

STUDY OF SHANKHAPUSHPI ON SPONTANEOUS LOCOMOTOR ACTIVITY
AND ANOXIC STRESS IN RODENTS

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Summary

Shankhapushpi is an Indian medicinal plant used in traditional Indian system of medicine for treatment of stress. The objective of present investigation was to evaluate the effect of *Ghana* of two varieties of shankhapushpi on spontaneous locomotor activity and anoxic stress in rodents. *Ghana* of all samples was prepared and labeled as *Ghana* A, B, C, D and E. The acute oral toxicity of *Ghanas* was carried out. The activity of acute and chronic treatment of *Ghanas* was evaluated in animals using two models 1) Spontaneous Motor Activity in rats 2) anoxic stress in mice. The effect of *Ghana* on brain monoamine levels in mouse was estimated by HPLC method.

Ghana C significantly decreased the spontaneous locomotor activity and significantly increased anoxic time. Noradrenaline but not 5-HT and dopamine levels were decreased after 28 days treatment as compared to vehicle control. *Ghana* C and D showed more anti-stress activity than *Ghana* A, B and E. It is concluded that *Ghana* C and D showed antistress effect by reduction in locomotion, increase in anoxic time and reduction in brain noradrenaline level.

Key words: - Anoxic stress, Brain monoamine, *Shankhapushpi*, Spontaneous motor activity.

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Introduction

In the present era of competition, stress has become integral part of human life. Approximately 45% of the cases in various age group visiting physicians are suffering from stress related disorders. In children, stress is manifested in the form of behavioral disorders like hyperactivity and attention seeking disorders, which has relevance in careers and personality of young generation in future. In acute stress, person experiences a physical threat, fear and helplessness, which can be real or imagined in response to emotional conditions. Stress affects several brain functions and leads to long term changes in multiple neural systems [1].

During stressful conditions certain biochemical changes take place for physiological adaptation. Long-term stress can induce a range of disorders like hypertension, coronary heart disease [2], gastric ulcers [3], immunosuppression [4], metabolic disorders like diabetes [5], mental depression, memory loss and host of other diseases [6]. Due to the nonspecific nature of the stress pathogenesis, a drug having central and peripheral activity is needed to combat stressful conditions. Since ancient times, therapeutic approach of Indian traditional system of medicine has involved utilization of substances from natural origin to combat stress. In *Ayurveda*, *Shankhapushpi* is used in the form of *Ghana* for the treatment. In Indian pharmaceutical market, 20 well known herbal preparations are available for the treatment of stress and related disorders. *Shankhapushpi* is one of the ingredients in 17 preparations among them.

In Indian Pharmaceutical market, seven different herbs viz *Convulvulus prostratus*, *Evolvulus alsinoidis*, *Clitoria ternatea*, *Canscora decussate*, *Canscora diffusa*, *Xanthium strumarium* and *Lavendulla bipinna* are used as *Shankhapushpi* [7]. *Convulvulus prostratus* and *Evolvulus alsinoidis* are the most commonly herbs used for the preparation of formulations [8] and proved for neuro-protective [9] and anti-stress activity [10] respectively.

It is reported that climatic conditions and environmental stressors like air, soil, water and temperature can affect the plant. Changes in biochemical and physiological conditions¹¹ may affect the therapeutic activity of the plant. However there is the paucity of data about the comparative pharmacological account of different plants used under name *Shankhapushpi*, collected from various locations for preparation of *Ghana*. The objective of present study was to evaluate and compare the antistress potential of *Ghana* of *Convulvulus prostrates* and *Evolvulus alsinoidis* collected from various locations.

