

www.jmscr.igmpublication.org

Impact Factor 3.79

ISSN (e)-2347-176x



Journal Of Medical Science And Clinical Research

An Official Publication Of IGM Publication

Isolation of Principle Active Compound of *Acorus Calamus*. In Vivo Assessment of Pharmacological Activity in the Treatment of Neurobiological Disorder (Stress)

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ABSTRACT

Acorus calamus (Araceae) is a plant widely used as food and medicine worldwide but isolation of principle active compound from hydroalcoholic extract and antistress activity compound is not reported. Dried and powder rhizome of Acorus calamus (Araceae) was used to isolate the chemical constituent using phase column chromatography and TLC purification. Structure was elucidated by spectroscopic methods. Elevated plus maze (EPM) model and forced swimming test (FST) were performed to assess antistress activity on albino mice. (Doses of the isolated chemical constituents: 25, 50 and 100 mg/kg orally) The crude hydroalcoholic extract of Acorus calamus (Araceae) was successively partitioned with n-hexane-EtOAc. The structure of the compound was determined by spectroscopic analysis. The results of elevated plus maze (EPM) model and forced swimming test (FST) revealed that isolated compound having antistress activity. This is the first report of antistress activity of β -Asarone and isolation by column chromatography separation, and TLC purification. The result also revealed that β -Asarone is a potent compound for the treatment of neurobiological disorder (stress). This compound may account for the use of Acorus calamus in folk medicine to treat the stress.

Keywords: Spectroscopic authentication, Elevated plus maze, Forced swimming test, Anti- stress therapeutics, Neurobiological disorder

INTRODUCTION

Empirical use of medicine derived from plants has been widely disseminated since ancient times to treat a wide range of diseases. In the last decades, the interest in alternative therapies has raised markedly in a worldwide shape [1]. In addition, medicinal plants playing significant role in the search of novel pharmacotherapy to treat psychiatric illnesses [2].

Acorus calamus is (Araceae) a popular traditional medicinal plant. The rhizomes were utilized extensively by the Chinese, Indians and American Indians as well as by other cultures, and many of these uses continue to this day including in Thai traditional medicine [3]. In Ayurvedic medicine, it is used for the treatment of insomnia, melancholia, neurosis, epilepsy, hysteria, loss of memory and remittent fevers. Ethanolic extract of rhizome of this plant having sedative, analgesic, moderately hypotensive and respiratory depressant properties [4]. β -Asarone (cis-2,4,5-trimethoxy-1-propenylbenzene) is a major active and principle compound [5]. Rhizome of *Acorus calamus* shows antidepressant activity [6]; sedative and hypothermic effects [7]. *Acorus* rhizome and its constituents, particularly α - and β -Asarone, possess a wide range of pharmacological activities such as sedative and behavior modifying along with CNS activity [8].

Stress is a common phenomenon that is experienced by every individual. When stress becomes extreme, it is harmful for the body and hence needs to be treated. Stress is involved in the pathogenesis of a variety of diseases including

hypertension, peptic ulcer, immunodepression, reproductive dysfunction and behavior disorder [9].

Drugs having antistress properties induce a state of non-specific resistance against stressful conditions. Drugs like benzodiazepines, certain CNS stimulants such as amphetamines and caffeine as well as some anabolic steroids are routinely used by people to combat stress. The incidence of toxicity and dependence has limited the therapeutic usefulness of these drugs [10]. The first drugs used to treat pathologic condition of the CNS were based on natural resources [11]. People from different area of world using herbal medicine to alleviate affective disorders [12]. In Mexico, several medicinal plants are used to alleviate insomnia, depressed mood and anxiety [13]. Various plants are being used in complementary and alternative medicines for management of stress [14]. The potential utility of safer and cheaper herbal medicines as antistress agents have been reported as they can withstand stress without altering the physiological functions of the body. Herbal medicines are known to act synergistically in combination [10].

The Present study was undertaken to isolate β -Asarone by column chromatography separation and TLC purification of fractions from hydroalcoholic extract and assess whether the isolated compound from rhizome of *Acorus calamus* has potential activity (antistress) on the central nervous system (CNS). Antistress activity was assessed by elevated plus maze test and forced swimming test.

