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Simultaneous Quantification of P-Methoxybenzoic Acid, 3, 4-Dimethoxycinnamic Acid and Ecdysteron in the Extract of *Trianthema Portulacastrum* Linn. And Its Marketed Polyherbal Formulation Using HPLC

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Abstract:
Trianthema portulacastrum Linn. belonging to the family aizoaceae is abundantly available weed which has enormous traditional uses due to different bioactive compounds present and thus used in different herbal formulations. A simple, reproducible and efficient reverse phase high performance liquid chromatographic method has been developed for simultaneous determination of p-methoxy benzoic acid, 3,4-dimethoxy cinnamic acid and Ecdysterone in the powder of Trianthema Portulacastrum. The Agiland 1100 system was used. The method involves use of a C18 column (Inertsil ODS , C18 15cm X 4.6 mm, 5µm) with a gradient mixture of acetonitrile and 0.01M NaH2PO4 as mobile phase. The developed method was then validated in accordance with the ICH guidelines for method validation. The validated HPLC method can be used for routine quality control analysis of formulations or dietary supplements containing leaf powder of Trianthema Portulacastrum Linn.

Keywords: *HPLC, Simultaneous quantification, p-methoxy benzoic acid, 3,4-dimethoxy cinnamic acid, Ecdysterone*

1. Introduction

Validation of analytical methods is mandatory in implementing a quality control system in any analytical laboratory. It provides an assurance of reliability during normal use and can be referred as a process of providing documented evidence of quality for several herbal and traditional drugs. Separation techniques such as chromatography and electrophoresis have been extensively used for quality control of herbal medicine because of their high efficiency and speed

Trianthema portulacastrum Linn. commonly known as Bishkhapra, belonging to the family Aizoaceae, is one of the common weed, which has enormous traditional uses against diseases and some bioactive compounds have been isolated from this weed[i]. It is an exotic weed. It is growing throughout most tropical countries, such as Baluchistan, Ceylon, and India[iii]. It is now naturalized throughout India in cultivated fields, river beds, waste ground, etc[ii]. Its infestation is very common in various agricultural and vegetable crops, such as mustard, maize, pigeon pea, mung bean, potato, onion, cotton, soybean, pearl millet, and sugarcane, especially during the rainy seasons[iv,v].

In recent years, there has been a growing interest in the chemical composition and biological activities of *Trianthema portulacastrum* L.. Among the various phytochemical found in *Trianthema portulacastrum* L., sterols and phenolic acids are widely regarded as major biologically active components which may contribute to their reputed and diversified health benefits.

Scientists have identified different components of T.portulacastrum to be responsible for its activity. Photochemical screening has revealed the presence of steroids, flavonoid, fats, terpenes, carbohydrates, tannins, and alkaloids. It contains Ecdysterone[vi,vii], Trianthenol[viii], 5-hydroxy-2-methoxybenzaldehyde[viii] , 3-acetylaleuritolic acid[viii] , p-methoxybenzoic acid8, 5-2'-dihydroxy-7-methoxy-6-8-dimethyl flavone[ix], 5,7-dihydroxy-6-8-dimethylchromone(Leptorumol)[ix], 3-4-dimethoxycinnamic acid[x], Nicotinic Acid[xi], Ascorbic acid[xi]. Alcoholic extract of aerial parts of T.portulacastrum showed significant hepatoprotective activity[xii,xiii]. The chloroform extract of the plant showed moderate antifungal activity. The alcoholic and water extracts of whole plant and fresh juice of leaves demonstrated diuretic activity[xiv]. The alcoholic extract of the plant possessed significant positive ionotropic and chronotropic activities in isolated frog heart[xv]. Ethanolic extract of the plant showed significant anti-pyretic[xvi], analgesic[xvi] , anti-inflammatory[xvi], antibacterial[xvi] and central nervous depressant properties[xvi]. P-methoxybenzoic acid appears to have important