Material and methods

Collection and authentication of plant material

The information regarding the geographical distribution of *Shankhapushpi* was obtained from Botanical Survey of India. Total five samples of *Shankhapushpi* were collected, among which three were directly collected from field. (Varanasi, Delhi and Vadodara) and two purchased were from dealers in Gujarat and Uttar Pradesh. The plant materials were collected during flowering and fruiting stage in the month of April-June 2003. These samples were authenticated from Agharkar Research Institute (ARI), Pune, and voucher specimens were deposited at AHMA, the herbarium deposition facility of ARI. Authenticated samples were assigned codes as follows: Plant A (*Convulvulus prostrates* Forssk. Convulvulaceae Gujarat commercial sample, Voucher No. AHMA WP017), Plant B (*Evolvulus alsinoides* L. Convulvulaceae U. P. commercial sample, Voucher No. AHMA WP018), Plant C

(*Convolvulus prostrates* Forssk. Convolvulaceae Varanasi collected sample, Voucher No. AHMA WP019), Plant D (*Convolvulus prostrates* Forssk. Convolvulaceae Delhi collected sample, Voucher No. AHMA WP020) and Plant E (*Convolvulus prostrates* Forssk. Convolvulaceae Vadodara collected sample, Voucher No. AHMA WP021).

Preparation of *Ghana*

***Ghanas* of all five samples were prepared by following method**

The coarsely powdered air dried plant material (50 g) was boiled in water (800 ml) until total volume reduce to 1/4th (200 ml) of original volume and then filtered through cloth to obtain extract. The extract was dried at 60 °C on water bath to obtain *Ghana*. Percentage yield of *Ghana* of plant A, B, C, D and E was 12.44%, 11.10%, 12.56%, 12.70% and 10.48% respectively. *Ghana* was stored in air tight glass container at 4-8 °C.

Following drugs and chemicals were used in the study

Sodium acetate (Qualigen Fine Chemical Ltd., Mumbai); methanol (Merck (I) Ltd., Mumbai); heptane sulfonic acid (Loba Chem Ltd., Mumbai); Di-butylamine (S.D.Fine Chem Ltd, Mumbai); orthophosphoric acid (Qualigen Fine Chemical Ltd., Mumbai); dopamine Injection (Charles Pharma Ltd., USA) ; 5-Hydroxytryptamine, Isoprenalin, Norepinephrine (Sigma Chemical Co., St. Louis, MO, USA).

Preparation of dosage form

The dosage form was administered to animal by oral route (volume not exceeding 1ml/100 g body weight of animals) in all the experiments. The oral liquid dosage forms of *Ghana* for administration to animals were freshly prepared on the day of experiment. *Ghana* was dissolved in distilled water to prepare solution.

Protocol approval for the Experimental study

The animal experiments were started after obtaining the approval from the Institutional Animal Ethical Committee (IAEC) of Poona College of Pharmacy, Pune, constituted as per the guidelines of "Committee for Purpose of Control and Supervision of Experimental Animals" (CPCSEA), India.

Experimental animals

Wister rats of either sex weighing 100-150g and Swiss male albino mice of 18-22g were purchased from National Toxicology Centre, Pune. Animals were housed in polypropylene cages at temperature 24 °C ± 1 °C and relative humidity of 45-55 % and 12:12 hr dark: light cycle. Animals had free access to food (Standard chaw pellet, Chakan oil mills, Sangli) and water made available *ad libitum*. The animals were quarantined for 7 days before starting the experiments. Food but not water was withdrawn from rats 12 hr before and from mice 3hr before commencement of experiment. The experiments were carried out during 10 a.m.-4 p.m.

Study design

The acute oral toxicity study was performed as per the OECD guideline no. AOT 425. Limit test was performed at dose 5000 mg/kg and main test was performed at the doses 175, 550, 1750 and 5000 mg/kg in mice as per the guideline. Animals were observed after dosing individually at least once during the 30 minutes for 4 hrs, periodically during the first 48 hours and daily thereafter for 14 days for signs of toxicity and mortality, if any.

