Pharm Methods, 2016; 7(2): 121-126
A multifaceted peer reviewed journal in the field of Pharm Analysis and Pharmaceutics
www.phmethods.net | www.journalonweb.com/phm

Original Article

Gas Chromatographic Method for Analysis β-Asarone in Rhizome extracts of *Acorus calamus* and Their Microbiological Evaluation

Kavyashree Banda Vijayakumar, Gurupadayya Bannimath*, Venkata Sairam Koganti, Vishwanathan Balasubramanya Iyer

Department of Pharmaceutical Chemistry, JSS College of Pharmacy, JSS University, Mysuru-570015, INDIA.

ABSTRACT

Introduction: The aim of study was to develop a simple, sensitive and precise gas chromatographic method for the analysis of β-asaronein ethanolic, ethyl acetate, aqueous extracts from the rhizomes of *Acorus calamus* and validate according to current ICH guidelines. Followed by evaluation of antimicrobial activity of prepared extracts in comparison with penicillin disc. **Methods**: The β-asarone was one chief active constituent from the rhizomes of *Acorus calamus*. The ethanolic, ethyl acetate, aqueous extract of rhizomes was prepared and further evaluated for its antimicrobial activity against *Streptococcus Mutants* by agar well diffusion method. The GC method was used for the analytical determination of β-asarone. The sample was estimated using gas chromatography with flame ionization as a detector. Nitrogen at a flow rate of 1.18 mL/min was used as a carrier gas and total run time was 10 minutes. The injection port and detector temperature were set to 225˚C and 270˚C, respectively. The retention time of β-asarone was found to be 6.9 minutes. **Results**: The linearity of the developed method was tested in the range of 100 ng/mL-500 ng/mL for β-asarone, limit of detection and limit of quantification was found to be 22.78 and 69.05 ng/mL respectively and the percentage recovery was from 99.63- 100.64%. **Conclusion:** A simple, precise and accurate GC-FID method has been developed for the determination of β-asarone in ethanolic, ethyl acetate and aqueous extracts of rhizomes.

Key words: *Acoruscalamus*, β-Asarone, Alcoholic, Aqueous, Ethyl acetate, Extracts, GC- FID, Microbiological evaluaiton, Stability studies.

Correspondence:
B. M. Gurupadayya, Department of Pharmaceutical Chemistry, JSS College of Pharmacy, JSS University, Sri Shivarathreeswara Nagar, Mysuru-570 015, Karnataka, INDIA.
Telephone: +91-821-2548353
Fax Number: +91-821-2548359
Mobile Number: +91-7411006136

E-mail: bmgurupadayya@jssuni.edu.in
DOI : 10.5530/phm.2016.7.18

INTRODUCTION

Acorus calamus is an aromatic medicinal plant which is commonly known as 'Sweet flag'. It has a very good medicinal values.[1] It is been used fromolden times of use in both traditional Indian and Chinese systems of medicine.[2] In earlier days it was used as a brain tonic for rejuvunation of the brain and nervous system. It showed wide variety of pharmacological actions in ayurvedic system of medicine like anti-hemorrhoidal, used for reliving cold sensation on skin, used as an adjunct to decoction enemas, it is also used as natural substances that remove fat from the body and also used in cleansing agent in nasal therapy.[3] *Acorus calamus* was even used in traditional folk medicine of America and Indonesia for diseases like diabetes and diarrhea.[4] It is also used in the treatment of various ailments like appetite loss, bronchitis, cough, chest pain, cramps, diarrhea, digestive disorder, depression, fever, hemorrhoids, inflammation, numbness, rheumatisim, skin diseases, tumors and as antidotes for several poisoning.[5-7]

Various parts of *Acorus calamus* have its own pharmacological importance in treatment of different diseases. Of all the parts most of the experimental studies have been carried on leaves, roots and stem of the plant.[8] The rhizomes of the plant has a long history of usage in many countries, it been used almost from 2000 years in India and China. Chinese have used this rhizome for the treatment of swelling and constipation. Native Americans have also used this plant rhizome as an anesthetic for toothache and headache.

