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Protective effect of *Habenaria intermedia* tubers against acute and chronic physical and psychological stress paradigs in rats

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Article

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Abstract: The present study was undertaken to evaluate the adaptogenic activity of ethanol (EtHI), ethyl acetate (EAHI) fractions of *Habenaria intermedia* D. Don, Orchidaceae (HI), tubers using immobilization induced acute stress (AS), chronic stress (CS) and swimming induced stress in experimental animals. The tested doses of EtHI (100 and 200 mg/kg, *p.o*.) and higher dose of EAHI (200 mg/kg, *p.o*.) normalized altered serum biochemical parameters and the severity of ulcers in both AS and CS. EAHI and EtHI restored the hyperthrophy of adrenal gland and atrophy of spleen and thymus gland in AS and CS. Greater swimming time was noted in the mice pretreated with EtHI and EAHI. Levels of adrenal ascorbic acid and cortisol were restored significantly. EAHI exhibited prominent scavenging effect of DPPH, hydroxyl radical and lipid peroxidation in vitro. Phytochemical studies resulted in the isolation of scopoletin and gallic acid as marker compounds. Our results proved the traditional claim of HI as anti-stress/adaptogen in Ayurvdea.

Introduction

Stress can be described as the sum total of all the reactions of the body, which disturb the normal physiological condition and result in a state of threatened homeostasis. Stress has been postulated to be involved in the etiopathogenesis of a diverse variety of diseases ranging from psychiatric disorders such as anxiety and depression, immunosuppression, endocrine disorders including diabetes mellitus, male sexual dysfunction, cognitive dysfunctions, peptic ulcer, hypertension and ulcerative colitis (Elliott et al., 1982). Benzodiazepines and anxiolytics, despite having significant anti-stress activity, have not proved effective against chronic stress induced adverse effects on immunity, behavior cognition, male sexual function, during pregnancy and lactation. Additionally, the problem of tolerance and physical dependence on their prolonged use, limits the clinical utility of these drugs When we study the history of ancient alternative systems of medicine, Ayurveda and Traditional Chinese Medicine (TCM) are on the forefront (Stuart, 1984) Therefore there is a need for an effective herbal anti-stress agent in the therapy of stress induced disorders (Muruganandam et al., 2002; Rai et al., 2003). Rasayanas of ayurveda may be effective anti-stress agents, because they appear to prolong, Selye's propounded second phase of the "General adaptation syndrome", the stage of resistance to stress, and prevent the final and third phase of exhaustion. (Rege et al., 1999). Plant adaptogens like *Panax ginseng* (Sang et al., 2006), *Elutherococcus senticosus* (Goulet and Dionne., 2005), *Withania somnifera* (Bhattacharya et al., 2001; Singh et al., 2003), *Bacopa monnieri* (Deepak et al., 2003) fractions and their constituents have been extensively studied for anti-stress/adaptogenic activity.

Habenaria intermedia D.Don, Orchidaceae, is an important member of rasayana herbs in Ayurveda. It is commonly known as Vrddhi in Indian system of medicine. The edible tubers are sweet, emollient, and used as intellect promoting, aphrodisiac, depurative, anthelmintic, rejuvenating and tonic. Tubers are also useful in asthma, leprosy and skin diseases. This plant is an important ingredient of *Chyavanprasha*, a well known polyherbal rejuvenator (Warrier et al., 1994; Kirtikar & Basu 1994). Traditionally it is used in many herbal preparations for its rejuvenating properties. Antioxidant activity of polyherbal formulation containing tubers of *Habenaria intermedia* was investigated in nitric oxide scavenging activity (Jagetia et al., 2004). Literature survey indicates

that *Habenaria* species are not explored so far for their chemical constituents and biological activity. Therefore, we attempt to investigate the anti-stress potentiality of different fractions of *Habenaria intermedia* using different stress models in rodents.