Spontaneous locomotor activity in rats

Single day treatment of Ghana

Treatment: Wister rats were divided in 11 groups, six animals in each group. Group 1, received vehicle (distilled water p.o), Group 2, 3, 4, 5 and 6 received *Ghana* A, B, C, D and E (500 mg/kg, p.o) respectively. Group 7, 8, 9, 10 and 11 received *Ghana* A, B, C, D and E (1000 mg/kg, p.o) respectively.

Procedure: Before administration of drug, two animals were placed in actophotometer for 5 minutes and the actophotometer count was noted as 0 h reading. The respective doses were administered orally. After 1 h of treatment, animals were again placed in the actophotometer. The spontaneous locomotor activity was recorded for 5 mins duration at an interval of 5, 30, 60, 90, 120 and 180 minutes.

Evaluation: The percent count of spontaneous locomotor activity (SLA) of treated group was compared with the vehicle control group and statically analyzed by ANOVA followed by Dunnett's test.

Seven day treatment of Ghana

Ghana A, B, C, D and E (1000 mg/kg, p.o) was administered to groups 7, 8, 9, 10 and 11, once a day for seven days respectively. SLA was recorded in actophotometer on first day before treatment and on seventh day one hour after treatment as described above.

Anoxic stress in mice**Twenty eight days treatment with Ghana**

The *Ghana* A, C and D showed significant reduction of SLA in rats in actophotometer test and therefore were selected for evaluating the effect of anoxic stress in mice. Swiss albino mice were divided in four groups consisting six animals in each group. Group 1, received vehicle (distilled water p.o), Group 2, 3, 4 received *Ghana* of A, C and D (1000 mg/kg, p.o) respectively for 28 days continuously.

Procedure: Glass bottle of 250 ml capacity were used for the study. The bottle was made airtight using rubber cork, before the start of the experiment. Male albino mice weighing 25 ± 2 g was then kept in the airtight bottle and the time taken for the first convulsion (anoxic tolerance time) was noted. The animal was removed immediately from the vessel and resuscitated if needed. On first (0th) day before treatment and on 7th, 14th, 21st and 28th day, 30 minute after the drug treatment anoxic tolerance time were noted for each animal.

The results are expressed as percentage change in anoxic time [12].

Estimation of brain monoamine levels after anoxic stress in mice

The *Ghanas* A, C and D were evaluated for its effect on brain monoamine levels after subjecting to anoxic stress.

Treatment: Swiss albino mice were divided in six groups containing six mice in each group. Group 1 and 2 were normal without treatment, group 3 received vehicle (distilled water p.o), group 4, 5 and 6 received *Ghana* A, C and D (1000 mg/kg) respectively for 28 days.

Procedure: On 28th day animals from group 2, 3, 4, 5 and 6 but not group 1 were subjected to anoxic stress after 30 min of drug treatment and then sacrificed by cervical dislocation. Immediately brains were isolated weighed and homogenized in 0.1 M perchloric acid and processed.

Apparatus: The HPLC system consists of a delivery pump (Model Jasco Pu-1580, Intelligent HPLC pump, Jasco, Japan), a reversed phase analytical column (Dim 250 x 4.6 mm; Lot 6586, Partical size 5 μ , ODS Hypersil, SN= 11323250) supplied by thermo Quest Hypersil Div., Mumbai, India. protected by guard column (7.5 x 4.6mm 5 μ , Hypersil ODS), Auto sample injector (Intelligent sampler, AS-1555, Jasco, Japan) and a fluorescence spectrophotometer (FP-1520, Intelligent fluorescence detector, Jasco, Japan).

Chromatographic conditions: The mobile phase consisted of sodium acetate (0.02 M), methanol (16 %), heptane sulphonic acid (0.055 %), EDTA (0.2 mM), dibutylamine (0.01 %, V/V). The solution was adjusted to pH 3.92 with o-phosphoric acid, filtered through a 0.45- μ m membrane filter and degassed. The flow rate was set to 0.9 ml/min.