Even in Ayurveda system of medicine rhizomes of *Acorus calamus* are considered to possess aphrodisiac, aromatic, antispasmodic, anthelmintic, bitter tonic, carminative, diuretic, emetic, laxative, expectorant, and stimulant properties. *Acorus calamus* are even used in the management of various ailments like asthma, abdominal tumors, bronchial catarrh, chronic diarrhea, cough and dysentery.[9]

Acorus calamus rhizomes are even used in traditional system of medicine like in case of new born children it is given in form of vasambu(rhizome paste) along with honey for proper brain development, better visual power, increased seminal power and proper speech ability. After the birth of newborns it is applied on the tongue in the form of paste along with the ghee, gold and water to improve the grasping power. It also stimulates the nervous system, giving relief from depression. It is useful in improving speech in stammering.[10] Vacha taken along with milk improves intellectual, grasping and memory power.[11]

A detailed literature review indicated that there are few analytical methods reported like HPTLC, HPLC, GC-MS and LC-MS[12-15] for quantification of various constituents of *Acorus calamus*. But till date there are no reported methods for quantification of β-asarone (Figure 1) in ethanolic, ethyl acetate, aqueous extracts from the Rhizomes of *Acorus calamus*. So in this concern,we have first extracted β-asarone from its rhizomes and its antimicrobial evaluation by well diffusion method. Followed by its quantification through GC-FID method. The GC-FID method employed was very simple, highly sensitive, does not require any derivitization of the sample and also short runtime.

Chemical Constituents

Many number of chemical constituents have been reported from the leaves, rhizomes along with essential oil of *Acorus calamus*.[16] The oil of *Acorus calamus* rhizomes were found to contain different concentration of alcohol, aldehyde, ester, furan, hydrocarbon, ketone, N-containing miscellaneous like α-asarone, β-asarone, α- pinene, β- pinene, calamene,

calamenenol, calameone, eugenol, isoeugenol, methyl isoeugenol, eugenol methyl ether, asaronaldehyde, terpinolene, camphor, α- caryophyllene and hydrocarbons etc.[17-19] From all these constituents β-Asarone with 46.78% was found to be a major bioactive compound.[20]

MATERIALS AND METHODS

Materials and reagents

Standard β-asarone was purchased from Sigma-Aldrich (Bangalore, India). HPLC grade methanol solvents (acetonitrile and methanol) procured from Merck Ltd, Mumbai, India. Brain heart infusion agar was procured from Himedia. The strains of *Streptococcus mutants* were procured from Microbiology laboratory in JSS Dental College, Mysuru. *Acorus calamus* rhizome was procured from local Mysuru market. All other chemicals used are analytical reagent grade (AR grade) procured from Loba Chemie, Mumbai.

*Preparation of **Acoruscalamus** Root Extract*

Ethanolic extract and Ethyl acetate (Soxhlet extractor):

The shade dried coarsely powdered rhizome of *Acorus calamus* (150 g) was taken and wrapped in filter paper and loaded in to Soxhlet extractor. Around 600 mL of ethanol/ethyl acetate was added in to the extractor. The extractor is assembled with water bath and condenser. The bottom of the extractor was connected to 1 lt round bottom flask and this was placed into water bath.

Initially the dried powder was packed with filter paper and loaded to thimble. This was arranged in such a way that the condensed alcohol/ethyl acetate should fall on packed material. The temperature of water bath was maintained below 100^{0}C throughout the extraction procedure. The extraction process was continued for 48 hrs with constant boiling. Further the extract was evaporated under reduced pressure in rotary evaporator to get a semi solid crude extract. This was transferred in to petri plate and stored in desiccator and followed in freezer with air tight pack for future use.