Material and Methods

Drugs and chemicals

Diagnostic kits for the estimation of glucose, triacylglycerides, AST, ALT were purchased from ERBA diagnostic Mannheim Ltd. (Germany), cholesterol (Span Diagnostics Ltd, India) and creatinine kinase (Agappe Diagnostics Ltd.) A gift sample of standardized *Withania somnifera* (WS) extract was obtained from Natural remedies, Bangalore, India. DPPH was obtained from Sigma Chemical Co. (St Louis, MO USA), DNPH (SD-fine chemicals, India), mannitol, thiourea, ascorbic acid, deoxyribose (Himedia, India).

Procurement and authentication of the plant material

Tubers of *Habenaria intermedia* D. Don, Orchidaceae, were obtained from Forest research institute, Dehradun, India and authenticated by qualified taxonomist, Department of Botany, Karnataka University, Dharwad. A herbarium specimen was kept in department of Pharmacognosy (SETCPD/Ph.cog/herb/36/2007). The collected tubers were washed with running water. The tubers were chopped in to small pieces and dried under shade. Dried tubers were coarsely powdered and used for extraction.

Preparation of test fractions

Coarse plant material was cleaned by passing the powder material through 120 mesh sieve to remove any fine dust or powder, and coarse powder was used for extraction. Dried powder (1 kg) of root was exhaustively extracted successively using ethyl acetate (EAHI) and ethanol (95%) (EtHI) respectively in a Soxhlet apparatus. The extracts were filtered to remove particulates concentrated using rotary flash evaporator (Suprtfit Rotovap, PBU-6) under reduced pressure and controlled temperature, followed by freeze drying (Heto FD 3 Dry winner) till dry powder was obtained and stored in a descicator. The yield of EtHI and EAHI freeze dried powder was found to be 10 and 8.5% respectively. Preliminary phytochemical screening and TLC analysis with suitable spraying reagents revealed the presence of coumarins, flavanoids, tannins and phenolic acids (Brain & Turner, 1975; Khandelwal, 2008).

Isolation of secondary metabolites from H. intermedia

Isolation of scopoletin

Ethyl acetate fraction (5 g) was dissolved in 10 mL of methanol and mixed with 5 g of silica gel (60-120 mesh size) and dried in vacuum oven at 45 ºC. The adsorbed material obtained was transferred to the column and elution was carried out with $CHCl_3$, $CHCl_3$-MeOH mixture in different proportions. A yellow colored compound was eluted from CHCl3-MeOH (90:10) fractions 60-69. Identification of isolated component was carried out on TLC plates (0.25 mm) precoated with silica gel GF254 (Merck, silica gel for TLC). Chromatogram was run using toluene:ether (1:1) saturated with 10% acetic acid as mobile phase. The TLC plates were checked under UV light (365 nm) and then sprayed with 10% ethanolic KOH reagent. Fractions with similar TLC pattern were pooled together and concentrated at reduced pressure and temperature. Pale yellow colored needles were obtained after recrystalisation with methanol. Completely dried components were weighed to calculate the total mass isolated. The identity of the compound was established by TLC and IR, ^{1}H NMR and MS spectroscopy and the data compared with those from literature.

Isolation of gallic acid

Ethanolic fraction (5 g) was subjected to acid hydrolysis with 2M HCl for 0.5 h. Extraction was carried with ether (10 mL aliquots, three times), evaporated to get light yellow colored residue. 2 g of residue was dissolved in 10 mL of MeOH and mixed with 2 g of silica gel (60-120 mesh size) and dried in vacuum oven at 45 ºC. The adsorbed material obtained was transferred to the column and elution was carried out by gradient method with ethyl acetate: benzene in different proportions. The elution rate was adjusted to 50 mL/min, for several 10 mL fractions up to 100 mL and then 18-20 drops/min up to 350 mL. A pale yellow colored compound was eluted from ethyl acetate: benzene (80:20) fractions 210-224.

Identification of isolated component was carried out on TLC plates (0.25 mm) precoated with silica gel GF_{254} (Merck, silica gel for TLC) using ethylacetate:benzene (9:11) as mobile phase. The TLC plates were then sprayed with Folin-Ciocalteu reagent. Fractions with similar TLC pattern were pooled together and concentrated at reduced pressure and temperature. The concentrated components were further dried in vacuum descicator. Completely dried components were weighed to calculate the total mass isolated. The identity of the compound was established by TLC and IR, ^{1}H NMR and MS spectroscopy and the data compared with those from literature.