MATERIALS AND METHODS

Collection and Authentication of Plant

material: The rhizomes of *Acorus calamus* was collected from the High Altitude Plant Physiology Research Centre (HAPPRC), Srinagar, Uttarakhand, India in the month of March 2011 and deposited in National Botanical Research Institute, Lucknow, India for taxonomical authentication. The rhizomes were air dried for 20 days and crushed into coarse powder with a grinder and passed through 40-mesh sieve. They were stored in a well closed container separately.

Animals: Swiss albino mice (20-25 g) were bought from the Animal House of Siddhartha Institute of pharmacy, Dehradun, Uttarakhand, India. The animal room was maintained on a 12-h light and dark cycle with a constant temperature and humidity. Standard pellet food and tap water were available ad libitum. All animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the Department of Pharmacology of the Siddhartha Institute of Pharmacy, Dehradun, Uttarakhand, India. The experiments were conducted in a sound proof laboratory. All the experimental procedures and protocols (IAEC / CPCSEA Proposal number 03) used in the study were reviewed by the Institutional Animal Ethics Committee.

Extraction and isolation of compound from

rhizomes of *Acorus calamus*: The air dried and powder rhizome (2 kg) of *Acorus calamus* was extracted with ethanol: water (60:40, v/v) under reflux at room temperature. After exhaustive

extraction the combined extracts were concentrated under reduce pressure [20]. A known quantity of the crude hydroalcoholic extract (919 g) was dispersed in distilled water (100ml). Further, this dispersion was transferred to separating funnel and extracted three times with 20 ml of n-hexane [21]. The mixture was shaken vigorously for 5 minutes, allowing the air to escape out. It was kept for about 30 min to separate out two layers. The upper layer of n-hexane was collected. Each fraction was evaporated to dryness under reduced pressure. The yield of n-hexane extract was found to be 296.2 g. The remaining aqueous layer was discarded. The n-hexane fraction was subjected to silica gel column 60-G (500 g Merck) [20] and successively eluted with a stepwise gradient of n-hexane-EtOAc (100/0, 95/5, 90/10, 75/25, 50/50 and 0/100, v/v). Column fractions were analyzed by TLC on silica gel 60-G (0.2 mm thick) [20] and fractions with a similar TLC pattern were pooled and concentrated. The active 90/10 elutes which shows three compounds in TLC (Rf: 0.56, 0.63, 0.73) was successively rechromatographed on a silica gel column with using solvents (Toluene: ethyl acetate: chloroform / 6:2:2, v/v/v). Finally obtained fractions was (18-20) having single compound confirmed by TLC (n-hexane: toluene: ethyl acetate / 1:1:0.1, v/v/v).

Spectroscopic authentication: Spectral analysis FTIR, ¹H NMR and Mass of isolated compound was performed at Sophisticated Analytical Instrument Facility, Central Drug Research institute, Lucknow, India to authenticate the

functional group, molecular weight and molecular formula. FTIR spectra were recorded on Perkin Elmer Spectrum RX1 using alcohol. ^1H NMR (400 MHz) spectra were recorded on Bruker Advance 400 in CDCl_3 with tetramethylsilane as internal standard. The FAB mass spectra were recorded on a Jeol SX102/Da-600 mass spectrophotometer/Data System using Argon/Xenon 6 kv, 10 mA0 as the FAB gas. The accelerating voltage was 10 kV.

Experimental groups: The experimental groups of mice were divided in to five groups. Group I was control and was given normal saline in a dose of 10 ml/kg, p.o. Group II was a positive control treated with standard drug, Diazepam (2 mg/kg, i.p.), suspended in the vehicle. Group III-V was treated as test groups and was given isolated β -Asarone at different dose. All the test solutions, standard drug and control were administered orally 30 minutes prior to experiment.

ASSESSMENT OF ANTISTRESS ACTIVITY:

Elevated plus maze test: Antistress activity was evaluated using the elevated plus maze model. The elevated plus maze consisted of two open arms (50 cm x 10 cm) crossed with two closed arms (50 cm x 10 cm x 40 cm). The arms were connected together with a central square (10 cm x 10 cm). The apparatus was elevated to the height of 70 cm in a dimly illuminated room. Mice were divided into groups of five, and received the compounds β -Asarone at different doses viz .25, 50 and 100 mg/kg, saline (10 ml/kg, p.o.) as control and diazepam (2 mg/kg i.p.) was used as

standard drug. One hour post administration, each mouse was placed individually at the center of the elevated maze. The time duration of the stay in open arm was noted [12].

