chromatography-electrospray ionization-mass spectrometry. Biomedical chromatography. 2011;25:547-554.
23. Fangdi H, Shilan F, Yuqiong W, Yingyan B, Cui F, Li Y and Chunming W. Development of high performance liquid chromatography method for costunolide and dehydrocostuslactone in mice plasma and tissues: Application to pharmacokinetic Study. Chin J Chem. 2010;28:2293-2300.