Animals

Albino Wistar rats (150-200 g) and Swiss albino mice (20-25 g) of either sex were used in the study. They were housed three to four per polypropylene cage at temperature 22±2 ˚C at 12:12 h, light:dark under controlled environment. Animals were fed standard laboratory food and water was given *ad libitum.* They were kept for seven days in laboratory for habituation. The principles of Laboratory Animals Care (PHS, 1986) and the instructions given by our institutional animal ethical committee (SETCP/IAEC/08/11) were followed throughout the experiment.

Acute toxicity studies

Acute toxicity study for EAHI and EtHI was carried out using Swiss albino mice (25-30 g) by up and down/staircase method as per CPCSEA guidelines. EtHI and EAHI fractions were orally administered to different groups of young and aged mice at doses of 50, 300, 1000 and 2000 mg/kg body weight respectively. Animals were observed for 48 h to study the general behavior of animals, signs of discomfort and nervous manifestations.

Preparation of drugs

EAHI and EtHI were suspended in 0.5% gum acacia, and a fine emulsion was made having uniform particle distribution. The emulsion of both the extracts was administered for orally daily for three days in case of acute stress (AS) and for seven days in case of chronic stress (CS). Both the drugs were prepared fresh daily before administration.

Stress protocol

Among the methods employed, immobilization has been used extensively and accepted widely for studying the stress induced physical and psychological alterations and consequences of the stress (Al-Mohaisen et al., 2000). In our experiments, the stress was produced by restraining the individual inside an acrylic hemicylindrical plastic tube (4.5 cm diameter, 12 cm long) for a period of 150 min once daily for three days in AS and once daily for seven consecutive days in CS as described earlier (Deepak et al., 2003). Freshly prepared emulsion of both the fractions (EAHI, EtHI-100 and 200 mg/kg *p.o*. respectively) was administered orally (*p.o*.) daily for three days in AS and for seven days in CS. After the stress protocol, blood was collected via retro-orbital plexus, serum was separated for biochemical estimations (glucose, ALT, AST, TG, TC and CK). The rats were sacrificed immediately under ether anesthesia, stomachs were split open along the greater curvature and the number of discrete ulcers was noted by using magnascope under 5X magnification ulcers were scored according and mean ulcer severity score was calculated as reported earlier, (Gupta et al., 1981), the abdomen and thorax were cut open, and the adrenals, spleen and thymus were dissected and weighed after removing the adhering tissues.

Swimming endurance test

Animals were divided in to normal control (unstressed), stress control, WS (100 mg/kg), and test groups (EAHI, EtHI-100 and 200 mg/kg *p.o.*, respectively). Treatment was given to mice for seven days. On 7th day, 1 h after drug administration the animals were forced to swim in glass chambers (30×30×15 cm) containing water at room temperature. The mice were allowed to swim till they got exhausted and the moment they drowned was considered as the endpoint. The mean swimming time for each group was noted. Adrenal glands were removed, weighed and the contents of ascorbic acid and cortisol in the adrenal gland were estimated as reported earlier (Kannur et al., 2006).

In-vitro *free radical scavenging activity*

Free radical scavenging effect of EAHI and EtHI was investigated spectrophotometrically using DPPH, hydroxyl and lipid peroxidation assays as per standard methods (Veerapur et al., 2007)

Statistical analysis

The results were expressed as mean±SEM The statistical significance was determined by two-way ANOVA followed by a post hoc Tukey's test. A probability p value of less than 0.05 was taken to indicate statistical significance.