Animals: Swiss albino Male mice (25 \pm 2 g) were divided in 8 groups with 6 mice in each group. Treatment schedule was as follows.

Sample preparation:-Forty-five min after vehicle or fraction 'C' and 30 min after diazepam mice were sacrificed and heads were dropped into ice-cold 0.1 M perchloric acid (PCA). Immediately after the sacrifice of mice brains were removed and transferred to ice chilled petridish. The tissue were weighed and homogenized in 2 ml of 0.1 M PCA containing isoproterenol at concentration of 30 ng/ml. After centrifugation at 14,000 g for 15 min at 4 0C, the supernatant was filtered through a 0.2 μ membrane filter and 100 μ l of the filtrate was injected onto a HPLC column. After separation, NE, DA, IP and 5-HT were detected at the excitement wavelength of 280 nm and an emission wavelength of 315 nm. The slit width was kept at 10/10 for excitation/emission, respectively.

Standards and calculations:-Stock solution of standards, 1 mg/ml, were prepared in 0.1 M hydrochloric acid and were stored at -20 0C. If they were not used within a month of preparation, they were discarded. The working standard solutions were prepared freshly in 0.1 M PCA for each experiment. The amount of standards per injection volume of 100 μ l were 3 ng each of NE, DA, IP and 5-HT. Monoamine peaks were identified by comparing their retention time in the sample solution with that of standard solutions. Each monoamine in the tissue was quantified by comparing peak heights in elution profile of samples with known standards [13](Lakshamana and Raju,1997).

Statistical analysis

The data was analyzed by one way analysis of variance followed by Dunnett's test using InStat GraphPad Version 3.06, 32 bit for windows, GraphPad Software, Inc, USA. The level of significance is p<0.05.

Results

Acute toxicity study

All *Shankhapuspi Ghana* samples were found to be safe at 5000 mg/kg limit dose study as per OECD guideline AOT 425. No signs of toxicity and mortality were observed at all doses of *Ghana*. The LD₅₀ value obtained was greater than 5000 mg/kg for *Ghana* (A, B, C, D and E).

Spontaneous locomotor activity in rats

Single day treatment of Ghana

Ghana A, B, C, D, and E did not show significant change in actophotometer count at 500 mg/kg dose (Figure 1), however, at 1000 mg/kg *Ghana* A, B, C, and D showed significant reduction in percentage change of actophotometer count at 180 minutes as compared with vehicle. *Ghana* D showed significant reduction in actophotometer count at 60 minutes time interval and non significant reduction at 5 minute to 120 min time interval (Figure 2).

Figure 1. Effect of single day treatment of *Ghana* A, B, C, D and E (500 mg/kg) on locomotor activity rats.