Forced swimming test: The FST is the most widely used pharmacological *in vivo* model for assessing antistress activity. The swimming test includes two exposures to a water tank, spaced 1 day apart. For these experiments, the tank sizes were 22 cm in diameter and 40 cm in height. The tank had a rounded lid and contained 20 cm, high fresh water at 25 °C. Mice were divided into groups of five, and received the compounds β -Asarone at different doses viz .25, 50 and 100 mg/kg, saline (10 ml/kg, p.o.) as control and diazepam (2 mg/kg i.p.) was used as standard drug. During the first exposure, mice not yet treated were placed in the tank and left there for 15 min. During the second exposure (test session), 30 min after the treatment, mice were placed in the tank and left there for 5 min during which their immobility time was observed. A mouse was considered immobile when it remained floating in the water, without struggling, making only very slight movements necessary to keep its head above the water [12].

Statistical analysis: All data are expressed as the mean \pm S.E.M and were obtained from four distinct experiments. Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Dunnett's test. The significant difference was set at $p < 0.05$.

RESULTS AND DISCUSSION

Extraction and isolation: The compound was extracted with ethanol: water (60:40) at room temperature. The quantity of extract obtained after concentration under reduce pressure was approximately 983 g (49.1 % based on the dry weight of whole plant). The physical appearance of concentrated extract was dark green syrupy.

The n-hexane fraction of dark green syrup was subjected to column chromatography. Single compound was isolated from fraction (18-20) confirmed by TLC. The TLC examination confirmed that it was a single compound (Fig. 1). The R_f value of isolated compound was found to be 0.63 [15].

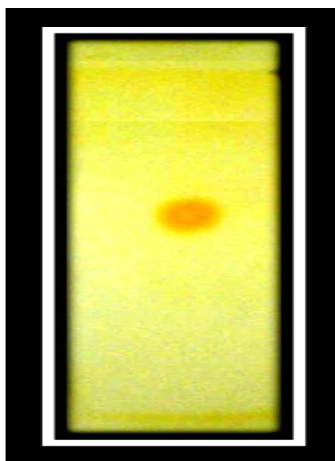


Figure 1. Isolated Compound From *Acorus Calamus* Rhizomes

Structure elucidation of isolated compound from rhizomes of *Acorus calamus*: The FTIR spectra of isolated compound showed sharp peaks at 2965.2 cm⁻¹ and 2926.2 cm⁻¹ indicating C-H stretching of alkanes. Medium and weak peaks were observed at 1608 cm⁻¹ and 1450 cm⁻¹

indicating C=C stretching of aromatic ring. Strong peak of ether (C-O stretching) was observed at 1050- 1300 cm⁻¹[16]. Sharp peak of tetra substituted benzene ring was observed at 802 cm⁻¹ (Fig. 2).