Aqueous extract (Simple distillation method)

The shade dried coarsely powdered rhizome of *Acorus calamus* (150 g) was taken and transferred into 500 mL round bottom flask. Nearly 400 mL water was added into the flask and placed in water bath. The temperature of water bath was maintained below 80^{0}C throughout the extraction procedure. Extraction was carried out for 24 hrs in water bath. The residue was filtered and the filtrate was condensed under water bath and further evaporated to dryness to get dark brown colored residue. The final residue was stored in desiccator and used for further analysis.

In Vitro Anti-microbial activity of Acorus calamus root extract against Streptococcus mutants by well diffusion method

20 mL of brain heart infusion agar medium was added to petriplates and seeded with bacterial strains for 24 hr. In this plates wells were made and 20 µL of each plant extracts i.e, aqueous, alcohol and ethyl acetate extracts were added. The plates were then incubated at 37°C for 24 hr. The antibacterial activity of the prepared extracts was determined by measuring the diameter of the inhibition zone formed around the well. Penicillin disc was used as a positive control.

Development and validation of GC method for the estimation of β-asarone in Acorus calamus root extract

Determination of β-asarone in *Acorus calamus* extracts was done by Gas liquid chromatography with flame ionization detector (GC-FID). GC studies were done on SHIMADZU model 2014 (Shimadzu Technologies, Japan) which runs by Shimadzu Lab solution software, coupled with a split/split less injector, operated in a split-mode and FID. In this study Rtx-5 capillary column (cross bond 5% biphenyl/95% dimethyl poly siloxane) which is of 30 meters length and with an internal diameter of 0.25 mm was used.

The optimized GC-FID parameters used in this method is based on boiling point of the β-asarone. β-asarone has a boiling point of about 236˚C. Based on that instruments injection port and detector temperatures were set to 225˚C and 270˚C, respectively. Manual injection of 1µL sample was given at the inlet temperature of 225˚C. After the injection, the oven temperature was rapidly increased from 110˚C to 250˚C at a rate of 20˚C per/min followed by allowing it to standby for 4 min. Nitrogen was used a carrier gas with the flow rate of 1.18 mL/min. Even synthetic air with a flow rate of 100 mL/min and Hydrogen at flow rate of 25 mL/min were also fed to the FID. All the gases used in this experiment were of pharmacopoeia standard purity.

Preparation of standard solution

20 µL of standard β-asarone was pipetted and transferred to 10 mL volumetric flask, it was diluted with methanol up to mark to obtain the main stock solution of concentration of 2000 ng/mL. From this further serial dilutions were made with methanol to get concentration of 100 to 500 ng/mL. From this working stock, 1 µL was injected to gas chromatography for further analysis.

Preparation and analysis of extracts

100 mg of each extract i.e alcoholic, aqueous and ethyl acetate extracts of *Acorus calamus* were weighed separately in 10 mL volumetric flask, dissolved and diluted up to the mark using methanol. From this solution 1 µL was injected in to gas chromatography for further analysis. The concentration of β-Asarone in extracts was calculated by calibration curve method.

RESULTS AND DISCUSSION

Preparation of Acorus calamus Root Extracts

- Practical yield obtained was 4.8 g for 100 g of *Acorus calamus* root for aqueous extract which appears light brown in color.
- Practical yield obtained was 8.8 g for 100 g of *Acorus calamus* root for ethyl acetate extract which appears reddish brown in color.
- Practical yield obtained was 8.2 g for 100 g of *Acorus calamus* root for ethanolic extract which appears dark brown in color.

