



RESEARCH PAPER

ISOLATION OF BETA-ASARONE AND FORSKOLIN AND THEIR IN VIVO ACTIVITY AGAINST STRESS

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Received: Mar 20, 2019 / Revised: Apr 29, 2019 / Accepted: Apr 30, 2019

Stress is known to result in impairment of learning and memory and precipitate several affective disorders including depression and anxiety. Drugs of natural origin are known to possess several effects on the central nervous system and are emerging as promising alternative. Beta-asarone is one of the main bioactive constituents in traditional Chinese medicine *Acorus calamus*. Forskolin, a labdane diterpene is the main active ingredient in the ayurvedic herb *Coleus forskohlii* (Labatiae) that has been used in India since ancient times. The present study was designed to evaluate the antistress activity of the isolated compounds. The compounds were isolated by normal phase column chromatography and the structures were elucidated by spectroscopic methods. Beta-asarone and Forskolin were tested using elevated plus maze (EPM) model and forced swimming test (FST) respectively in Swiss albino mice. Albino mice were treated with different doses of the extracts (50, 100 and 200 mg/kg orally) and behavior was observed on the EPM and FST. Results showed that isolated compounds Beta-asarone and Forskolin at the dose of 100 mg/kg markedly increased the average time spent in the open arms in EPM and the same compounds showed a significant decrease in the time spent immobile by mice in FST. These results provide support for the potential antistress activity.

Key words: Antistress, Beta-asarone, Forskolin, Elevated plus maze model, Forced swimming test.

INTRODUCTION

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 (Singh *et al* 2009), behavioral disorders like anxiety due to involvement of the central nervous system (CNS), endocrine system and metabolic system. 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 (Desai *et al* 2009).

Herbal formulations and medicinal plants have been in use for many years not only in Asian countries but also globally for human well-being as well as reported in literature for their biopotential (Dahiya *et al* 2019; Oloke *et al* 2017; Dahiya *et al* 2017; Senthil Kumar *et al* 2017; Viana *et al* 2017; Dahiya and Singh, 2017a; 2017b; 2017c). The herbal formulations claimed to enhance physical endurance, mental functions

and non-specific resistance of the body and have been termed as adaptogens (Desai *et al* 2009). Various plants are being used in complementary and alternative medicines for management of stress (Gupta *et al* 2010). 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 (Desai *et al* 2009).

Acorus calamus (Family: Araceae) is a popular traditional medicinal plant. In Ayurvedic medicine, it is used for the treatment of insomnia, neurosis, epilepsy, hysteria, loss of memory and many more diseases (Tiwari *et al* 2010). Beta-asarone (*cis*-2,4,5-trimethoxy-1-

propenylbenzene) is a major active principle found in *A. calamus* (Shenvi *et al* 2011).

Coleus forskohlii is an important plant in Indian Ayurvedic medicine. It is the only source for Forskolol among the plant kingdom. Forskolol has a unique property of activating almost all hormone sensitive adenylate cyclase enzymes in a biological system. Forskolol is reported to be useful in the treatment of congestive heart failure, glaucoma, asthma and certain type of cancers. It is an important plant used against various disorders in indigenous systems of medicine such as anti-aging, antioxidant, as a remedy for heart, abdominal and respiratory disorders (Khatun *et al* 2011). The chemical structures of Beta-asarone and Forskolol are presented in **Figure 1**.

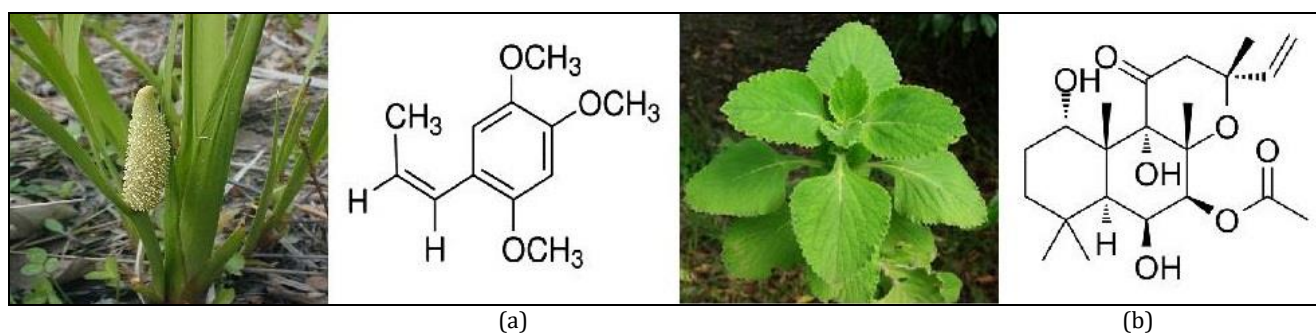


Fig. 1. Structures of (a) Beta-asarone (from *A. calamus*) and (b) Forskolol (from *C. forskohlii*)

MATERIALS AND METHODS

Plant material

The rhizomes of *Acorus calamus* and *Coleus forskohlii* were collected from the High Altitude Plant Physiology Research Centre (HAPPRC), Srinagar, Uttarakhand, India in the month of march 2018 and were taxonomically authenticated by the Dr. Alok Lehri, Scientist E-1, Central Instrumental Facility, National Botanical Research Institute, Lucknow, India. The rhizomes were air dried for 20 days, crushed into coarse powder with a grinder and passed through 40-mesh sieve. They were stored in a well closed container separately.