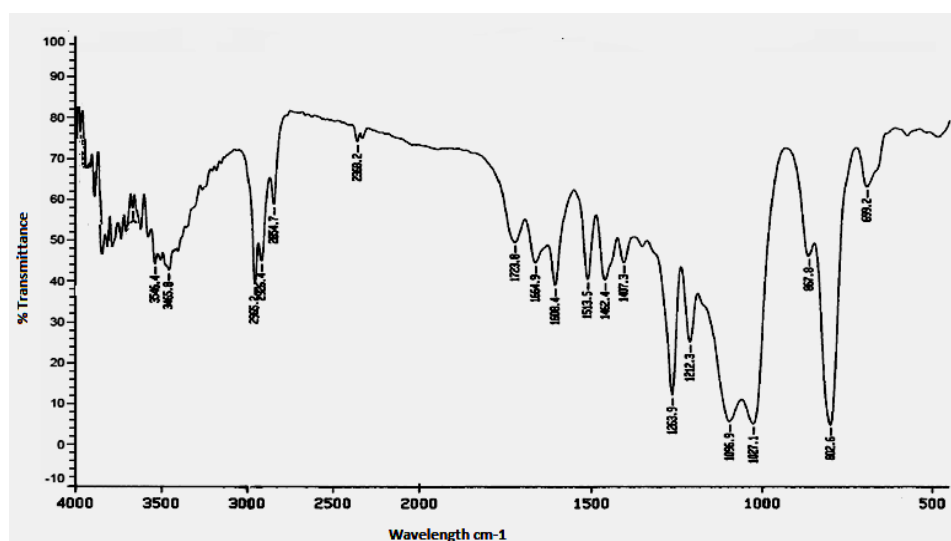


Figure 2. Ftir Spectra Of B-Asarone

^1H NMR spectra confirmed two singlet aromatic protons (δ 6.85 and 6.63), two olefinic protons (δ 6.50 and 5.61), three methoxyl protons (δ 3.83, 3.80, and 3.77), and methyl protons (δ 1.87) indicating sixteen protons signals (Fig. 3) [17].

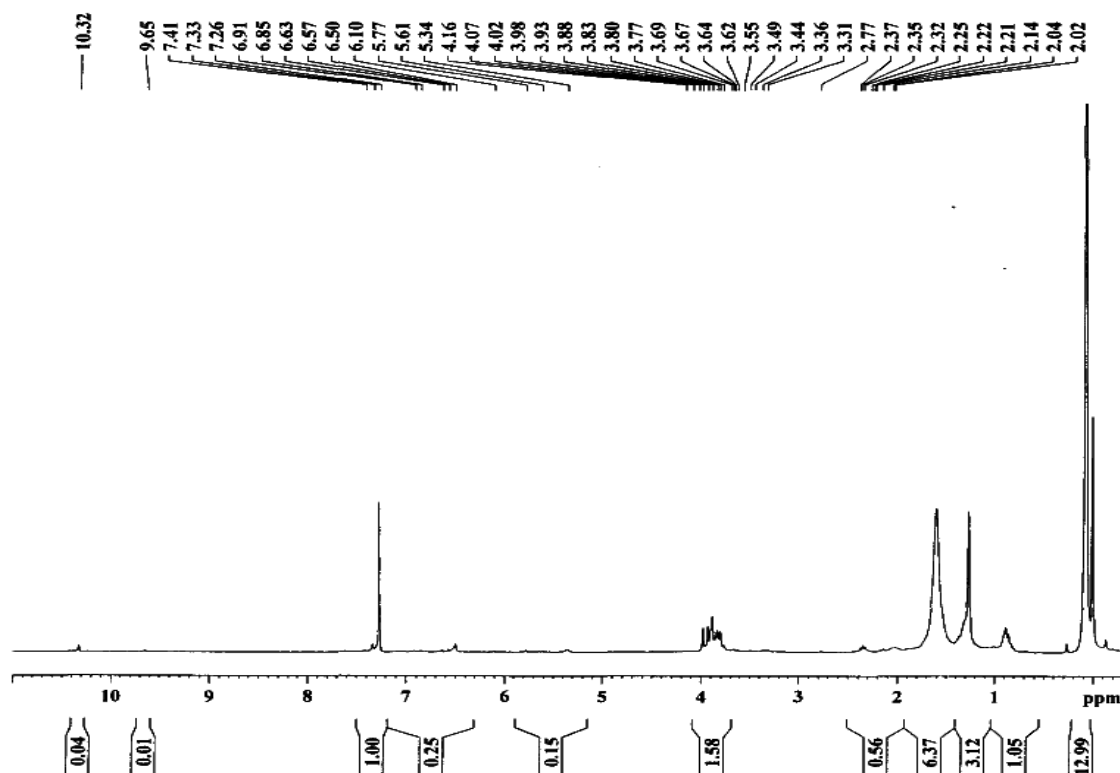


Figure 3. ^1H -Nmr Spectra Of B- Asarone

Mass spectra confirmed a molecular peak at m/z 208 (M^+). Fragment peaks were also observed at 193 ($\text{M}^+ - \text{CH}_3$), 165 ($\text{M}^+ - \text{C}_3\text{H}_7$) and 105 ($\text{M}^+ - \text{C}_6\text{H}_5 - \text{CO}$) [18] (Fig. 4).

