Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(3): 3074-3078
Received: 22-03-2019
Accepted: 24-04-2019

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Comparing extraction efficacy of different solvents to extract *Acorus calamus* by using HPLC

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Abstract
Acorus calamus L. (A. calamus) is an herbaceous perennial plant, cultivated in the wetland where the water stagnation is possible. The solvent extract of *A. calamus* rhizome has shown insecticidal activity against many insects. The β-asarone was the predominant bioactive compound responsible for insecticidal action. The aim of this study is to identify the efficiency of different solvent to extract β-asarone from *A. calamus* rhizome. The extraction was done using soxhlet apparatus with a different solvent like chloroform, acetone, methanol, hexane and ethyl acetate. The amount of β- asarone present in each extract was quantified with High Performance Liquid Chromatography– Photo Diode Array (HPLC-PDA). Among the solvent extract tested, the result revealed that extraction efficiency follows the order: methanol> ethyl acetate>hexane>acetone>chloroform. The β- asarone was highest in methanol extract 9,80,840 µg g^{-1} (98.08%). The minimum extraction efficiency was recorded in chloroform extract 88,872µg g^{-1} (8.88%). The extraction efficacy was high in polar solvent like methanol followed by dipolar aprotic solvents like ethyl acetate and acetone. The less extraction efficiency was observed in non-polar solvent like chloroform.

Keywords: *Acorus calamus,* β-asarone, solvent extraction, high performance liquid chromatography

1. Introduction
A. calamus is native to Central Asia, North America and Eastern Europe (Gilani *et al.*, 2006) [6]. In India, *A. calamus* rhizome has been used in Siddha, Ayurveda and Unani traditional medicines. In Tamil Nadu, the extract of *A. calamus* rhizome is given to babies while the bits of *A. calamus* rhizome are tied to emit a fragrance and repel insects. It is being proved to be anti-cancer in humans (Gaidhani *et al.*, 2009) [5].
From time immemorial, *A. calamus* leaves and rhizomes had been used to repel insects in India and many other countries. However, systematic research was initiated by Subramanyam (1942) at Coimbatore. Later the effects of sweet flag rhizome have been tested against more than sixty insects. The rhizomes have been found to act on insects in many ways as insecticide ((El-Nahal *et al.*, 1989); (Su, 1991), ovicide (Risha *et al.*, 1990); (Pajni *et al.*, 1995);) [4, 7], [12], (Shukla *et al.*, 2009) [18] oviposition deterrent (Rahman & Schmidt, 1999), chemosterilant (Bhaskar *et al.*, 1976) [1], antifeedant (Reddy & Reddy, 2000) [16] and repellent (Pierce & Schmidt, 1993); (Ignatowicz & Wesołowska, 1996) [8].
A number of bioactive constituents were identified and characterized from the leaves and rhizomes and their essential oils. The oil was found to contain varying concentrations of a asarone (1), b-asarone (2), c-asarone (3), calamene, calamenenol, calameone (4), a-pinene (5), b-pinene (6), camphene, p-cymene, eugenyl acetate, eugenol (7), isoeugenol (8), methyl isoeugenol (9), calamol, azulene (10), eugenol methyl ether, dipentene (11), methyleugenol, asaronaldehyde (12), terpinolene (13), 1,8-cineole (14), camphor (15), a-caryophyllene (16), and hydrocarbons. (Nigam *et al.*, 1990); (Mukherjee *et al.*, 2007) [10]. Major chemical constituents identified are alpha and β- asarone which is responsible for the therapeutic and medicinal properties of sweet flag species (Devi & Ganjewala, 2009) [3]. The different solvent based extract of A. calamus has been used for controlling many pest. The present study is aimed to quantify the amount of β- asarone content in the different solvent based extract of *A. calamus*.

2. Materials and methods
2.1 Materials
An analytical standard of β- asarone (70% purity) obtained from Sigma Aldrich (Germany) and solvents were purchased from and Merck (Mumbai, India).