hepatoprotectiveactivity17, 3,4-dimethoxy cinnamic acid has been reported to have anti-oxidant activity[xviii]. Major phytosterol Ecdysterone is responsible for Immunomodulatory as well as hepatoprotective activity[19-22]. From literature suevey it was found that *Trianthema portulacastrum* Linn. is used as an adulterant in different formulations of *Boerevia diffusa*[xxiii]. *Boerevia diffusa* L. does not contain p-methoxy benzoic acid and 3,4-dimethoxy cinnamic acid. Thus, they can act as a marker compounds to confirm presence of *Trianthema portulacastrum* L. in the formulation. In a view of these wide therapeutic effects and adulteration a need was felt for simultaneous quantification of p-methoxybenzoic acid, 3,4-dimethoxy cinnamic acid and Ecdysterone from the whole plant extract.
Literature survey revealed that HPLC methods have been reported for Identification and quantitative determination of ecdysterone[xxiv]. Also methods for quantitative determination of p-methoxybenzoic acid[xxv] and 3,4-dimethoxycinnamic acid[xxv] are reported. But no HPLC method has been reported for quantitation of ecdysterone, p-methoxybenzoic acid, 3,4-dimethoxycinnamic acid from *Trianthema portulacastrum* L individually or simulteneously.
In this research work, a simple, precise and accurate HPLC method has been established for simultaneous quantitation of ecdysterone, p-methoxybenzoic acid, 3,4-dimethoxycinnamic acid in the plant powder of *Trianthema portulacastrum* Linn. Further, the proposed method has been validated as per ICH guidelines and applied as a quality control tool for standardization of a commercially available hepatoprotective formulation containing plant extract of *Trianthema portulacastrum* L. Also with this method adulteration in the locally available formulation has also been confirmed. Such a study would not only facilitate standardization of the raw material and commercial products, but also facilitate future pharmacological studies and quality control.

2. Methods and Materials

2.1. Chemicals and Reagents

HPLC grade methanol, ethanol, toluene, n-hexane, ACN were procured from E.Merck, Mumbai, India. Reference standards of p-methoxybenzoic acid (Puriety >95%), 3,4-dimethoxycinnamic acid (Puriety >97%), Ecdysterone (purity >95%) and were purchased from Sigma-Aldrich Chemie (Aldrich Division; Steinheim, Germany).

2.2. Plant Material

Plant was collected from Vasai, Thane district. Herbarium samples of *T.portulacastrum* were prepared in duplicate and authenticated by Botanical Survey of India (BSI, Pune, India). Formulation 'Jigreen' was purchased from Humdard shop. Also local formulation made up of *Boerevia diffusa* L was also purchased from local vender to check if it is adulterated.

2.3. SamplePreparation

2.3.1. Plant

About 5gm of dried powder of *T.potulacastrum* was weighed into a round bottom flask,100ml of ethanol was added and was subjected for soxhlate extraction using soxhlate apparatus for 12 hrs. The extract was then filtered through Whatman filter paper no. 41(E.Merk, India) initially and then using syringe filters of mesh size 0.45µ.

2.3.2. Formulation

200 ml of liquid formulation was taken in a measuring cylinder and taken in a round bottom flask. It was mixed well with 100ml of ethanol. Then it was transferred to separating funnel. Extract was obtained using liquid-liquid extraction. 25ml of n-hexane was used at a time for extraction. No. of extractions were four.
Solid formulation was available in powdered form. 5gm powder of formulation was weighed into a round bottom flask, 100ml of ethanol was added and was subjected for soxhlate extraction using soxhlate apparatus for 12 hrs. The extract was then filtered through Whatman filter paper no. 41(E.Merk, India) initially and then using syringe filters of mesh size 0.45µ.

2.4 Chromatography

A sensitive and accurate high-performance liquid chromatographic (HPLC) method has been developed, validated and used for simultaneous quantitation of two phenolic acids (p-methoxy benzoic acid, 3,4-dimethoxycinnamic acid) and phytosterol (Ecdysterone) in plant powder of *Trianthema portulacastrum* Linn. The ethanolic extract of the powder was separated on HPLC column (Inertsil ODS , C18 15cm X 4.6 mm, 5µm) with gradient mixture of HPLC grade acetonitrile and 0.01M NaH_2PO_4 as mobile phase. The separated components were detected using Photo Diode Array (PDA) detector at 240 nm. Different Gradient programs were tried but following program was finalized.
Solution A: 0.01M NaH_2PO_4 pH 3 with OPA
Solution B: CAN