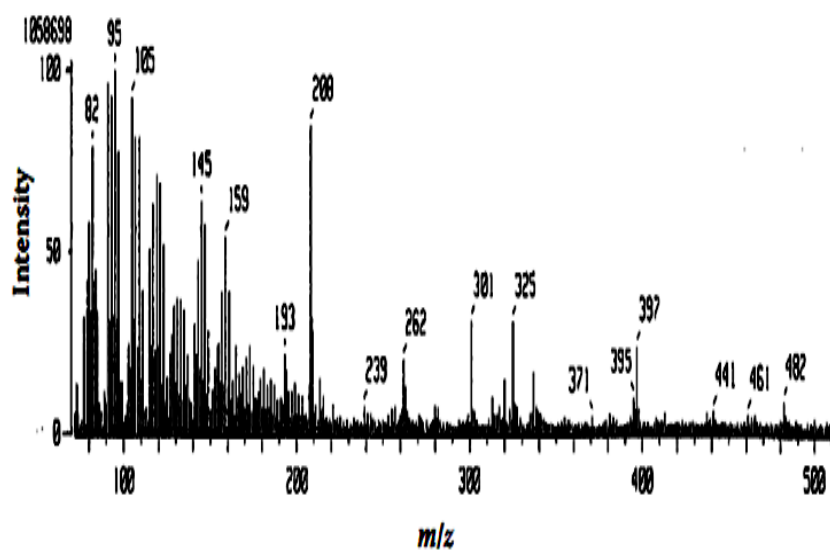


Figure 4. Mass Spectra Of B-Asarone

From the spectral data the structure of the isolated compound was elucidated as β -Asarone with molecular formula ($C_{12}H_{16}O_3$) (Fig. 5).

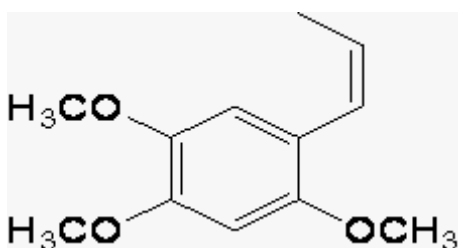


Figure 5. Chemical Structure Of B -Asarone

Elevated plus maze test: Mice received the standard diazepam showed significant ($p < 0.05$) time spent 311.5 ± 5.36 sec in open arm at dose of 2 mg/kg i.p. (Fig. 6). Time spent by mice in open arm treated with β -Asarone was 127.2 ± 3.82 ,

149.5 ± 2.52 and 178.5 ± 2.66 second at the dose of 25, 50 and 100 mg/kg respectively. While the time spent in open arm by control group was 78.5 ± 2.96 second (Table 1).

Table 1. Effect Of Isolated Compound B-Asarone In Elevated Plus Maze Model (Open Arm)

Time spent (s)	Control	Standard Diazepam (mg/kg)	β -Asarone (mg/kg)		
		2	25	50	100
Open arm	Open arm	Open arm	Open arm	Open arm	Open arm
	78.5 ± 2.96	$311.5 \pm 5.36^*$	127.2 ± 3.82	149.5 ± 2.52	$178.5 \pm 2.66^*$

Note: $n=6$, the observation are mean \pm S.E.M and data were analyzed using graph prism pad as statistical unit.

* $p < 0.05$ (ANOVA followed by Dunnett's test)

In the closed arm entry (Fig. 7) the time spent by mice treated with β -Asarone was ($p < 0.05$) 562.8 ± 6.81 , 546.8 ± 4.54 and 532.8 ± 8.49 second at

the dose of 25, 50 and 100 mg/kg respectively. In other hand time spent by control group was 613.2 ± 5.91 second while time spent by standard group was 512.7 ± 2.88 second (Table 2).

Table 2. Effect Of Isolated Compound B-Asarone In Elevated Plus Maze Model (Closed Arm)

Time spent (s)				
Control	Standard Diazepam (mg/kg)	β-Asarone (mg/kg)		
	2	25	50	100
Close arm	Close arm	Close arm	Close arm	Close arm
613.2 ± 5.91	512.7 ± 2.88*	562.8 ± 6.81*	546.8 ± 4.54*	532.8 ± 8.49*

Note: n=6, the observation are mean ± S.E.M and data were analyzed using graph prism pad as statistical unit.