2.2 Extraction of β- asarone by soxhlet apparatus
The dried rhizome powder was subjected to soxhlet's extraction by using different solvent viz., chloroform, acetone, methanol, hexane and ethyl acetate.

The rhizome powder (100 g) was packed in filter paper and loaded to the thimble. About 500 mL of solvent was poured into the round bottom flask and the temperature was maintained at 50 °C for 12h. The obtained extract was concentrated by rotary vacuum evaporator at 40- 60 °C.

2.3 Sample preparation

The 100 µl of the different solvent extract viz., chloroform, acetone, methanol, hexane and ethyl acetate of *A. calamus* was diluted with 900 µl of methanol: water (80:20, v/v) mixture. The solution was further diluted with methanol: water (80:20, v/v) about 10,000 times and injected in HPLC.

2.4 Instrumentation

A High-Performance Liquid Chromatography HPLC (Shimadzu LC- 20 AT) equipped with photodiode array detector (Shimadzu SPD- M20A) and online degasser (DGC-20 A5) was used in detection and quantification of β- asarone. A reversed Agilent 5 µm TC-C18 (2) (250X4.6 mm) column was used to separate the target analysis. The mixture of methanol: water (80:20, v/v) was used as mobile phase. The injection volume and flow rates were 20 µl and 1 ml min-1, respectively.

2.5 Preparation of standard stock solution

The 31.5 mg of the analytical standard of β- asarone (70% purity) was transferred into a 25 ml volumetric flask and the volume was made up to 25 ml with methanol (HPLC grade). The flask was then shaken well to get a homogenous stock solution of 882 mg L-1 concentration of β- asarone and the stock solution was stored at -200C.

2.6 Working standard solutions

The working standard solutions ranging from 0.005 to 1 mg L-1 was prepared by serial with methanol: water (80:20, v/v). These working standards were used to find out the retention time, calibration curve and quantitative determination of β- asarone samples.

β- asarone calculation formulas

$$\beta\text{- asarone } (\mu g\ g^{-1}) \text{ in } A.\ calamus = \frac{A_S \times C_{std} \times IV_{std} \times FV_{sam}}{A_{Std} \times Wt_{sam} \times IV_{sam}}$$

A_s	Sample area
C_{std}	Concentration of standard (ppm)
IV_{std}	Injected volume of standard (µl)
FV_{sam}	Final volume of sample (ml)
A_{Std}	Standard area
Wt_{sam}	Weight of sample (g)
IV_{sam}	Injected volume of sample (µl)

3. Result and discussion

3.1 Quantification of β- asarone in HPLC

The asarone was detected and quantified in HPLC (Shimadzu LC- 20 AT) equipped with diode array detector (Shimadzu SPD- M20A) and online degasser (DGC-20 A5). A reversed Agilent 5 µm TC-C_{18} (2) (250X4.6 mm) was used to separate the target analysis. The retention time of β- asarone at a wavelength of 251 nm was 5.43 minutes and run time was 10 minute. The 1ppm standard chromatogram was of β given in fig. 1.

