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Original Research Article

Comparative studies on variation of β-Asarone composition in *Acorus calamus* Linn. by GC and HPLC

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Abstract

The objective of this study was to assess the essential oil yields and chemical composition of the root of two varieties of *Acorus calamus*, namely the local and Jabalpur varieties. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) analysis identified β-Asarone as the major chemical constituent. Notable findings revealed that fresh samples exhibited higher essential oil yield and β-Asarone content compared to various drying methods. Specifically, powdering the Jabalpur material resulted in a significant percentage (2.0%) of essential oils, with negligible variations observed in β-Asarone content. Additionally, besides GC, a straightforward and effective reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed for β-Asarone analysis from *A. calamus* root extracts in different solvents. Interestingly, the spent material also contained a considerable amount of β-Asarone. Among the different solvent extractions, ethyl acetate extracts exhibited the highest percentage (62%) of β-Asarone, while the lowest amount was recorded in methanol extract (37.47%).

Keywords: *Acorus calamus*, Essential oil, GC, HPLC, β-Asarone.

Introduction

Sweet flag (*Acorus calamus* Linn.) is a member of the Acoraceae family and is characterized as a tall perennial wetland monocot plant. Traditionally, both the leaves and rhizomes of this plant have been utilized for medicinal purposes. The powdered roots possess a spicy flavor and are employed as a substitute for ginger, cinnamon, and nutmeg due to their aromatic properties (Balakumbahan *et al*., 2010). The propagation of the sweet flag primarily involves the *in-vitro* method of vegetative multiplication, which offers significant advantages for the medicinal trade and germplasm conservation. It is found growing wild or cultivated across the Himalayas, reaching altitudes of up to 6000 feet. This perennial herb is commonly found along stream banks and in damp, marshy areas.

The volatile oil and extracts of *A. calamus* are extensively utilized in pharmaceuticals, traditional medicinal systems, and perfume manufacturing. This plant is recognized for its diverse therapeutic properties, including anti-inflammatory effects (Kim *et al*., 2009), anti-cholinesterase activity (Mukherjee *et al*., 2007), cardiac suppression (Shah *et al*., 2010), insulin sensitization (Wu *et al*., 2009), radioprotective properties (Sandeep and Nair, 2010), anticonvulsant and analgesic properties (Jayaraman *et al*., 2010), neuromodulatory effects (VengadeshPrabu *et al*., 2009), potential in murine cancer treatment (Bains, *et al*., 2005), anxiolytic activity (Kaushik, 2020) and neurological and metabolic disorders (Sharma *et al*., 2020)

A. calamus is recognized as a valuable source of essential oil, which accounts for its insecticidal and medicinal properties, along with its bitter principle (Venakutonis and Dagilyte, 2003), with β-Asarone identified as the major chemical constituent. The essential oil was initially isolated in Noricum (Gildemeister and Hoffmann, 1900). It comprises various phyto-molecules such as glycosides, flavonoids, saponins, tannins, polyphenolic compounds, and mucilage. Additionally, three monocyclic sesquiterpenes, shyobunone, epishyobunone, and 2,6-diepishyobunone have been isolated from the rhizomes (Iguchi *et al*., 1968). Numerous chemical constituents have been previously reported from the rhizomes, leaves, and roots, including β-Asarone, α-Asarone, elemicine, cis-iso-elemicine, cis and trans-isoeugenol, their methyl ethers, camphene,

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P-cymene, α-selinene, β-gurjunene, β-cadinene, camphor, terpinen-4-ol, α-terpineol, acalacorene, acorone, acrenone, acoragermacrone, 2-deca-4,7-dienol, shyobunones, linalool, and preisocalamendiol. Additionally, compounds such as acoradin, galangin, 2,4,5-trimethoxy benzaldehyde, 2,5-dimethoxy benzoquinone, calamendiol, spathulenol, sitosterol and phenylpropionates (Hao *et al*., 2023) have been identified.

Due to its significant industrial importance in medicinal and essential oil production, our study delves into assessing the volatile oil yields and chemical composition of fresh and dried roots from two *A. calamus* varieties. This investigation employs gas chromatography (GC) and high-performance liquid chromatography (HPLC) techniques alongside an exploration of antioxidant activity in different solvent extracts, including those derived from spent material.

Materials and Methods

Chemicals, standards

The chemicals employed, including methanol, acetonitrile, and water, were of HPLC grade sourced from Merck (India). Hexane, chloroform, ethyl acetate, and acetone for extraction were purchased from the same supplier. The standard β-Asarone of analytical grade was procured from Natural Remedies Pvt Ltd. (Bengaluru, India). Prior to use, the solvents were freshly prepared and degassed.