* $p < 0.05$ (ANOVA followed by Dunnett's test)

Forced Swimming Test: The control animals remained immobile for most of the time during the test session and immobility time of control group was 184.1 ± 0.22 second. Immobility time (Fig. 8) of mice treated with β-Asarone was ($p < 0.05$) 195.3 ± 0.31 , 279.5 ± 0.29 and 289.7 ± 0.37

second at the dose of 25, 50 and 100 mg/kg respectively. While mice treated with standard diazepam was 520.8 ± 0.21 second at the dose of 2 mg/kg (Table 3).

Table 3. Effect Of Isolated Compound B-Asarone In Forced Swimming Test

Immobility Time (s)				
Control	Standard Diazepam (mg/kg)	β-Asarone (mg/kg)		
	2	25	50	100
184.1 ± 0.22	520.8 ± 0.2*	195.3 ± 0.31	279.5 ± 0.29	289.7 ± 0.37*

Note: n=6, the observation are mean ± S.E.M and data were analyzed using graph prism pad as statistical unit.

In the EPM (Fig. 7, 8), besides decreased open arm exploration, β -Asarone exhibited significant ($p < 0.05$) decreased closed arm entries and increased time spent in the open arm of Swiss albino mice. This suggests the highest anti stress effect was exhibited by β -Asarone at a dose of 100 mg/kg. Moreover the compound showing dose dependent activity. All these results suggested that the extract is having anti stress activity.

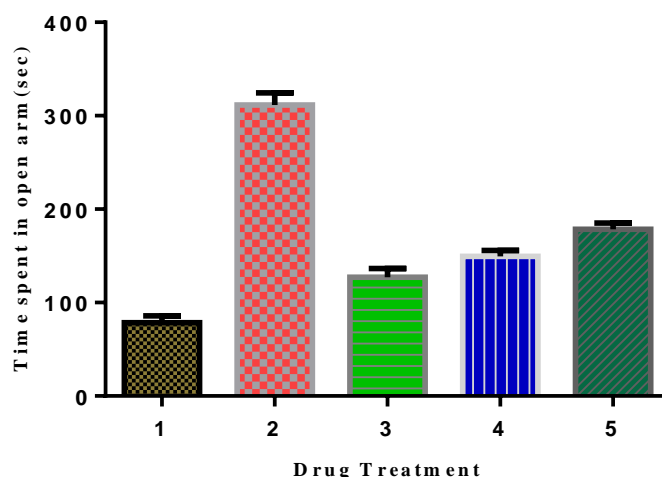


Figure 6. Time Spent (Sec) In Open Arm Of Elevated Plus Maze Model (Bar 1: Control; Bar 2: Standard 2mg/Kg; Bar 3: B Asarone 25mg/Kg; Bar 4: B Asarone 50 Mg/Kg; Bar 5: B Asarone 100mg/Kg).Value Represent Mean \pm S.E.M, $P < 0.05$, N=6