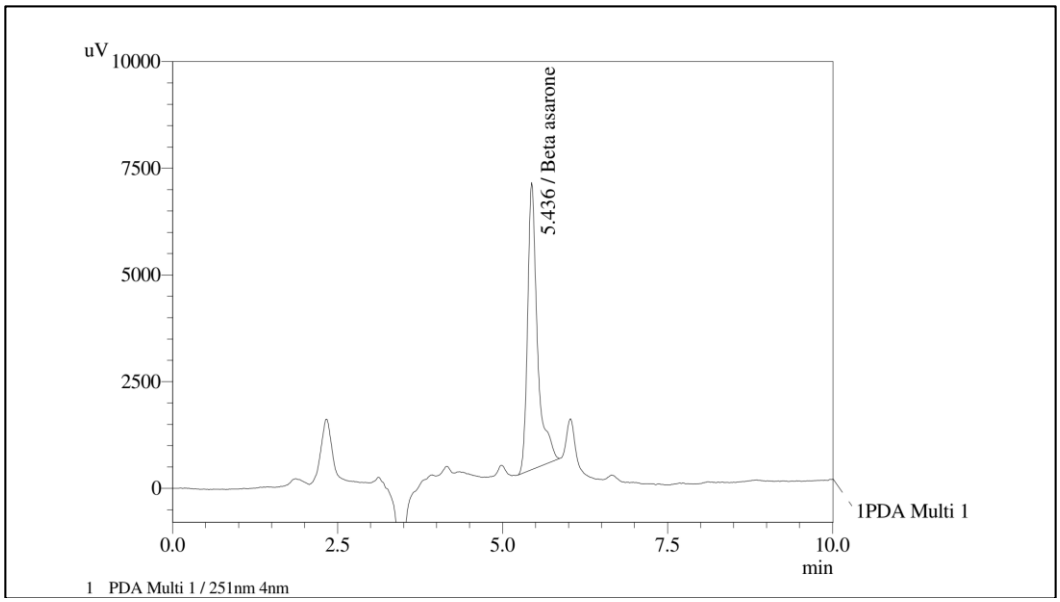

Fig 1: Chromatogram of β- asarone (1ppm)

3.2 The efficiency of the analytical method

The linearity of the calibration curves was established for method validation by plotting the area against the concentrations (0.005, 0.0 1, 0.05, 0.1, 0.5 and 1 mg L^{-1}). The coefficient of determination (R^2) was 0.999, which indicates good linearity for the target. (Fig 2).