Results

Spectral data of scopoletin

IR spectrum of scopoletin showed characteristic absorption band at 3338 cm^{-1} due to hydroxyl group. Another band at 1703 cm^{-1} attributed to stretching frequency of carbonyl group. The CH=CH stretching peak appeared at 2944 cm^{-1}. Proton NMR spectrum showed a doublet at δ 7.8-7.9 (1-H) which integrated for one proton and was assigned to C3 proton. One more doublet at δ 6.20-6.22 (1-H) which corresponds to one proton was attributed to C_4 proton. Two singlets which appeared at δ 7.21 and δ 6.77 were assigned to C_8 and C_5 protons. Hydroxyl proton at C_7 resonated as singlet at 10.30. A singlet at δ 3.80 which integrated for three protons was due to –OCH_3 group. The Mass spectrum of Scopoletin showed a molecular ion peak at *m/z* 193, which was due to its molecular formula ($C_{10}H_8O_4$) and

molecular weight.

Spectral data of gallic acid

IR spectrum of gallic acid exhibited a characteristic peak at 1612 cm^{-1} which was due to carbonyl peak of -CO_2H group. A broad peak at 3387 cm-1 was due to hydroxyl groups. ^1H NMR spectrum displayed a singlet at δ 6.9 (2-H) was assigned to two aromatic protons at C_2 and C_6. Three hydroxyl groups comes to resonate at δ 9.5, broad singlet corresponds to three -OH groups at C_3, C_4 and C_5. The carboxylic acid -OH group also appeared as broad singlet at δ 12.3 respectively. The mass spectrum of gallic acid showed a molecular ion peak at m/z 170, which was due to its molecular formula ($C_7H_6O_5$) and molecular weight.

Effect of drug treatment on acute stress (AS) and chronic stress (CS) induced alterations in biochemical parameters

AS and CS resulted in a significant increase in the serum glucose, total cholesterol and triacylglyceride compared to AS and CS control. Pretreatment with EAHI 200 mg/kg, EtHI 100 mg/kg, EtHI 200 mg/kg and WS 100 mg/kg (p<0.001) significantly decreased the elevated levels of glucose, total cholesterol and triacylglyceride level in AS and CS. Exposure to AS and CS resulted in the significant increase in serum AST, ALT and CK level as compare to respective control. Pretreatment with EAHI 200 mg/kg, EtHI 100 mg/kg, EtHI 200 mg/kg and WS 100 mg/kg (p<0.001) significantly reduced the elevated levels of AST, ALT and CK in AS and CS. (Table 1 and 2).

Effect of drug treatment on acute stress (AS) and chronic stress (CS) induced alterations in ulcer index and organ weight

Effect of EtHI, EAHI on weight of adrenal gland, spleen and thymus in AS and CS is presented in Table 3. Rats exposure to AS and CS resulted in the significant increase the adrenal gland weight. Pretreatment with EAHI 200 mg/kg (p<0.001), EtHI 100 mg/kg (p<0.001), EtHI 200 mg/kg (p<0.001) and WS 100 mg/kg (p<0.001) significantly reduced the increased adrenal weight in AS where as in CS, EtHI 100 mg/kg (p<0.001), EtHI 200 mg/kg (p<0.001) and WS 100 mg/kg (p<0.001) significantly restored the adrenal weight. A significant decrease was found on exposure to AS (p<0.01) and CS (p<0.001) in spleen weight. The weight was increased by EAHI, EtHI and WS 100 mg/kg (p<0.05) in AS and EAHI 200 mg/kg (p<0.05), EtHI 100 and 200 mg/kg (p<0.01) and WS 100 mg/kg (p<0.001) in CS. Chronic stress resulted in significant decrease in the weight of thymus (p<0.001). In AS EAHI 200 mg/kg (p<0.01), EtHI 200 mg/kg and WS 100 mg/kg increased the thymus weight. The weight was restored by the EtHI 100 and 200 mg/kg (p<0.001) and WS 100 mg/kg (p<0.001) in CS.

The severity of ulcers was scored after histological confirmation as 0 = no ulcers, 1 = changes limited to superficial layers of mucosa with no congestion, 2 = half the mucosal thickness shows necrotic changes, 3 = more than two thirds of mucosal thickness shows necrotic changes and 4 = complete destruction of mucosa with hemorrhage. Acute and chronic immobilization stress resulted in a significant increase in score of ulcer index. Pretreatment with EAHI 200 mg/kg (p<0.001), EtHI 100 mg/kg (p<0.001), EtHI 200 mg/kg (p<0.001) and WS 100 mg/kg (p<0.001) significantly decreased the ulcer index as compared to AS and CS control respectively (Table 4).