TIME	%B
0.00	10
05.00	10
15.00	60
20.00	60
20.10	10
27.00	10

Table 1

2.4.1. Chromatograms

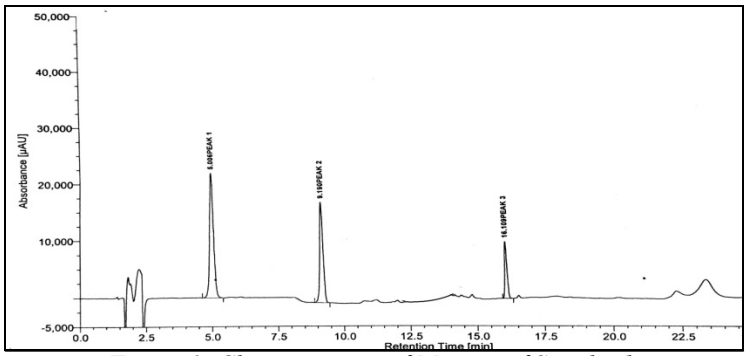

Figure 1: Chromatogram of Mixture of Standards

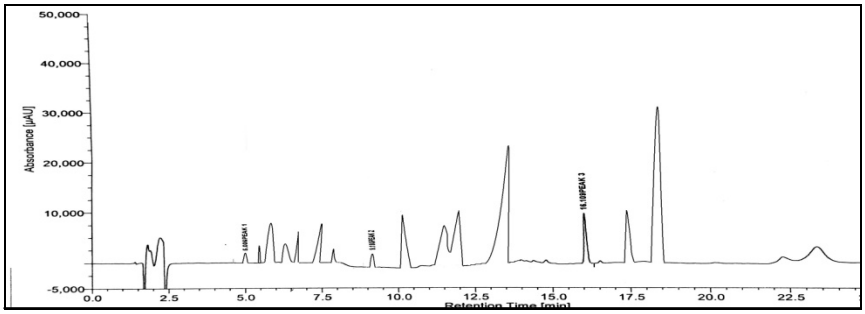

Figure 2: Chromatogram of ethanolic extract of Trianthema portulacastrum

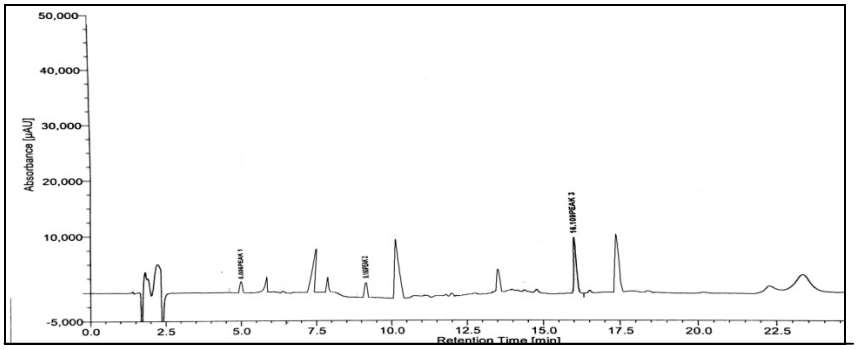

Figure 3: Chromatogram of ethanolic extract of Formulation
Peak I- p-methoxy benzoic acid : 5.00 mins Peak II-3,4-dimethoxy cinnamic acid: 9.10mins
Peak III-Ecdysterone: 16.10mins

2.5. Method Validation

ICH harmonized tripartite guidelines were followed for the validation of the developed analytical method

2.5.1. Specificity

The specificity of the proposed HPLC method was ascertained by analyzing standard compounds and samples. Interference at the Rt of standards was not found in a blank injection.

2.5.2. Linearity

Working standard solutions of p-methoxybenzoic acid (0.8-400 µg/mL), 3,4-dimethoxycinamic acid (0.5-600 µg/mL) and Ecdysterone (1-600 µg/mL), were injected, in triplicate. The chromatogramms were then acquired and the peak areas were recorded for each concentration of standard. RSD of standard peak areas for each linearity level were less than 2%.