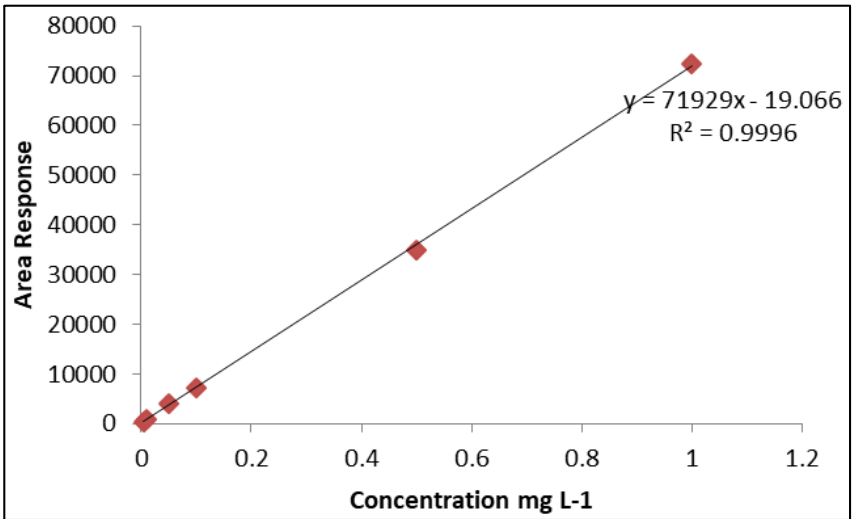

Fig 2: Calibration of the curve of β- asarone

3.3 Extraction efficiency of different solvent

The HPLC analysis revealed that concentration of β- asarone was maximum of 9, 80,840 µg g-^{1} (98.08%) in methanol based extract of sweet flag rhizome whereas in chloroform based extract it was only 88,872µg g^{-1} (8.88%). The concentration of β- asarone in ethyl acetate, hexane and acetone based extract were 9,35,011.8 µg g^{-1} (93.50%), 8,32,046.1 µg g^{-1} (83.20%) and 7,79,942.7 µg g^{-1} (77.99%) respectively (Table 1). The *A. calamus* rhizome was commonly extracted by using different solvents like chloroform, acetone, methanol, hexane and ethyl acetate. The insecticidal action of *A. calamus* oil and β- asarone was reported in many households (El-Nahal *et al.*, 1989) [4]. The bioefficacy of *A. calamus* extract differs with the solvent used for extraction (Hossain *et al.*, 2008) [7]. Toxic effect of β-asarone was reported against stored product insects like *C. chinensis* and *S. granaries* (El-Nahal *et al.*, 1989) [4]. (Park *et al.*, 2003) [13] tested the rhizome extract against the adults of *S. oryzae* and reported that (*Z*)-asarone caused 70% and 90% mortality against *S. oryzae* adults. The HPLC analysis of different extract revealed that the highest retrieving capacity of β- asarone by methanol. The extraction efficiency followed the order: methanol> ethyl acetate>hexane>acetone>chloroform. The present finding can be corroborated with the findings of (Hossain *et al.*, 2008) [7] who reported that the efficacy of *A. calamus* solvent extracts followed the order : petroleum ether > methanol> acetone and that the *S. oryzae* adult mortality was found to be 99% after 24 hours at a dosage of 0.157 mg/ cm^2 and 99.17mg/cm^2 for petroleum ether and acetone respectively. The methanol based extract had 9,80,840 µg g^{-1} (98.08%) of β- asarone whereas acetone based extract had 7,79,942.7 µg g^{-1} (77.99%).

The difference in insecticidal activity was due to the extraction efficacy of β- asarone by the different solvents. Petroleum ether extract *A. calamus* was times less toxic than Malathion against larvae of *C. cephalonica* (Chauhan *et al.*, 1987). The difference in efficiency was because of the presence of higher amount of β- asarone in *A.* calamus extract. There is a correlation between the insecticidal activity and quantity of β- asarone present in rhizome extract.(Kim *et al.*, 2003) reported that over 90 per cent mortality of adults of *S. oryzae* at 3 or 4 days after treatment was achieved using methanol extracts of *A. calamus* var. *angustatus* rhizome and *A. gramineus* rhizome. The petroleum ether extract of *A. calamus* was as toxic as Malathion to *S. oryzae* (Teotia & Pandey, 1979). Chromatograms of β- asarone in different solvent-based extract of sweet flag rhizome is illustrated in Fig 3.

Table 1: β – asarone in different solvent- based extract of *A. calamus*

Sample	RT(min)	β – asarone (µg g^{-1})
Methanol	5.42	980804.00
Ethyl acetate	5.42	935011.80
Acetone	5.42	779942.70
Hexane	5.42	832046.10
Chloroform	5.42	88872.00