***In-vitro* anti-microbial activity of *Acorus calamus* root extracts**

Ethanol and ethyl acetate extracts of *Acorus calamus* root were examined for antimicrobial properties. The extracts were tested from initial concentration of 100 mg/mL to final concentration of 1000 mg/mL showed good *in-vitro* antimicrobial activities against the clinical isolates of *Streptococcus Mutants*. The results of agar well diffusion method showed that the growths of the organisms were inhibited by only alcoholic and ethyl acetate extract (Figure 2A and 2B) and there were no zone of inhibition in aqueous extract. However, slight inhibitory effect was observed (1.5 mm) in ethanoic extract in compared with standard penicillin disk zone (Table 1). No growth was observed with aqueous extract and shows ineffective against *Streptococcus mutans*.

Method validation[21-23]

The method was validated for different parameters like linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ), robustness**.**

Linearity

Linearity (Figure 3) for the above method was carried out by preparing standard solutions at different concentrations (Table 2). Linearity was constructed between the peak area responses versus concentration for β-asarone at five different concentration levels i.e, 100-500 ng/mL

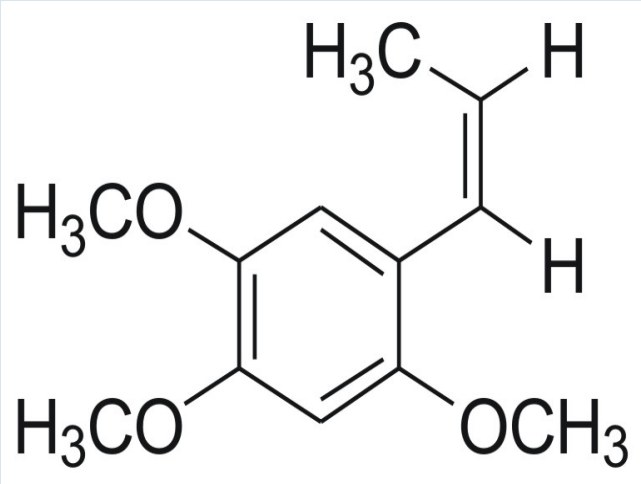


Figure 1: Chemical structure of *β-asarone.*

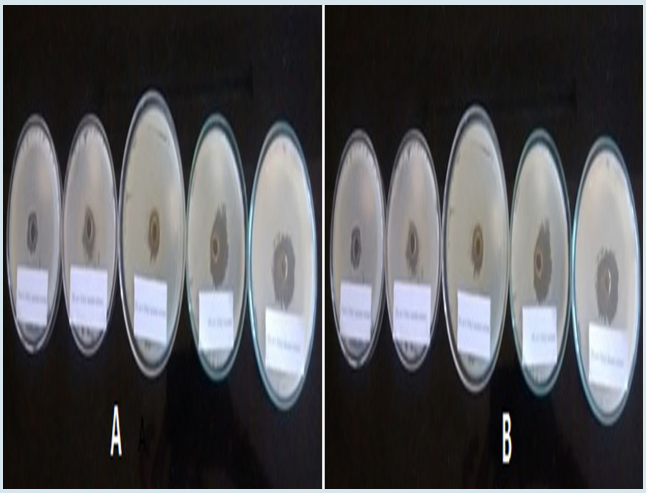


Figure 2A: Antimicrobial activity of ethanolic extract. **2B:** Antimicrobial activity of ethyl acetate extract.

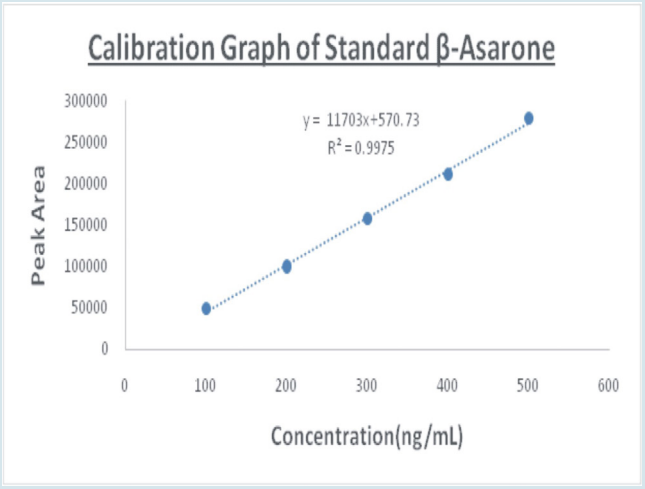


Figure 3: Graph Showing linearity of β-asarone.