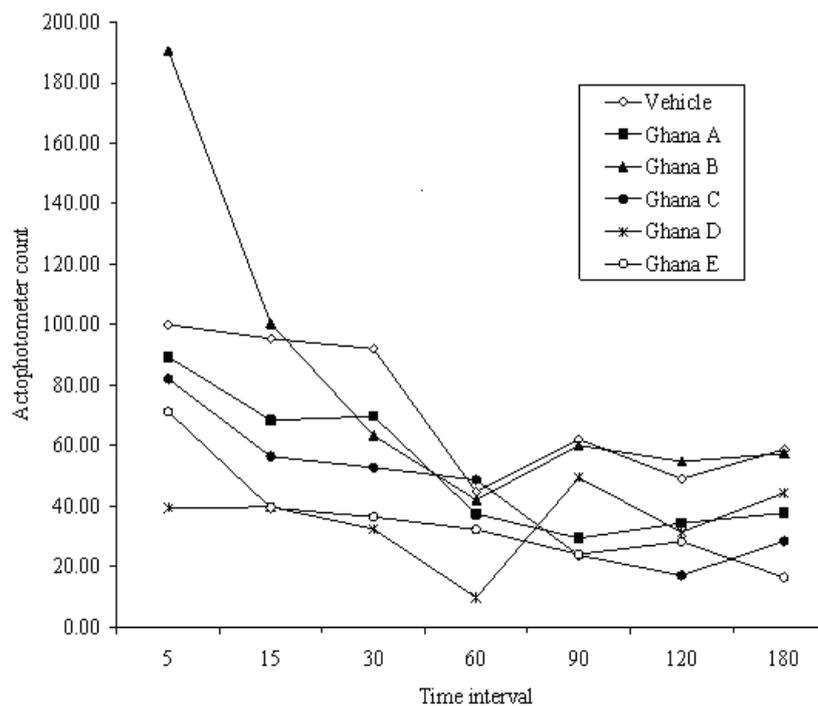
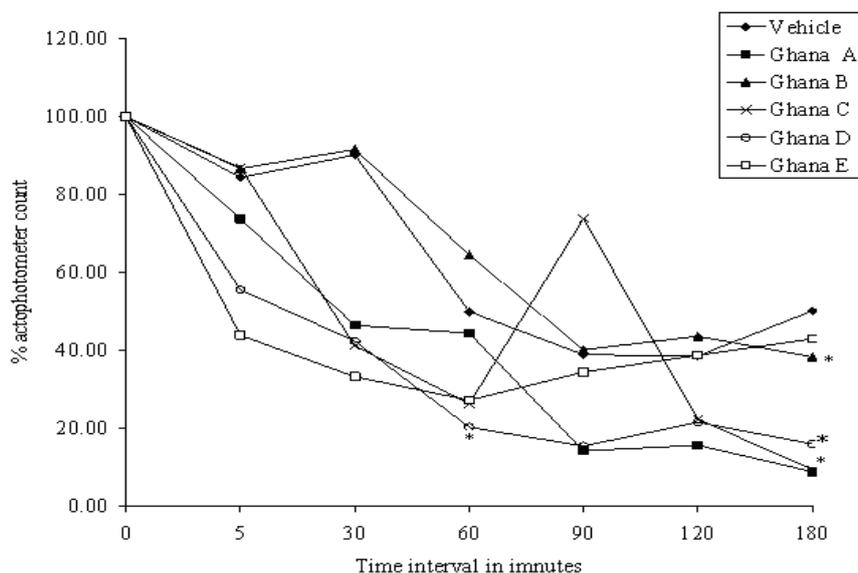


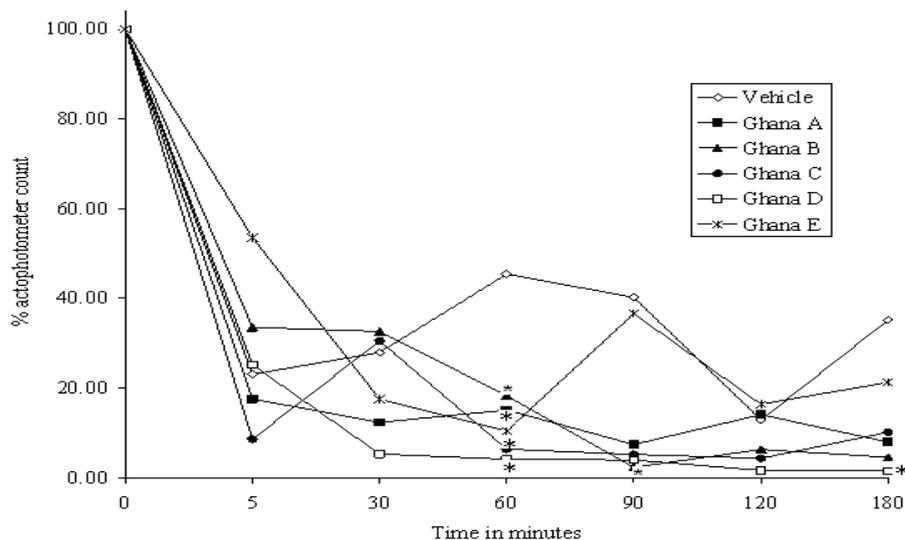
Figure 2. Effect of single day treatment of *Ghana* A, B, C, D and E (1000 mg/kg) on locomotor activity in rats.