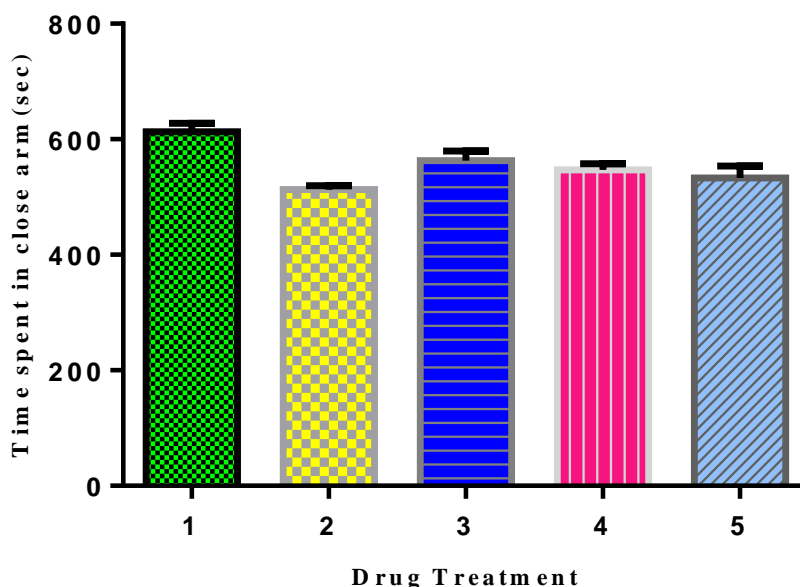


Figure 7. Time Spent (Sec) In Close Arm Of Elevated Plus Maze Model (Bar 1: Control; Bar 2: Standard 2mg/Kg; Bar 3: B Asarone 25mg/Kg; Bar 4: B Asarone 50 Mg/Kg; Bar 5: B Asarone 100mg/Kg).Value Represent Mean \pm S.E.M, $P < 0.05$, N=6

In the swimming endurance test (Fig. 8), the mice were forced to swim in a restricted space from which they cannot escape. This induces a characteristic behavior of immobility. It has been well-demonstrated that drugs with antistress activity increase swimming endurance [19]. Results of the swimming endurance test indicate

clearly that the β -Asarone was the most active constituent that reducing the immobility time significantly ($p < 0.05$) at a dose of 100 mg/kg. In the FST all the doses administered were able to reduce immobility time and simultaneously to enhance swimming.

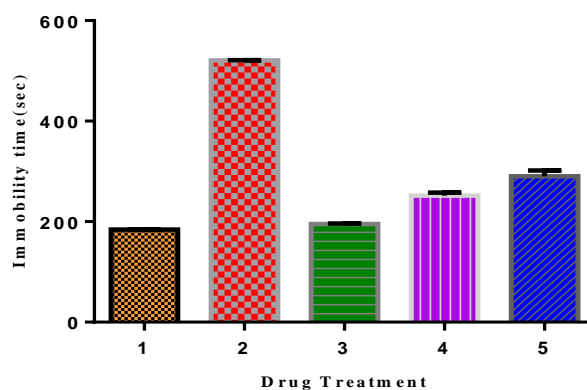


Figure 8. Immobility Time (Sec) In Forced Swimming Test (Bar 1: Control; Bar 2: Standard 2mg/Kg; Bar 3: B Asarone 25mg/Kg; Bar 4: B Asarone 50 Mg/Kg; Bar 5: B Asarone 100mg/Kg). Value Represent Mean \pm S.E.M, $P < 0.05$, N=6

CONCLUSION

The present study investigated the pultative behavioral effects of β -Asarone. In conclusion, although β -Asarone has been used as an anti-stress remedy in folk medicine. β -Asarone was successfully isolated from hydroalcoholic extract of rhizome using column chromatography, TLC purification. Spectral analysis result also revealed that isolated compound is β -Asarone. Results of both model revealed that β -Asarone having higher antistress activity at the dose of 100 mg/kg which discover the antistress activity of β -Asarone. This potent compound from *Acorus calamus* may have potential for future development of anti- stress therapeutics.

REFERENCES:

1. P.S. Dalsenter, J.C. Muller, G.G.K. Botelho., A.C. Bufalo, A.C. Boareto, Y.D. Rattmann, E.S. Martins, D.A. Cabrini, M. Otuki, *In vivo* and *in vitro* reproductive toxicology, *J. Ethnopharmacol.* 121, 2009; 229-233.
2. Z.J. Zhang, Effects of herbal extracts and constituents in animal models of psychiatric disorders, *life Sci.* 75, 2004; 1659–1699.
3. S. Phongpaichit, N. Pujenjob, V. Rukachaisirikul, M. Ongsakul, Crude methanol extract of *Acorus calamus* Linn, *J. Sci. Technol.* 27, 2005; 517-523.