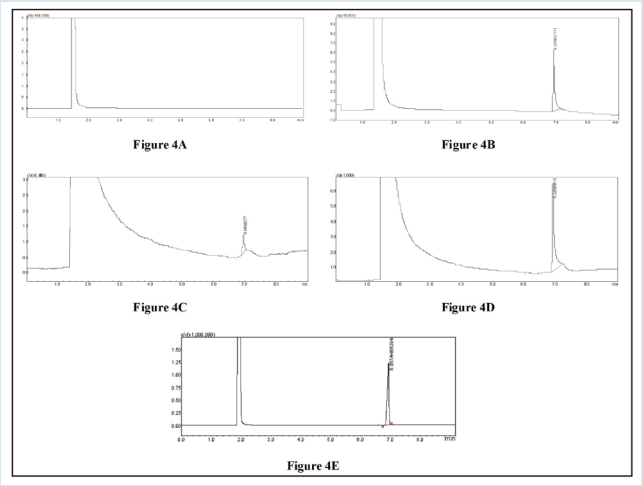


Figure 4: GC Chromatogram of β- Asarone **4a)** Blank chromatogram (Methanol Rt at 2 min) **4b)** GC Chromatogram of β- Asarone (standard-500 ng) Rt 6.9 min. **4c)** Chromatogram of β- Asarone in aqueous extract. **4d)** GC Chromatogram of β- Asarone in Ethanoic extract. **4e)** GC Chromatogram of β-Asarone in Ethyl acetate extract.

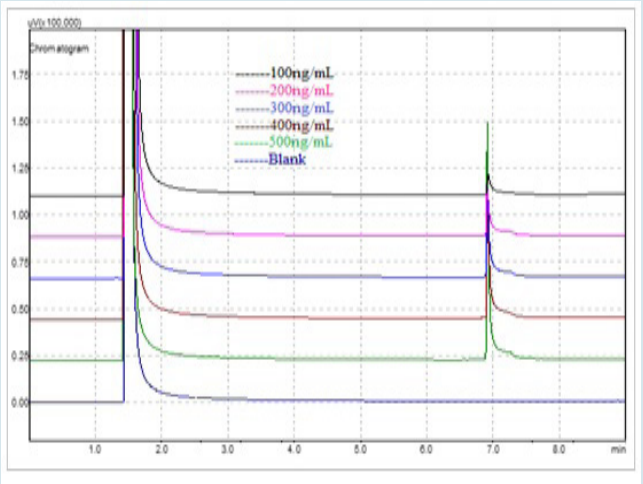


Figure 5: Overlay of β-asarone for linearity range.

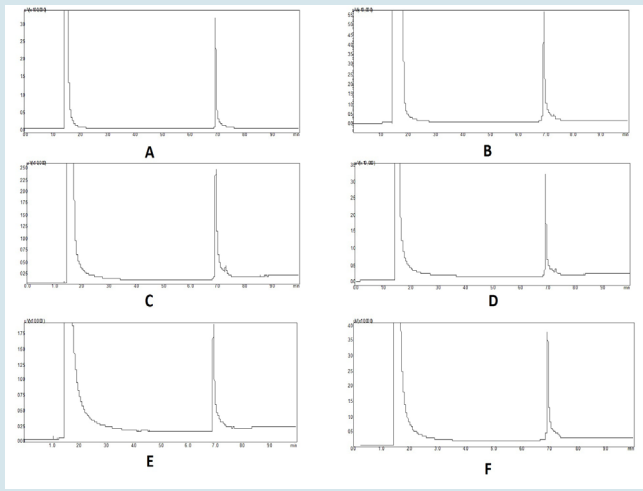


Figure 6: GC Chromatogram of force degradation. **6A)** Chromatogram of unstressed β-asarone at 500 ng. **6B)** Chromatogram of 0.1N NaOH Stressed Sample. **6C)** Chromatogram of 0.1N HCl Stressed Sample. **6D)** Chromatogram of Peroxide Stressed Sample. **6E)** Chromatogram of UV-Light Exposed Sample. **6F)** Chromatogram of Thermal Stressed Sample.