Collection of plant samples

Root samples of two distinct varieties (Local and Jabalpur) of *A. calamus* were gathered from Nizamabad District, Telangana, India, in December 2020. The plant specimens underwent taxonomic identification and authentication by Dr. Venkat Ramana, Assistant Professor in the Department of Botany at Nizam College, Osmania University, Hyderabad, India. A voucher specimen (CIMAP-2020/AC) was deposited and stored at the CIMAP Research Centre in Hyderabad, India.

Sample preparation

The fresh *A. calamus* root samples from both varieties were divided into five portions. One portion underwent individual hydrodistillation using a Clevenger apparatus. The remaining three portions were subjected to different drying techniques (shade, sun, and oven at 45°C) before undergoing hydrodistillation. The final portion was coarsely powdered and then hydrodistilled.

Essential oil extraction/distillation

The roots of two distinct varieties of *A. calamus*, both fresh and dried, underwent hydrodistillation using a Clevenger-type apparatus for a duration of 5 hours. The essential oils extracted were subsequently dried over anhydrous sodium sulfate and stored at 4°C until they were analyzed using GC.

Gas chromatography analysis

The essential oils were analyzed using a Varian CP-3800 model gas chromatograph equipped with a flame ionization detector (FID) and an electronic integrator controlled by the Galaxy software system. Separation of compounds was achieved using a Varian CP-Sil 5CB capillary column (ID: 50 m X 0.25 mm; film thickness 0.25 μm). Nitrogen served as the carrier gas, maintaining a constant flow rate of 0.4 mL/min. The column temperature was programmed to increase from 100°C (held for 2 minutes) to 240°C (held for 15 minutes) at a rate of 5°C/min. The injector and detector were set to temperatures of 250 and 300°C, respectively. Samples of 0.2 μL were injected with a 20:50:20 split ratio. Retention indices were generated using a standard solution of n-alkanes (C6-C19). The composition was reported as a relative percentage of the total peak area without FID response factor correction.

Chemical compounds identification

The identification of β-Asarone was done based on their retention indices (RI), determined with reference to a homologous series of n-alkanes (C_9–C_{24}, Polyscience Corp., Niles, IL, USA) under identical experimental conditions in both polar and non-polar columns, co-injection with standards (Natural Remedies, India), a mass spectra library search and by comparison with the mass spectral literature data. The relative amount of β-Asarone was calculated based on computer-calculated GC peak areas without correction for flame ionization detection response factors.

Preparation of HPLC sample solution

Preparation of sample solution

About 5 g of *A. calamus* rhizomes, the plant material was powdered and placed into a conical flask. Subsequently, 30 mL of methanol was added, and the mixture underwent sonication at 45°C for 30 minutes. The extraction was then filtered through filter paper into a round bottom flask, with the residue returned to the conical flask. This extraction process was repeated twice more. The combined methanol extractions were concentrated under reduced pressure using a rotary evaporator. Accurate measurement of 25 mg of the resulting extract was transferred into a 25 mL volumetric flask and made up to volume with methanol. Prior to HPLC injection, each sample solution was filtered through a 0.45 μm membrane filter into an HPLC sample vial.

Instrumentation

The HPLC analysis was performed using a Waters modular HPLC system consisting of two pumps (model

1525), an automatic gradient controller, an injector (model 717 plus), a photodiode array detector (PAD model 2996) and Empower software. The injector, gradient controller and chromatography manager were integrated together to give reproducible results. A Phenomenex Luna C_{18} column (4.6 X 250 mm, 5 µm particle size) was used for the analysis, and spectral acquisition was performed at 218 nm after scanning the maxplot of standard.

Chromatographic conditions

The solvent system consists of (A) acetonitrile and (B) water. Elution occurred at room temperature using an isocratic mode (A = 50: B = 50%) for 40 minutes at a flow rate of 0.6 mL/min. Standard β-Asarone was identified at 218 nm with a retention time of 22 minutes. An injection volume of 20 µL was selected. Peak identification relied on matching the retention time and comparing it to the injected authentic reference standard.

Comparative studies on different varieties and spent material

The roots of two distinct *A. calamus* varieties were air-dried and subsequently turned into powder. The resulting powdered material underwent methanol extraction using a sonicator, followed by analysis through RP-HPLC utilizing the aforementioned method. Both the Jabalpur and local root materials were divided into four batches. The first batch underwent hydrodistillation and drying. The remaining three batches were subjected to various drying techniques (shade, sun, and oven at 45°C) before undergoing hydrodistillation. These samples were powdered, prepared as HPLC samples, and analyzed using the established method. Additionally, the Jabalpur variety root material was subjected to extraction using solvents with increasing polarity and subsequently analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC).