2.5.3. Inter-Day and Intra-Day Precision

Variability of the method was studied by analyzing quality control samples p-methoxybenzoic acid (1.0, 10, 300 µg/mL), 3,4-dimethoxycinnamic acid (1.0, 10, 300 µg/mL) and Ecdysterone (1.0, 10, 200 µg/mL) on the same day (intra-day precision) and on different days (interday precision) and the results were obtained in acceptance limit i.e less than 2%..

2.5.4. Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were calculated using S/N ratio according to ICH guidelines. The value of limit of detection for p-methoxybenzoic acid, 3,4-dimethoxycinnamic acid and Ecdysterone was found to be 0.26 µg/mL, 0.16 µg/mLand 0.33 µg/mL respectively, whereas the limit of quantification for p-methoxybenzoic acid, 3,4-dimethoxycinnamic acid and Ecdysterone was found to be 0.8 µg/mL, 0.5 µg/mLand 1 µg/mL respectively.

2.5.5. Recovery

Recovery tests were carried out to further investigate the accuracy of the method by adding three concentration levels (80, 100 and 120%) for p-methoxy benzoic acid, 3,4-dimethoxycinnamic acid and Ecdysterone to known amounts (Zero Level) plant and formulation prior to extraction. The resultant samples were then extracted and analyzed with the described method. For p-mthoxy benzoic acid it was found to be 93.91 in plant and 93.63 in formulation extract. For 3,4-dimethoxy cinnamic acid it was found to be 94.55% in plant and 94.97 in formulation extract. For Ecdysterone it was found to b 94.33 in plant and 93.58 in formulation Extract.

2.5.6. Rubustness

Ruggedness of the method was studied by determining the effects of small variations, of mobile phase composition (±2%), chamber saturation period, and scanning time (10 % variation of each) on the Rt and response of the quality control samples.

2.5.7. Stability

The stability of the stock solutions of all the three standards was evaluated by storing the solutions in refrigerator at 2-8^{o}C for 72 hours and then comparing the results against freshly prepared stocks for each standard.

2.6. Use of the Validated Method for Quantification of p-methoxybenzoic acid, 3,4-dimethoxycinnamic acid and Ecdysterone in plant and formulation containing whole plant powder of Trianthema portulacastrum (ASSAY)

The extract of plant and formulation was injected seven times. p-methoxybenzoic acid, 3,4-dimethoxycinnamic acid and Ecdysterone peak areas were recorded for each peak, and the amount of all standards was calculated by use of the calibration plot. The procedure was repeated six times, with a new sample of formulation each time.

ASSAY	p-methoxy benzoic acid	3,4-dimethoxy cinnamic acid	Ecdysterone
PLANT	0.32	0.66	0.91
FORMULATION	0.32	1.09	0.90

Table 2

3. Results and Discussion

p-methoxy benzoic acid and Ecdysterone are marker components as they are present only in *Trianthema portulacasstrum* Linn. and not in other plants used in polyherbal formulation. *Boeravia Diffusa* Linn. does show presence of ecdysterone but it does not contain p-methoxy benzoic acid. In case of finding out adulteration of *Trianthema portulacastrum* in the formulation made up of Boeravia Diffusa, p-methoxy benzoic acid acts as a marker. Presence of p-methoxy benzoic acid confirmed adulteration in locally available formulation in present work.

4. Conclusion

The developed method in this research work is precise, accurate and reproducible. It is suitable for Qualitative and Quantitative analysis of p-methoxy benzoic acid, 3,4-dimethoxy cinnamic acid and Ecdysterone in the ethanolic extract of. *Trianthema portulacastrum* Linn. Also it can be used as a quality control method for other market formulations or dietary supplements containing powder of *Trianthema portulacastrum* Linn.