Swimming endurance test

The survival time of swimming mice increased significantly in dose dependent manner by pretreatment with EAHI 200 mg/kg (p<0.05), EtHI 100 mg/kg (p<0.05) and EtHI 200 mg/kg (p<0.001) compared to normal (non-drug treated) (Figure 1). Pre-treatment with EAHI 200 mg/kg (p<0.001), EtHI 100 mg/kg (p<0.05), EtHI 200 mg/kg (p<0.001) and

Table 1. Effects of EtHI and EAHI on the serum biochemical parameters in AS induced rats.

Groups/dose (mg/kg)	AST (IU/L)	ALT (IU/L)	TG (mg/dl)	TC (mg/dl)	CK (IU/L)	Glucose (mg/dl)
Normal control	66.83±6.66***	20.06±0.49***	33.68±2.38***	43.88±3.91***	138.7±3.57***	78.92±1.18***
Acute stress control	162.7±5.92	44.20±0.88	77.50±5.04	75.55±5.71	261.5± 8.87	163.5±4.38
EAHI 100	125.4±12.71	38.09±1.88	55.85±1.49	65.99±2.45	268.2±11.15	149.6±8.55
EAHI 200	116.0±8.04*	32.83±2.30*	48.64±1.66*	64.03±2.16	218.7±4.27**	112.6±6.46***
EtHI 100	94.83±4.188***	30.37±1.31***	44.74±3.02**	51.31±2.23***	165.8±2.81***	107.0±6.01***
EtHI 200	81.61±37.63***	26.93±1.76***	42.82±3.79**	52.69±6.25***	155.7± 0.84***	92.31±1.03***
WS100	66.01±3.28***	27.24±2.38***	42.23±1.987**	47.33±0.87***	160.8±6.39***	89.12±1.37***

Results are represented as mean±SEM (n=6). *p<0.05, **p<0.01 and ***p<0.001as compared with acute stress control group.

Table 2. Effects of EtHI and EAHI on the serum biochemical parameters in CS induced rats.

Groups/dose (mg/kg)	AST (IU/L)	ALT (IU/L)	TG (mg/dl)	TC (mg/dl)	CK (IU/L)	Glucose (mg/dl)
Normal control	34.68±1.55***	27.03±2.77***	36.18±5.03***	33.61±3.92***	147.0±5.69**	85.75±4.26***
Chronic stress control	86.78±20.76	42.35±2.23	88.09±15.98	67.49±3.22	202.7±8.79	137.1± 4.23
EAHI 100	76.69±4.19	40.62±2.59	39.77±2.229	48.64±1.30*	197.3±16.16	134.4±10.50
EAHI 200	72.79±7.23	29.62±0.526***	30.83±1.19***	45.31±31.32**	170.3±11.12	107.4± 5.15
EtHI 100	22.48±0.653**	27.84±1.76***	43.27±3.26**	48.49±1.94*	156.7±5.98***	95.40± 12.12***
EtHI 200	21.20±0.77***	26.91±2.31***	38.13±7.56***	46.56±3.64**	158.2±4.28***	91.36±10.41***
WS100	20.88±0.83*	26.63±0.97***	36.82±0.10***	45.17±2.46***	147.7±4.25***	89.12±5.07***

Results are represented as mean±SEM (n=6). *p<0.05, **p<0.01 ***p<0.001 as compared with chronic stress control group.

Table 3. Effects of EtHI and EAHI on the weight of adrenal gland, spleen and thymus on AS and CS induced rats.