Seven day treatment of Ghana

Mice treated with *Ghana* B (1000 mg/kg) showed significant reduction in spontaneous locomotor activity recorded as percentage change of actophotometer count at time interval of 60, 90 and 180 minute. *Ghana* D (1000 mg/kg) at 60 and 180 minute showed significant reduction in spontaneous locomotor activity. *Ghana* A and C (1000 mg/kg) showed significant reduction in spontaneous locomotor activity at 60 minute time interval. (Figure 3)

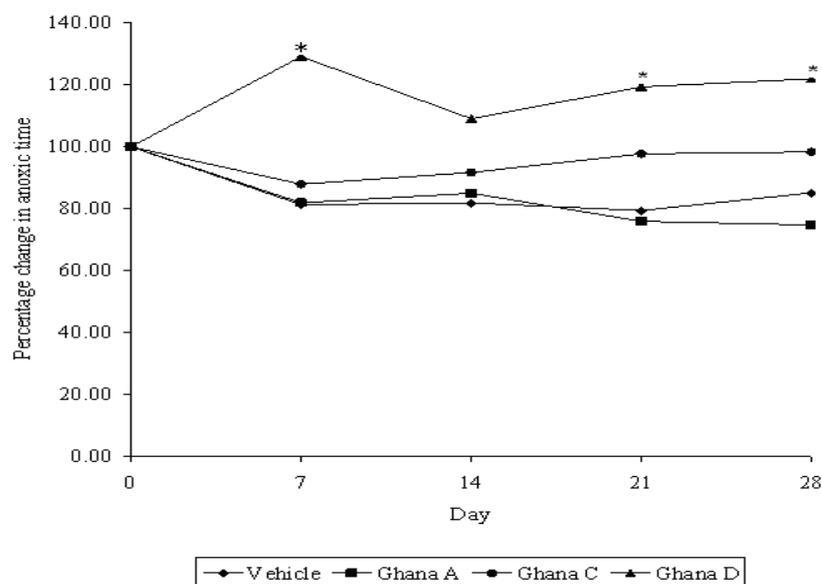
Figure 3. Effect of seven day treatment of Ghana A, B, C, D and E (1000 mg/kg) on locomotor activity in rats.



Anoxic stress in mice

Ghana D (1000 mg/kg) on 7th, 21st and 28th days showed significant increase in percentage change in anoxic time. *Ghana* C showed non-significant increase in percentage change in anoxic time on 7th, 14th 21st and 28th day. (Figure 4).

Figure 4. Effect of 28 day treatment of Ghana A, C and D (1000 mg/kg) on anoxic stress in mice.



Brain monoamine levels in mice

Pretreatment with *Ghana* A and C resulted in non-significant decrease in brain NA as compared to vehicle group while *Ghana* D showed non-significant increase in brain NA level. Anoxic stress in the group of *Ghana* A and C treated animals resulted in non-significant decrease in brain DA levels while *Ghana* D pretreatment showed non-significant increase brain DA levels. *Ghana* A showed non-significant decrease while *Ghana* C and D showed non-significant increase in 5-HT levels in a brain as compared to vehicle treated group (Table -1). Anoxic stress did not alter 5-HT levels compared to unstressed normal animals.

Table 1. Effect of *Ghana* pretreatment (28 days) on brain monoamine levels determined after anoxic stress.