Table 1: Results of anti-microbial activity

Concentration (µg/mL)	Zone of inhibition			
	Ethanolic extract	Ethyl acetate extract	Aqueous extract	Pencillin disc-Control
100	0.3 mm	0.5 mm		
200	0.5 mm	0.7 mm		
300	0.6 mm	0.8 mm	0.0 mm	50 mm
400	0.9 mm	1.4 mm		
500	1.2 mm	1.5 mm		
Media used:			Brain heart infusion agar	
Incubation period:			48 hrs	

Table 2: Linearity results of β-asarone by Gas chromatography

Sl No	Concentration of β-asarone (ng/mL)	Peak area	Theoretical Plates
01	100	49535	098451
02	200	99884	121361
03	300	157571	177044
04	400	211489	214841
05	500	279095	264589
Regression Equation		Y=11703x+570.73	

Table 3: System suitability parameters of the proposed analytical method of β-asarone

Parameters		β-asarone
Linearity range (ng/mL)		100-500
Regression equation		Y=11703x+570.73
Slope		11703
Intercept		570.73
Correlation coefficient (R^2)		0.997
Retention Time (Rt) min		6.92
LOD (ng/mL)		22.78
LOQ (ng/mL)		69.05
Tailing factor		1.315
Theoretical plates		98451
Precesion	Intraday % RSD	0.996
	Interday % RSD	0.997

(Figure 4 and 5). The linearity of the method was evaluated by its correlation coefficient. The calibration equation from three replicate experiments, y = 11703x+ 570.73 (r^2 = 0.99), demonstrated the linearity of the method.

Precision

Precision for the optimized analytical method was carried out at three concentration levels (100 ng, 300 ng and 500 ng) for each analyte on both intra-day and inter-day. %RSD values were calculated on both intraday and interday results are summarized in the Table 3.

Accuracy

For the optimized analytical methods sample solution in triplicates was spiked along with the test solutions of β-asarone at 60%, 100% and 140% of the specification were prepared separately and injected into GC system according to the test procedure. The 'amount of drug added', 'amount of drug found' and average % recovery for β-asarone spiked levels were

Table 4: Recovery values of β-Asarone

Level of % recovery	Amount of Standard β-Asarone added (ng/mL)	Amount of Extract added (ng/mL)	Total amount of β-Asarone (ng/mL)	Total amount of drug found	% Recovery	Average recovery in %	%RSD
				156.95	98.09		
60	60	100	160	162.03	101.26	100.64	1.006
				164.34	102.71		
				195.94	97.97		
100	100	100	200	198.84	99.42	99.63	0.996
				203.02	101.51		
				233.90	97.45		
140	140	100	240	239.91	99.96	100.25	1.00
				248.06	103.35		

Table 5: Results of Robustness

Condition		Taling factors	Theorectical plates	%RSD
As such condition in optimized method		1.315	098451	0.89
Column temperature	Increased (+5^{0}C)	1.45	102152	0.62
	Decreased (-5^{0}C)	1.38	096584	1.62

calculated and the results are summarized in the Table 4.

Robustness

A minor, but deliberate variation were made in the optimized analytical method to check for the efficiency of the developed analytical method to remain unaffected. The characteristic variations studied under this parameter is change in column temperature and the results are shown in Table 5 respectively.