Table 1. Hydrodistillation and GC analysis report of *A. calamus* essential oils

Sample name	Oil yield (%)	β-Asarone (%)
Local variety	0.5	91.471
Jabalpur variety	1.25	92.293

Results and Discussion

Comparative studies of essential oils by GC

Hydrodistillation was performed on the rhizomes of two different varieties of *A. calamus*, and the resulting essential oils were subjected to GC analysis. The predominant component, β-Asarone, was identified in the oil. As indicated in the hydrodistillation report (Table 1), the Jabalpur variety demonstrated a higher essential oil percentage (1.25%) and a greater concentration of the major chemical constituent, β-Asarone (92.293%), in comparison to the local variety (91.471%). The higher oil percentage and increased concentration of β-Asarone in the Jabalpur variety compared to the local variety may be attributed to several factors such as genetic variations, environmental conditions, and cultivation practices, etc.

Effect of drying on essential oil yield and β-Asarone content

The objective of the study was to examine variations in volatile oil yields employing three distinct drying methods (Shade, Sun, and Oven). In the Jabalpur variety, the volatile oil content decreased in the dried samples compared to the fresh sample (1.25%), with a notable reduction observed in the oven-dried sample (1.0%). This decline is likely linked to the swift loss of moisture and volatile components during rapid drying. In contrast, for the local variety, the oil yield increased in the oven-dried sample, while the content remained relatively consistent in the other drying techniques (Figure 1).

Upon analyzing the chemical composition of the oils, it was observed that the predominant chemical constituent, β-Asarone, exhibited higher concentrations in the fresh sample (92.293% in the Jabalpur variety) in contrast to the other dried samples. In contrast, for the local variety, the highest concentration was documented in the oven-dried samples (92.541%), while the lowest was noted in the sun-dried samples (51.460%) (Figure 2). When evaluating physical parameters such as root

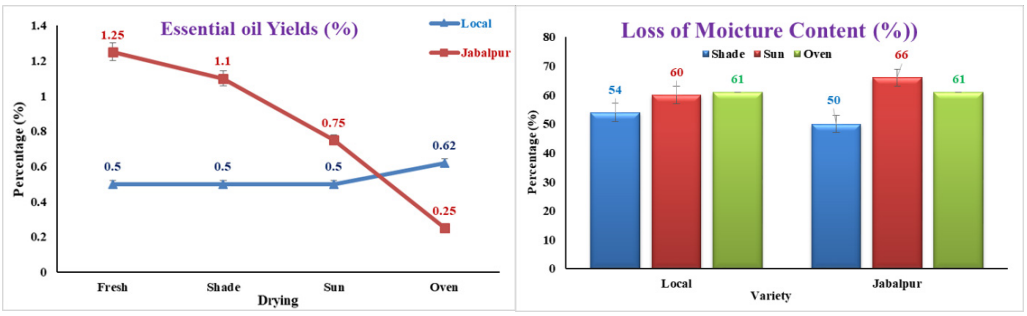

Figure 1. Essential oil yields and loss of moisture content

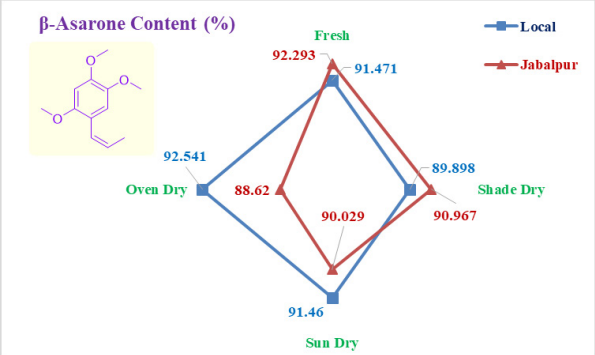

Figure 2. Effect of drying techniques on β-Asarone content

size and length, it was noted that the Jabalpur material exhibited larger dimensions compared to the local variety.

Typically, larger plant structures may undergo faster moisture loss, potentially leading to the evaporation of volatile oils. As a result, this phenomenon contributed to reduced essential oil yields in the dried Jabalpur samples. Consequently, a decrease in the concentration of the major constituent is anticipated, alongside the reduction in essential oil yields. Contrastingly, in the case of the local variety samples, both essential oil yields and β-Asarone content increased in the oven-dried samples. This increase can be attributed to the smaller size and length of the roots in the local variety compared to the Jabalpur roots.