5. References

i. Vol. 1. New Delhi: Indian Council of Medical Research; 2003. Anonymous. Quality Standards of Indian Medicinal Plants; pp. 261–70.
ii. Kumar A, Saluja AK, Shah UD, Mayavanshi AV. Pharmacological potential of Albizzia lebbneck: A review.Pharmacogn Rev. 2007;1:171–4
iii. KR, Basu BD. Indian Medicinal Plants. Vol. 2. Allabahad: Lalit Mohen Basu; 1975. pp. 1180–1184.
iv. Balyan RS, Bhan VM. Emergence, growth and reproduction of horse purslane (Trianthema portulacastrum) as influenced by environmental conditions. Weed Science. 1986;34:516–9.
v. Randhawa MA, Khan MA, Khan NH. Influence of Trianthema portulacastrum infestation and plant spacing on the yield and quality of maize grain. Int J Agric Biol. 2009;11:225
vi. Banerji A. et.al, Isolation of ecdysterone from Indian plants, phytochemistry 1971;10;2225-2226
vii. Ravishankar G.A. Mehta A.R., Control of ecdysterone biogenesis in tissue culture of trianthema portulacastrum. J. Nat. Prod 1979; 42;152;158
viii. Nawaz HR, Malik A, Ali MS, Trianthenol an antifungal tetraterpenoid from Trianthema portulacastrum. Phytochemistry 2001;56-99-102(for antifungal activity also)
ix. Kokpol U et.al, Isolation of c-ethyl flavones from Trianthema portulacastrum. Phytochemistry 1997;44:719-722
x. Singh BP et.al, Flavonoids of some Azioaceae and Molluginaceae of India1982;4: 157-163
xi. Khare CP. Indian Medicinal Plants, an Illustrated Dictionary. Berlin/Heidelberg: Springer-Verlag; 2006. p. 96. 668.
xii. Mandal A. et.al Trianthema portulacastrum affords anti-hepatotoxic activity against carbon tetrachloride-induced chronic liver damage in mice; Reflection in subeellular levels; Phytochemistry res 1997; 11: 216-221.
xiii. Mehta RS, et.al. Hepatoprotective activity of Trianthema portulacastrum, Indian Drugs 1999; 36: 29-32
xiv. Murthy AR et.al. Diuretic effects if Trianthema portulacastrum J. Res Ayur Siddha 1996; 36: 29-32 .
xv. Gupta SK, Priliminary pharmacodynamic studies on Trianthema portulacastrum. J. Res Ayur Siddha 1978; 2: 333-339.
xvi. Vohra SB et.al, Studies on Trianthema portulacastrum, Planta Med. 1983; 47: 106-108.
xvii. Chayya Gadgoli et.al, Antihepatotoxic activity of p-methoxy benzoic acid from capparis spinosa, Journal of Ethnopharmacology, Vol 66, Issue 2, August 1999, 187-192.
xviii. Antioxident activity of caffeic acid (3,4-dimethoxy cinnamic acid), toxicology, Volume 217, Issues 2–3, 16 January 2006, Pages 213–220.
xix. Pablo Kizelsztein et.al, 20-Hydroxyecdysone decreases weight and hyperglycemia in a diet-induced obesity mice model. American journal of psychology, Endocrinology and Metabolism, March 2009 Vol. 296 no. 3,E433-E439.
xx. José M. Zubeldia et.al, In Vitro Characterization of the Efficacy and Safety Profile of a Proprietary Ajuga Turkestanica Extract. Scientific Research, Vol.3 No.4, December 2012.
xxi. H. Chiang, J. Wang and R. Wu, "Immunomodulating Effects of the Hydrolysis Products of Formosanin C and Beta-Ecdysone from Paris formosana Hayata," Anticancer Research, Vol. 12, No. 5, 1992, pp. 1475-1478.
xxii. Syrov V et. al,Effect of Ecdysterone on the hematopoietic activity on the laboratory animals with experimental anemia. Issues Obstetr Gynecol **1**: 62–63, 1976.
xxiii. BM Goyal et.al, Pharmacological Potential of Boerhaavia diffusa: An Overview. International Journal of Pharmaceutical Sciences and Drug Research 2010; 2(1): 17-22
xxiv. Zimmer AR et.al, HPLC method for the determination of ecdysterone in extractive solution from Pfaffia glomerata, J Pharm Biomed Anal. 2006 Feb 13;40(2):450-3. Epub 2005 Aug 24.
xxv. Liu H. et.al, A universal HPLC method for the determination of phenolic acids in compound herbal medicines, J Agric Food Chem, 2005, Aug 24;53(17):6624-9.