Groups/dose (mg/kg)	Acute immobilization stress (AS)			Chronic immobilization stress (CS)		
	Weight of adrenal gland (mg)	Weight of spleen (mg)	Weight of thymus gland (mg)	Weight of adrenal gland (mg)	Weight of spleen (mg)	Weight of thymus gland (mg)
Normal control	14.00±0.36***	644.5±15.05**	667.7±9.28***	17.00±0.93**	664.0±16.08***	651.5±19.33***
Acute stress control	23.17±1.25	473.7±33.59	619.3±27.93	----	-------	------
Chronic stress control	---	--	----	21.33±0.91	444.0±39.21	386.3±10.48
EAHI 100	20.67±0.95	559.5±13.61	578.2±22.97	21.50±0.84	459.2±18.47	446.3±8.81
EAHI 200	17.33±0.55***	617.5±5.00*	599.2±3.19**	19.33±1.25	623.3±9.73**	437.7 ±8.82
EtHI 100	16.33±0.33***	605.0±6.90	563.0±12.68	16.33±0.55*	603.8±48.79*	579.8 ±7.44***
EtHI 200	15.33±0.49***	622.5±7.11*	604.5 ±7.22**	15.17±0.47***	630.3±57.76**	607.0 ±14.39***
WS100	14.83 ±0.47***	620.0±14.17**	608.3±8.11**	15.67± 0.76***	667.3±40.36***	617.3 ±10.86***

Results are represented as mean±SEM (n=6). *p<0.05, **p<0.01 ***p<0.001 as compared with stress control group for AS and CS respectively.

WS 100 mg/kg (p<0.01) prevented the increase in adrenal weight significantly (Figure 2). Exposure to swimming stress causes hypertrophy of adrenal gland (p<0.05) which is associated with significant depletion of adrenal contents of ascorbic acid (p<0.001) (Figure 3) and cortisol (p<0.001) (Figure 4) when compared to non swimmer group. Depletion of adrenal ascorbic acid was attenuated significantly by pretreatment with EAHI 200 mg/kg (p<0.05), EtHI 100 (p<0.001), EtHI 200 mg/kg (p<0.001) and WS 100 mg/kg (p<0.01). Pretreatment with EAHI 200 mg/kg (p<0.05), EtHI 100 mg/kg (p<0.001), EtAS 200 mg/kg (p<0.001) and WS 100 mg/kg (p<0.001) also significantly increased cortisol contents of adrenals.

In vitro *free radical scavenging activity*

Reaction with DPPH radical

Ethanolic extract (EtHI) of *H. intermedia* showed DPPH scavenging activity with IC50 value of 35.46 µg/mL as compared with ascorbic acid (2.94 µg/mL), where as EAHI showed DPPH scavenging activity with IC50 value of 32.88 µg/mL (Figure 5A and 6A).

Table 4. Effects of EtHI and EAHI on AS and CS induced gastric ulceration in rats.

Groups	Ulcer index	
	Acute stress(AS)	Chronic stress(CS)
Normal control	0.0±0.0	0.0±0.0
Stress control	11.00±0.89	14.33±1.1
EAHI 100	9.500±0.61	14.17±0.47
EAHI 200	7.00±0.68***	8.50±0.42***
EtHI 100	5.50±0.42***	5.83±0.60***
EtHI 200	3.50±0.42***	3.167±0.47***
WS100	2.83±0.47***	2.83±0.47***

Results are represented as mean±SEM (n=6).***p<0.001 as compared with stress control group for AS and CS respectively.

Lipid peroxidation (LPO) assay

The inhibition of LPO by EtHI was found to be 122.62 µg/mL where as EAHI inhibit LPO with IC50 42.75 µg/mL (Figure 5B and 6B).

Reaction with hydroxyl radical

The ability of the EtHI and EAHI to scavenge the hydroxyl radical was found to be 52.38 µg/mL and 11.28 µg/mL respectively and IC50 value of standard mannitol was found to be 4.99 µg/mL (Figure 5C and 6C).

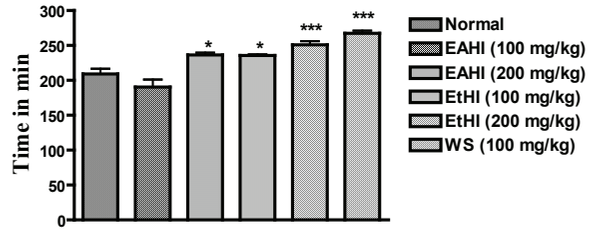

Figure 1. Effect of EtHI and EAHI on mean swimming time (min) on swimming induced stress in mice. The bars indicate mean±SEM (n=6). Statistically significant differences *p<0.05, ***p<0.001 with respect to control according to one-way ANOVA, followed by Tukey's post hoc test.