Effect of powdering on essential oil yield and chemical composition

Another experiment was conducted to investigate the impact of powdering on the essential oil yield and chemical composition of *A. calamus* root. Typically, hard materials such as roots stems, and bark are pulverized into smaller pieces to facilitate the extraction of maximum volatile oil yields in a shorter duration. In this study, the *A. calamus* root material was powdered before undergoing essential oil extraction.

In the Jabalpur variety, a significant increase in essential oil yield (0.7%) was observed in the coarsely powdered material (2.0%) compared to the non-powdered material (1.3%). Conversely, in the local variety, the essential oil yield decreased in the coarsely powdered material (0.9%), and the difference between the powdered and non-powdered material was minimal (0.1%), rendering it negligible. Similarly, the β-Asarone content was slightly lower in the powdered material compared to the non-powdered material in both varieties (Jabalpur: 0.447% and Local: 0.575%), with the difference also being negligible (Figure 3).

However, from an economic perspective for farmers, both the duration of hydrodistillation and the resulting essential oil yields are crucial factors affecting income. Typically, the extraction of essential oils from *A. calamus* roots requires a hydrodistillation time of 5 hours. However, our current study reveals that it only takes 4 hours to extract the maximum amount of essential oil from coarsely powdered material. Hence, to optimize financial returns for farmers, it is recommended to use coarsely powdered material for essential oil extraction, as it allows for a shorter extraction time while still maximizing yield.

Comparative studies of β-Asarone from A. calamus by HPLC

In addition to GC, we also developed an RP-HPLC method to quantify the β-Asarone content in the roots of two varieties (Local and Jabalpur), both in dried and spent materials. This method utilized a Phenomenex Luna C18 column (4.6 x 250 mm, with 5 µm particle size) and an acetonitrile and water (1:1) solvent system

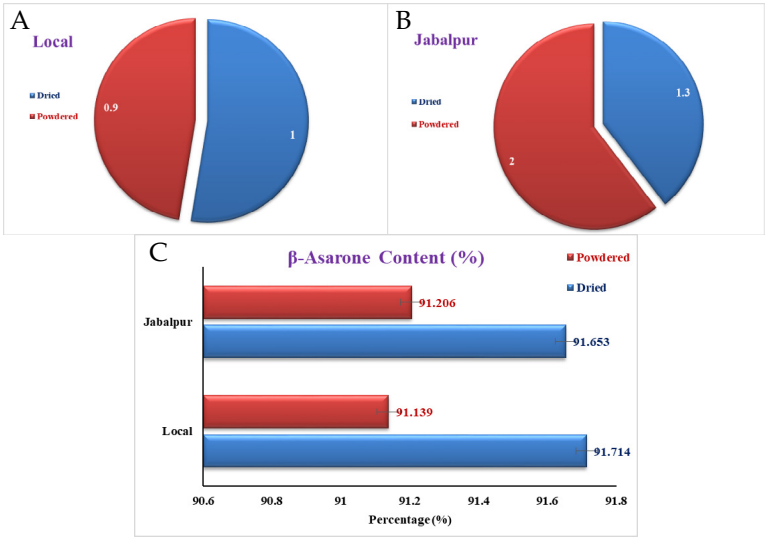

Figure 3. Effect of powdering on essential oil yield (A and B) and β-Asarone content (C)

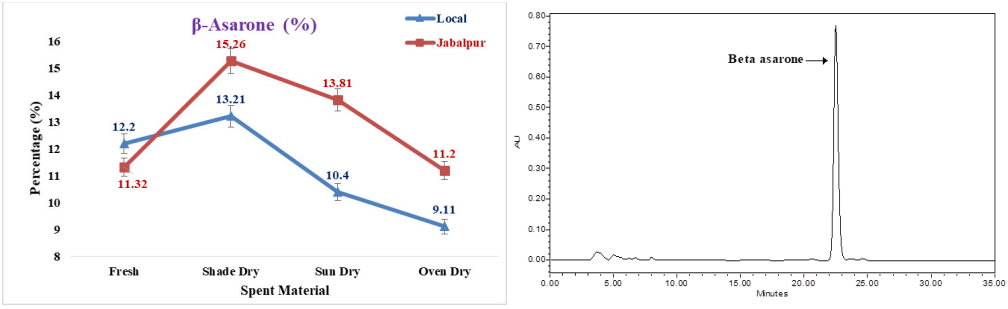

Figure 4. β-Asarone content in spent material [Fresh: hydro-distilled and dried; shade, sun and oven: dried and hydro-distilled]