Forced Degradation Studies of B-Asarone

Forced degradation studies was performed on β-asarone drug under stress conditions like hydrolytic stress (acid and alkali), oxidative stress (peroxide), UV and thermal stress conditions to demonstrate that the method is having stability indicating characteristics like specificity, peak purity etc. It is carried out under the following stress conditions and all stressed samples were analyzed by optimized GC method (Figure 6B-F). Recovery results of stressed samples showed that all the samples passes forced degradation test. For all these stability study, the formation of degradable product was confirmed by comparing with the chromatogram of the solution kept under normal unstressed conditions (Figure 6A).

ACKNOWLEDGEMENT

Authors are thankful to The Principal, JSS College of Pharmacy, JSS University, Sri Shivarathreeshwara nagara, Mysuru for providing research facilities.

CONFLICT OF INTEREST

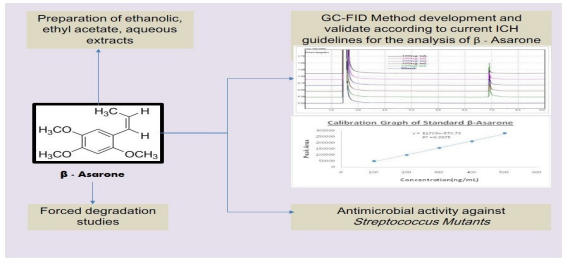
The authors of the article declare no conflict of interest.

REFERENCES

1. Lee MH, Chen YY, Wang SC, Watanobe T, Tsai YC. Inhibitory effect of β-asarone, a component of *Acorus calamus* essential oil, on inhibition of adipogenesis in 3T3-L cells.Food Chem.2011;126(1):1-7. http://dx.doi.org/10.1016/j.foodchem.2010.08.052; http://dx.doi.org/10.1016/j.foodchem.2010.08.052.
2. Wu HS, Zhu DF, Zhou C. Insulin sensitizing activity of ethyl acetate fraction of *Acorus calamus* L. in vitro and in vivo. J of Ethnopharmacol. 2009;123(2):288-92. http://dx.doi.org/10.1016/j.jep.2009.03.004; PMid:19429374 http://dx.doi.org/10.1016/j.jep.2009.03.004.
3. Sharma PV.Caraka-samhita: Text with English Translation, 1st ed. Chaukhambha Orientalia, India. 2000.
4. Gilani AU, Shah AJ, Ahmad M, Shaheen, F. Antispasmodic effect of *Acorus calamus* is mediated through calcium channel blockade. Phytotherapy Research. 2006;20:1080-84. http://dx.doi.org/10.1002/ptr.2000; PMid:17009206.
5. Nadkarni, KM. Indian MateriaMedica, 1st ed. Popular Prakashan, India.1998.
6. Vaidyaratnam, PS.Varier's Indian Medicinal Plants, 1st ed. Oriental Longman Ltd., India.1994
7. Kirtikar KR,Basu BD. Indian Medicinal Plants, 4th ed. Mahendra Pal Sing Publishers, India.1987.
8. Balakumbahan R, Rajamani K, Kumanan. K. *Acorus calamus*: An overview. J Med Plants Res. 2010;4(25):2740.
9. Asha DS, Deepak G. Antimicrobial activity of *Acorus calamus* (L.) rhizome and leaf extract. Acta Biol. Szegediensis. 2009;53(1):45-9.
10. JadhavT. SushrutSamhita, 1st ed. ChaukhambhaSurbharatiAcadamy, India. 1994.
11. Shah NC. Bharat BhaishajyaRatnakar. B. Jain Publishers Pvt. Ltd., India.2005.
12. Malik AS, Kurniawan A, Najib A. Comparative Study of HPTLC fingerprint of β-Asarone content between leaves and rhizome of *Acorus calamus* L. Int J PharmTech Res. 2014;6(2):829-33.
13. Mythili MN, Immanuel SC, Rajasekharan PE, Rajasekaran C, Tharachand C, Rao VK, *et al.* HPLC profiling of B-Asarone content and cytogenetic studies of medicinally important Indian *Acorus calamus* l. Accessions. Int J Pharm Sci Rev Res. 2013;22:73-8
14. Xuemeng, Xinfeng, Shixiang W, Jia, Yajun B,Sha L, *et al.* Simultaneous determination of volatile constituents from *Acorus tatarinowii* schott in rat plasma by gas chromatography-mass spectrometry with selective ion monitoring and application in pharmacokinetic study. Journal of Analytical Methods in Chemistry. 2013;2(1):78-82