4. V.K. Singh, S. Mehrotra, K.P. Mishra, R. Maurya, R.C. Srimal, V.S. Yadava, R. Pandey, Anticellular and immunosuppressive properties of ethanolic extract of *Acorus calamus* rhizome, Int. Immunol. 3, 2003; 53–61.
5. S. Shenvi, V.R. Hegde, A. Kush, G.C. Reddy, A unique water soluble formulation of β -asarone from sweet flag (*Acorus calamus* L.) and its *in vitro* activity against some fungal plant pathogens, J. Med. Plants Res. 5, 2011; 5132-5137.
6. V.S. Pawar, A. Akhade, S. Baokar, H. Shivkumar, Antidepressant-like effect of *Acorus calamus* in forced swimming and tail suspension test in mice, Asia-Pac. J. Biomed. Trop. 2012; S17-S19.
7. P. Zanolli, R. Avallone, M. Baraldi, Sedative and hypothermic effects induced by β -asarone a main component of *Acorus calamus*, Phytother. Res. 12, 1998; S114–S116.
8. P.K. Mukherjee, V. Kumar, M. Mal, P.J. Houghton, *Acorus calamus*: scientific validation of ayurvedic tradition from natural resources, Pharm. Biol. 45, 2007; 651-666.
9. M. Asad, A.K. Singh, S.S. Dhamanigi, Anti stress activity of hydroalcoholic extract of *Eugenia caryophyllus*(clove) buds, Indian J. Pharmacol. 41, 2009; 28-31.
10. S.K. Desai, S.M. Desai, S. Navdeep, P. Arya, T. Pooja, Antistress activity of *Boerhaavia diffusa* root extract and a polyherbal formulation containing *Boerhaavia diffusa* using cold restraint stress model, Int. J. Pharm. and Pharm. Sci. 3, 2011; 130-132.
11. N.G.M. Gomes, M.G. Campos, J.M.C. Orfao, C.A.F. Ribeiro, Plants with neurobiological activity as potential targets for drug discovery, Prog. Neuro-Psychopharmacol. Boil. Psychi. 33, 2009; 1372-1389.
12. S. Mora, R. Millan, H. Lungenstrass, G. Diaz-Veliz, J.A. Moran, M. Herrera-Ruiz, J. Tortoriello, The hydroalcoholic extract of *Salvia elegans* induces anxiolytic and antidepressant like effects in rats, J. Ethnopharmacol. 106, 2006; 76-81.
13. C. Zolla, Traditional medicine in Latin America with particular reference to Mexico, J. Ethnopharmacol. 2, 1980; 37-41.
14. V. Gupta, P. Bansal, P. Kumar, R. Shri, Anxiolytic and antidepressant activities of different extracts from *Citrus paradisi* var. *Duncan*, Asia-Pac. J. Chem. Eng. 3, 2010; 98-100.
15. A.K. Sinha, Process for the preparation of 1-propyl-2, 4, 5- trimethoxybenzene from toxic beta-asarone of *Acorus calamus* or from crude *calamus* oil containing beta-asarone, Patent application publication,

United State, Pub. No. US 2002/0035299
A1.

16. D.L. Pavia, G.M. Lampman, G.S. Kriz, Introduction to spectroscopy, 3rd edition, Thomson brooks Ltd, United State, 2001, pp. 40-44.
17. B.K. Hwang, J.Y. Lee, B.S. Yun, Antifungal Activity of α -Asarone from Rhizomes of *Acorus gramineus*, J. Agric. Food Chem. 52, 2004; 776-780.
18. R. Oprean, M. Tamas, L. Roman, Comparison of GC-MS and TLC techniques for Asarone isomers determination, J. Pharm. Biomed. Anal. 18, 1998; 227-234.
19. G. Varsha, P. Bharatkumar, P. Nikunj, Antistress activity of *Argyreia speciosa* roots in experimental animals, J. Ayurveda Integr. Med. 3, 2011; 129-136.
20. G. Burton, M.V. Dansey, P.H.D. Chenna, A.S. Veleiro, Z. Kristofikova, H. Chodounska, A. Kasal, Synthesis and GABA_A receptor activity of A-homo analogues of neuroactive steroids, Eur. J. Med. Chem. 45, 2010; 3063-3069.
21. Young-Joon, P. Chan, L. Hee-Known, Insecticidal activities of asarones identified in *Acorus gramineus* rhizome against *Nilaparvata lugens* (Homoptera: Delphacidae) and *Plutella xylostella* (Lepidoptera: Yponomeutoidae), Appl. Entomol. Zool. 37 (2002) 459-464.