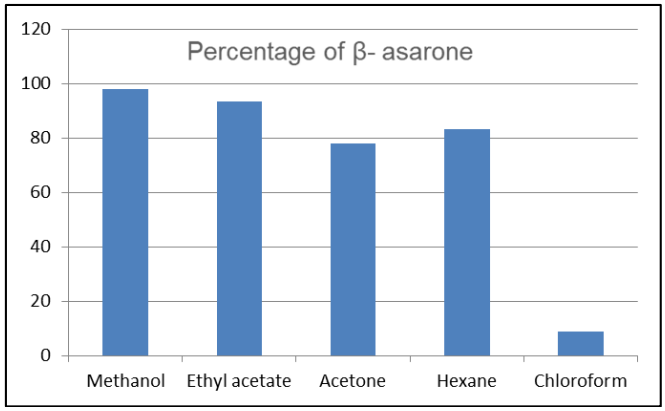

Fig 3: Percentage of β- asarone in different solvent

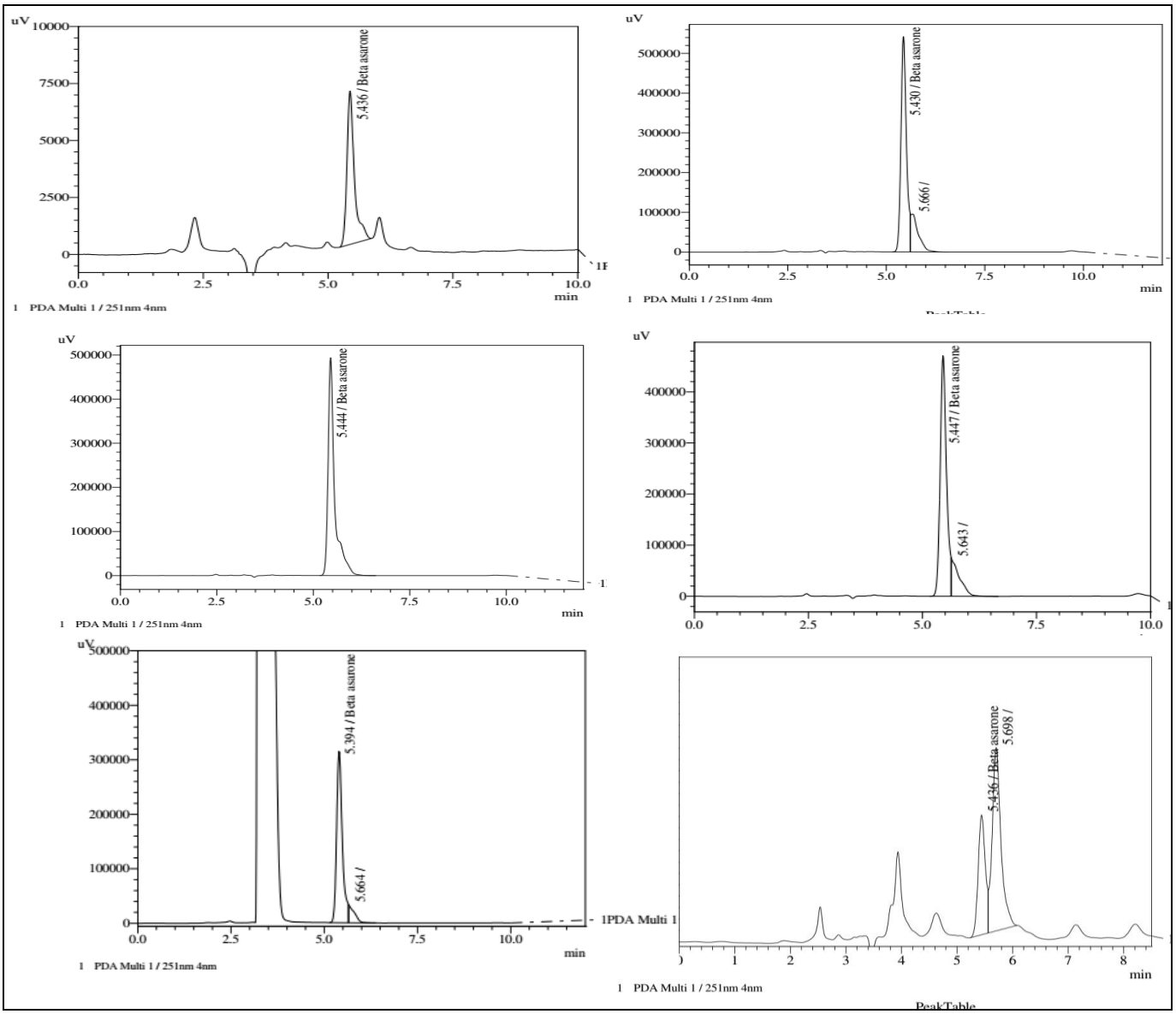

Fig 4: (A) Standard chromatogram of β- asarone; (B) Chromatogram for β- asarone in Ethyl acetate extract of sweet flag rhizome powder; (C) Chromatogram for β- asarone in hexane extract of sweet flag rhizome powder;(D) Chromatogram for β- asarone in acetone extract of sweet flag rhizome powder; (E) Chromatogram for β- asarone in methanol extract of sweet flag rhizome powder; (F) Chromatogram for β- asarone in Chloroform extract of sweet flag rhizome powder

4. Conclusion

In this research, the efficacy of different solvents to retrieve β-asarone from *A. calamus* rhizome was detected. The present finding will lead to an appropriate solvent selection for extraction of β-asarone and development of new formulations.

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