Isolation of compound 1 from rhizomes of *A. calamus*

The powder rhizomes were dried in the dark at room temperature. Dried and powdered material (950 g) was taken in a round-bottom flask and was first defatted with petroleum ether twice. The marc was then extracted with ethanol:water (60:40) twice. After 48 h, the extract was filtered. The filtrate was evaporated and the dark green crude extract was obtained

with a yield approximately 270 g. The concentrated hydro-alcoholic extract (15 g) was subjected to silica gel column (60-120 mesh, 500 g) and successively eluted with a stepwise gradient of chloroform / methanol (100/0, 95/5, 90/10, 75/25, 50/50, and 0/100, v/v). Column fractions were analyzed by TLC (silica gel G), and fractions with a similar TLC pattern were pooled and concentrated.

The active 90/10 elutes which showed three compounds in TLC (R_f : 0.56, 0.63, 0.73) was successively re-chromatographed on a silica gel column with using solvents (Toluene:ethyl acetate:chloroform / 6:2:2). Finally obtained fractions was (18-20) having single compound AC-1 confirmed by TLC (*n*-hexane: toluene: ethyl acetate / 1:1:0.1). The R_f value of compound AC-1 was found to be 0.63.

Structure elucidation of compound 1

The FTIR spectra showed sharp peaks at 2965.2 cm^{-1} and 2926.2 cm^{-1} indicating C-H stretching of alkanes.(7) 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} . Sharp peak of tetra substituted benzene ring was observed at 802 cm^{-1} . ^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.

Mass spectra (FAB) confirmed a molecular peak at m/z 208 (M^+). Fragment peaks were also observed at 193 ($M^+ - \text{Me}$) and 105 ($M^+ - \text{C}_6\text{H}_5 + \text{CO}$). From the spectral data the structure of the compound AC-1 was elucidated as β -asarone with molecular formula $\text{C}_{12}\text{H}_{16}\text{O}_3$.

Isolation of compound 2

1.5 kg dried rhizomes of *Coleus forskohlii* were defatted with petroleum ether at room temperature. The marc was then extracted with ethanol:water (60:40) twice. After 48 h, the extract was filtered. The solvent was removed in vacuum. The residues (395 g) were dissolved in H_2O . The aqueous solution was extracted with petroleum ether, CHCl_3 and *n*-butanol, the CHCl_3 extract was evaporated to give 85 g of residues. The residues were subjected to CC silica gel, eluted with petroleum ether-acetone (from petroleum ether to petroleum ether-acetone 1:1). The fractions were combined by monitoring with TLC to obtain fractions B 1~B 22.

The fraction B 8 (4 g) was chromatographed repeatedly on silica gel eluted with CHCl_3 -acetone and recrystallized from petroleum ether-acetone to afford 175 mg of compound 2. The R_f value of compound AC-1 was found to be 0.48.

Structure elucidation of compound 2

FTIR spectroscopy analysis were carried out to identify the constituents responsible for the antistress activity. *Coleus forskohlii* alcoholic extract shows absorption peaks located at 1035.71, 1383.73 and 1624.72 in the region 500–4000 cm^{-1} .

The peaks corresponding to presence of fatty acids, carbonyl groups, flavanones and amide band of proteins. The ^1H NMR spectrum of compound 2 displayed a doublet of doublets centered at 3.35 ppm ($J_{9, L1} = 10.00$, $J_{9,8} = 5.00$ Hz) for the H-9 proton, and a doublet of doublets at 3.06 ppm ($J_{5,6} = 10.00$, $J_{5,6,,} = 3.50$ Hz) for the ring junction proton. Forskolol with a mass-to-charge ratio of 411, 393 [$\text{MH}^+ - \text{H}_2\text{O}$], 375 [$\text{MH}^+ - 2\text{H}_2\text{O}$] and 315 [$\text{MH}^+ - 2\text{H}_2\text{O} - \text{AcOH}$].

Test animals

The experimental animals [Swiss albino mice (20-25 g) of either sex] were procured from the Animal House, Siddhartha Institute of Pharmacy, Babhnan, Gonda. The animals were given standard laboratory feed and water ad libitum. The experiments were performed between 8.00 am to 1.00 pm. The experiments were conducted in a sound proof laboratory. All the experimental procedures and protocols used in the study were reviewed by the Institutional Animal Ethics Committee.

Assessment of antistress activity

Elevated plus maze test

Anxiolytic activity was evaluated using the elevated plus maze. The elevated plus maze consisted of two open arms (50×10 cm) crossed with two closed arms (50×10×40 cm). The arms were connected together with a central square (10×10 cm). The apparatus was elevated to the height of 70 cm in a dimly illuminated room. Mice were divided into groups of six and received the compounds C1 and C2, the vehicle or the standard reference drug diazepam (2.0 mg/kg *i.p.*). One hour post-administration, each rat was placed individually at the center of the elevated maze. The duration of the stay in open arm were noted.