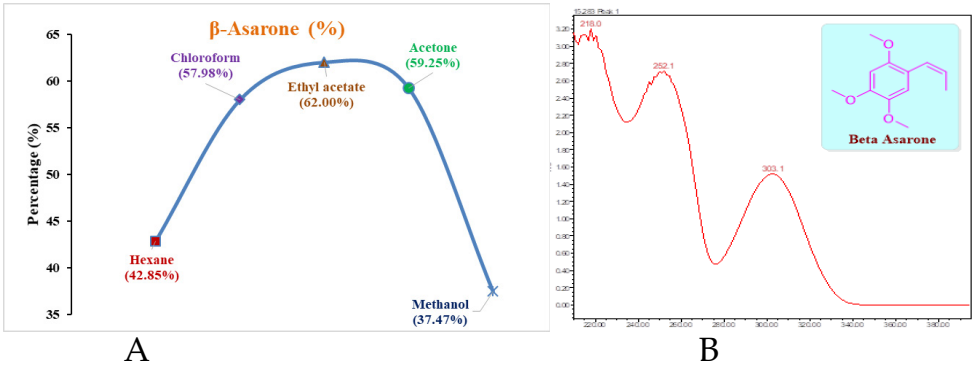

Figure 5. HPLC results of different solvent extracts and UV-spectrum of β-Asarone

at a flow rate of 0.6 mL/min. Interestingly, there were no discernible differences in the β-Asarone concentration between the two types of dried samples (without hydrodistillation), with Jabalpur exhibiting 24.6% and local showing 24.2%.

Comparative studies on spent material

In the spent materials, higher β-Asarone content was observed in shade-dried materials in both varieties (Jabalpur: 15.26% and Local: 13.21%) compared to other samples (Figure 4). Notably, in oven-dried materials, a higher percentage of β-Asarone was lost compared to other drying techniques, likely due to the circulation of hot air in the oven. Overall, considerable quantities of β-Asarone were present in the spent materials, alongside other enriched phytochemicals. These spent methanol extracts could serve in meeting various market demands through continuous bioactivity screening investigations and may serve as a useful baseline for comparing results in the literature.

Comparative studies of β-Asarone in different solvent extracts

In addition to conducting HPLC analysis on both the raw material and spent material, we examined the fluctuations in β-Asarone content across various simultaneous solvent extracts (with increasing polarity) using RP-HPLC. According to the HPLC results (Figure 5), the highest percentage of β-Asarone content was observed in the ethyl acetate extract (62%) compared to other solvent extracts. Each extract contains β-Asarone along with additional enriched phytochemicals, which may potentially offer enhanced activity compared to β-Asarone alone.

Conclusion

In conclusion, our study demonstrates that the Jabalpur variety of *A. calamus* exhibits higher essential oil yields and β-Asarone content compared to other varieties. However, different drying techniques had a negative impact on essential oil yields, although a slight increase was observed in β-Asarone content with the oven-dry technique, albeit negligible. Overall, root drying is recommended for essential oil extraction in *A. calamus*. Furthermore, coarse powdering of the root material yielded positive effects on essential oil yields, particularly in the Jabalpur variety. Although there was a slight reduction in β-Asarone content, it was negligible. Considering the economic perspective for farmers, hydrodistillation time and essential oil yields significantly influence income. Our study indicates that it takes one hour less to extract the maximum amount of essential oil from coarsely powdered material compared to non-powdered material. Therefore, to optimize farmers'

financial returns, coarse powdering of material is recommended for essential oil extraction due to its shorter extraction time.

Additionally, HPLC analysis of spent materials revealed a considerable quantity of β-Asarone alongside other enriched phytochemicals. These spent methanol extracts could address diverse market demands through continuous bioactivity screening investigations and serve as a useful baseline for comparing results from the literature. Moreover, our investigation into variations of β-Asarone in different simultaneous solvent extracts highlights the presence of additional enhanced phytochemicals in each extract, which may offer superior activity compared to β-Asarone alone.

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Author Contribution

M Sumalatha: Formal Analysis; A. Niranjana Kumar: Experimentation, Analysis, Observations; J. Kotesh Kumar: Methodology, Investigation, Interpretation of Results, Manuscript Preparation; K.V.N. Satya Srinivas: Investigation, Interpretation of Results; M. Vijay Kumar: Recording of drying observations; K. Vinutha: Observations.

Conflict of Interest

The authors declare that they have no conflict of interest.

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