Treatment	Concentrations ng/g of brain tissue		
	Nor-adrenaline	Dopamine	5-Hydroxy-tryptamine
Normal	363.58± 84.65	170.91± 45.57	199.6± 44.23
Normal (stress)	332.10± 95.04	154.57± 42.64	198.22± 58.83
Vehicle (stress)	403.31± 94.82	164.77± 42.00	185.85± 27.78
<i>Ghana</i> A (stress)	301.87± 28.57	162.02± 73.96	180.16± 56.32
<i>Ghana</i> C (stress)	345.73± 80.64	143.84± 36.03	195.96± 48.21
<i>Ghana</i> D (stress)	405.84± 43.34	180.84± 22.73	203.10± 18.23

Values are expressed as Mean ± S.D, Concentration in ng/g of brain n=6, Data analyzed by one way ANOVA followed by Dennett's test.

Discussion

Convulvulus prostrates and *Evolvulus alsinoidis* are traditionally used plants for the antistress activity. It is reported that isolation from their home cages induces stress [14,15] and stress increases the locomotor activity in animals [16]. In the present study, rats in control group showed increase in spontaneous locomotor activity. *Ghana* D (1000 mg/kg) in single day treatment study showed significant reduction in spontaneous locomotor activity at 60 min time interval. However at time interval 120 and 180 min the activity of *Ghana* A, C and D are equivalent. *Ghana* B and E were comparatively less active. Seven day treatment with *Ghana* B and D showed significant reduction in spontaneous locomotor activity. Reduction in activity after seven day was found to be more than reduction activity observed after single day treatment. Activity of *Ghana* D was more than *Ghana* B. Thus *Ghana* A, C and D showed were selected for evaluating the effect of *Ghana* treatment in anoxic stress model in mice.

Anoxia is a very severe stressor. All the body functions including cellular respiration depend on oxygen supply to them. Any lack of this vital element will play havoc on all body mechanisms and increase in adaptation during this stress by any drug could be considered as its major antistress effect [17]. *Ghana* D showed significant increase in anoxic time however, activities of *Ghana* D was observed on 7th day and remained so 28th till day. The brain is master control of interpretation of stressful conditions and responds to it by producing behavioral and/or physiological changes. The brain appears to handle repeated stress over weeks by showing adaptive plasticity in which local neurotransmitters as well as systemic hormones interact to produce structural as well as functional changes [18]. A typical

neuroendocrine response involved initially, within seconds is the increased secretion of catecholamine (noradrenaline and adrenaline) from the sympathetic nervous system and adrenal medulla. A general activation of the noradrenaline neurons has been described in response to different stressors in rats and cats.

In the present study increase in brain noradrenaline (NA) levels after anoxic stress in vehicle treated group appears to be due to stimulation of sympathetic outflow as result of stress. Pretreatment with *Ghana* A and C decreased NA levels after anoxic stress. Noradrenaline may play a role in increasing percent anoxic time. While correlating the brain dopamine levels with anoxic time it is obvious that a threshold increase in anoxic time is necessary for such correlation. Increase in brain NA and dopamine levels after treatment with *Ghana* indicate stimulation of sympathetic outflow as a result of stress. In the behavioral parameter however significant prolongation of anoxic time indicated that even marginally increased NA and dopamine levels may prepare the animals for coping up with emergency situations produced due to anoxic stress.

Pretreatment with *Ghana* A, C and D did not significant increase brain serotonin levels. 5-Hydroxytryptamine is widely distributed monoamine in brain and involved in mood and impulse control. Different stressors like immobilization or restrain stress, foot shock lead to increased synthesis/metabolism of 5-Hydroxytryptamine in limbic regions [19]. Measurement of brain monoamines indicated that *Ghana* C and D showed significant antistress activity.

It is concluded that *Ghana* C and D of showed antistress effect by reduction in locomotion, increase in anoxic time and reduction in brain noradrenaline. *Ghana* C prepared from *Convulvulus prostrates* Forssk collected from Varanasi showed potent antistress activity and therefore confirms the ethanobotanical claims of the antistress activity of *Shankhapushpi*.

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