Forced swimming test

The FST is the most widely used pharmacological *in vivo* model for assessing anti stress activity (Gupta *et al* 2010). 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. 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.

Treatment

Animals were divided in to eight 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 and was given standard drug, Diazepam

(2mg/kg, *i.p.*), suspended in the vehicle. Group III-V were treated as test groups and were given isolated Beta-asarone and Group VI-VIII were treated as isolated Forskololol at different doses *viz.* 50, 100 and 200 mg/kg respectively. All the test solutions, standard drug and control were administered orally 30 min prior to experiment.

Statistical analysis

The results are given as mean±SEM. The data obtained was analyzed by one-way analysis of variance (ANOVA) followed by Turkey's test. Differences were considered significant at the 5% level.

RESULT

Elevated plus maze test

Based on previous studies that were carried out on active constituents and in order to determine

the effective dose on the EPM, Beta-asarone and Forskololol doses of 50, 100 and 200 mg/kg were tested. Mice exhibited decreased closed arm entries and increased time spent in the closed arm. Mice received the standard diazepam showed a significant ($P < 0.05$) 311.5±5.36 sec increase in the time spent in open arms in the dose of 2mg/kg, *i.p.* when compared to control. C1 and C2 significantly ($P < 0.05$) increased the time spent in the open arms relative to the control at doses of 50, 100 and 200 mg/kg. Result indicates in **Table 1** that the most pronounced effect of C1 and C2 was produced at the dose of 200 mg/kg. In this case, time spent of C1 and C2 in the open arms was 157.8±4.78 sec and 152.5±1.87 sec respectively compared to a value of 78.5±2.96 sec for control. Moreover, both compounds C1 and C2 produce dose dependent activity.

Table 1. Effect of isolated compounds C1 and C2 at Elevated plus maze model

Treatment (dose: mg/kg)	Time spent(s) (mean±SEM)	
	Open arm	Close arm
Vehicle	78.5±2.96	211.2±7.42
Diazepam	311.5±5.36	71.3±2.04
C1(50)	136.3±3.88	164.7±7.35
C1(100)	151.5±3.40	158.5±2.18
C1(200)	157.8±4.78	160.2±5.16
C2(50)	129.8±2.12	171.2±6.31
C2(100)	141.0±1.09	154.7±3.32
C2(200)	152.5±1.87	161.5±3.84

n=6, the observation are mean±SEM * $P < 0.05$, ANOVA followed by Dunnett's test, C1: Beta-asarone, C2: Forskololol

Forced swimming test

Table 2 shows the antistress effect of C1, C2 and diazepam in the experimental animals. The control animals remained immobile for most of the time during the test session. C1 and C2 (50, 100 and 200 mg/kg, *p.o.*) induced a dose-dependent significant reduction in the immobility time of rats ($P < 0.5$) as compared to

the control group. In the same experimental conditions, the antistress activity of the reference drug diazepam (2 mg/kg, *i.p.*) was clearly evident ($P < 0.05$). Antistress effect produced by C1 and C2 (200 mg/kg, *p.o.*) was comparable to that of diazepam. This shows that C1 and C2 has significant antistress activity in a dose of 100 mg/kg.

Table 2. Effect of isolated compounds C1 and C2 at Forced swimming test

Treatment (Dose: mg/kg)	Survival time (s)
vehicle	184.1±0.22
diazepam	520.8±0.21
C1 (50)	195.3±0.31
C1 (100)	279.5±0.29
C1 (200)	289.7±0.37
C2 (50)	184.7±0.19
C2 (100)	194.9±0.48
C2 (200)	198.7±0.60

n=6, the observation are mean±SEM * $P < 0.05$, ANOVA followed by Dunnett's test, C1: Beta-asarone, C2: Forskololol

DISCUSSION

In the search for new therapeutic products for the treatment of neurological disorders (like stress) and other conditions, global medicinal plants research has progressed constantly, demonstrating pharmacological effectiveness of different plant species in a variety of animal models (Shafeen *et al* 2012; Dahiya, 2007; Dahiya and Kaur, 2008). This is an important finding since herbal drugs are thought to be a good alternative or supplement in the therapy of stress disorders. However, it has to be borne in mind that there are not enough controlled double-blind clinical trials (Deng *et al* 2007). The isolated active compound β -asarone has been isolated previously by others from *Acorus calamus* but has not been evaluated antistress activity before (Graw *et al* 2002). Forskolol, a labdane diterpene is the main active ingredient in the ayurvedic herb *Coleus forskohlii* (Labatiae) that has been used in India since ancient times. The root portion of the plant has been traditionally used for medicinal purposes and contains the active constituent, Forskolol (Patel *et al* 2011). Beta-asarone and Forskolol isolated were analyzed and characterized by IR, NMR and MASS spectroscopy.

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