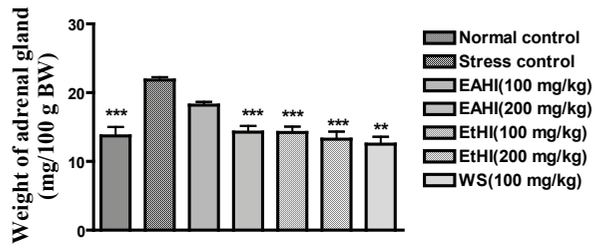

Figure 2. Effect of EtHI and EAHI on weight of adrenal gland mg/100g bodyweight on swimming induced stress in mice. Statistically significant differences, **p<0.01, ***p<0.001 with respect to control according to one-way ANOVA, followed by Tukey's post *hoc* test.

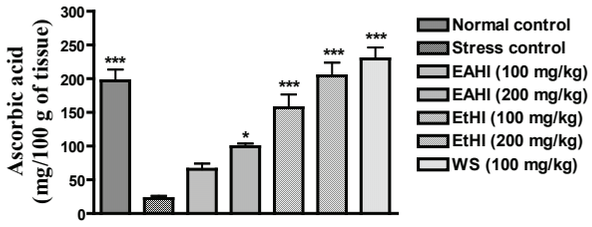

Figure 3. Effect of EtHI and EAHI on level of in adrenal ascorbic acid (mg/100 g of tissue) on swimming induced stress in mice. The bars indicate mean±SEM (n=6). Statistically significant differences *p<0.05, ***p<0.001 with respect to control according to one-way ANOVA, followed by Tukey's post hoc test.

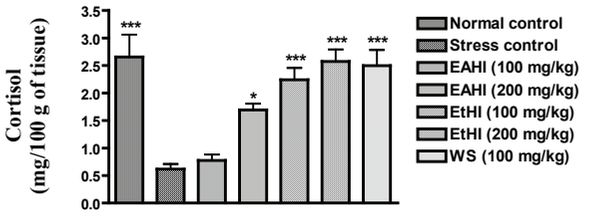

Figure 4. Effect of EtHI and EAHI on level of adrenal cortisol (mg/100 g of tissue) on swimming induced stress in mice. The bars indicate mean±SEM (n=6). Statistically significant differences *p<0.05, ***p<0.001 with respect to control according to one-way ANOVA, followed by Tukey's post hoc test.