15. Nair S, Menon S, Shailajan S. A rapid HPLC-ESI-MS/MS method for determination of β-asarone, a potential anti-epileptic agent, in plasma after oral administration of *Acorus calamus* extract to rats. Biomed Chrom. 2012;27:318-26
16. Wang HZ,Chag YG, Fan CS.Review of studies on chemical constituents and pharmacology of genus Acorus. Acta. BotYunnanica. 1998;5:96-100.
17. Raina VK, Srivastava SK, Syamasunder KV. Essential oil composition of *Acorus calamus* L. from then lower region of the Himalayas. FlavourFragr J. 2003;18: 18-20. http://dx.doi.org/10.1002/ffj.1136.
18. Nigam MC, Ahmad A, Misra LN. GC-MS examination of the essential oil of *Acorus calamus*. Indian Perfum. 1990;34:282-85.
19. Mukherjee PK. Quality Control of Herbal Drugs–An Approach to Evaluation of botanicals. New Delhi, India, Business Horizons, 2002;692-4.
20. Gyawali R. Kim K. Volatile organic compounds of medicinal values from Nepalese *Acorus calamus* L. Kathmandu University Journal of Science, Engineering and Technology. 2009;5:51-65.
21. ICH, Q2 (R1), Validation of analytical procedures: Text and Methodology, 1995.
22. ICH, Q2A, Text on validation of analytical procedures, 1995.
23. ICH, Q2B, Validation of analytical procedures: Methodology, 1996.

PICTORIAL ABSTRACT



SUMMARY

- Ethanolic, ethyl acetate, aqueous extracts from the rhizomes of *Acorus calamus* was prepared
- Simple, sensitive, precise and stability gas chromatographic method for the analysis of β-*asarone* in ethanolic, ethyl acetate, aqueous extracts was developed and validated according to current ICH guidelines.
- The prepared extracts were evaluated for its antimicrobial activity against *Streptococcus Mutants* by agar well diffusion method

ABOUT AUTHORS

Kavyashree Banda Vijayakumar: Is a post graduate student in the Department of Pharmaceutical chemistry at the JSS college of Pharmacy, JSS University, Mysuru. Her research area of interests are analytical method development and validation for synthetic and herbal extracts.

Gurupadayya Bannimath: Currently working as a Professor in the Department of Pharmaceutical chemistry at the JSS College of Pharmacy, JSS University, Mysuru. He has obtained his Ph.D. Degree in 1996 from Kuvempu University, Shankaraghatta, Shimoga. His main research interests are in the areas of drug/herb interaction, chiral and herbal compound analysis.

Venkata Sairam Koganti: Is a Research scholar at JSS University; JSS College of Pharmacy Mysuru, Where he graduated in Bachelor and Master of Pharmacy. His main research interests are in the areas of Analytical and Bio-analytical method developments for the pharmaceutical drugs and its validation. He has published widely in international journals and conferences.

Vishwanathan Balasubramanya Iyer: Working as Assistant professor for Pharmaceutical Chemistry department at JSS University; JSS College of Pharmacy Mysuru. He has has obtained his Ph.D. Degree from JSS University. His research area of interests are Drug design, Docking, Synthesis and Pharmacological evaluation of newly synthesized drugs.