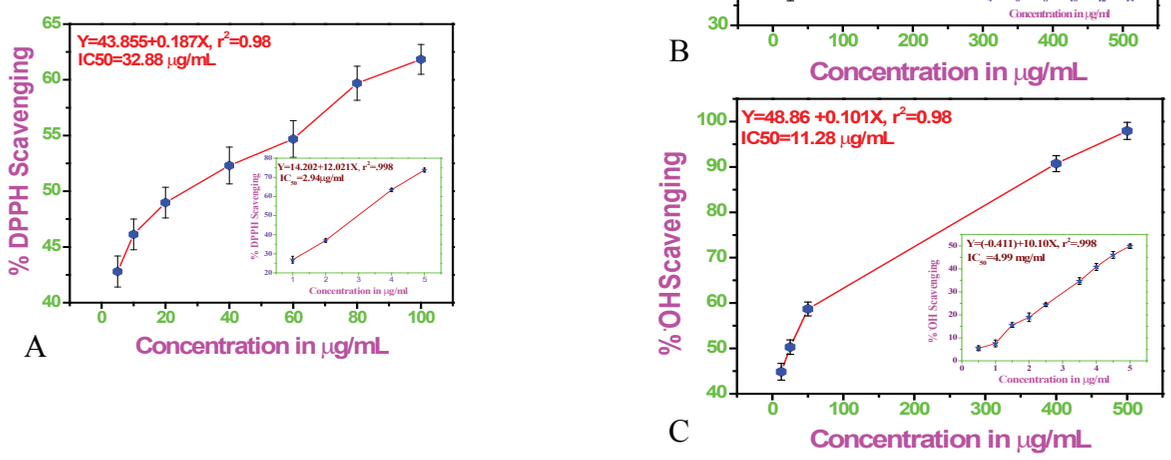

Figure 5. A. Free radical scavenging effect EAHI using DPPH; B. Scavenging effect of EAHI on LPO; C. Free radical scavenging effect of EAHI using hydroxyl radical. Each point represents the mean of three experiments and SEM; Internal line graph represents the IC50 values of respective standards.

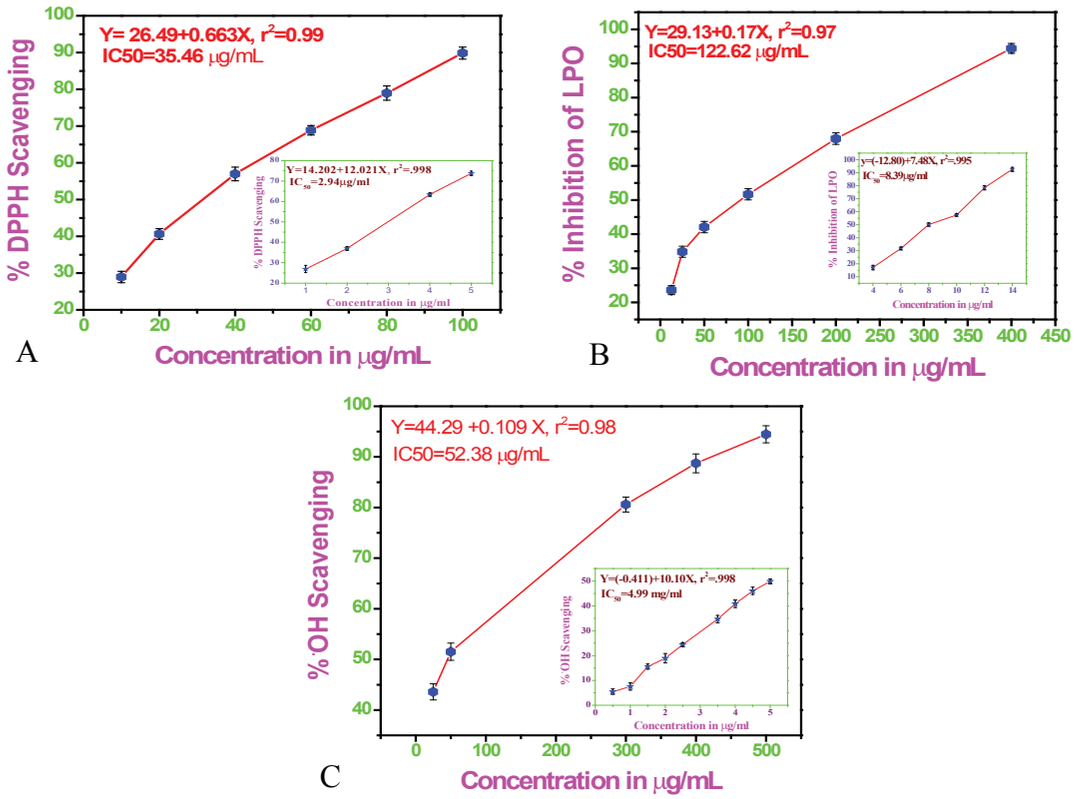

Figure 6. A. Free radical scavenging effect of EtHI using DPPH; B. Scavenging effect of EtHI on LPO; C. Free radical scavenging effect of EtHI using hydroxyl radical. Each point represents the mean of three experiments and SEM; Internal line graph represents the IC50 values of respective standards**.**

Discussion

Biological stress is a response to physical, chemical, biological and emotional changes, consisting of a pattern of metabolic and behavioral reactions that helps to strengthen the organism. The management of unusual stress therefore has acquired enormous significance in day-to-day life (Selye, 1976).Various attempts have been made to counter the aversive effects of stress, ranging from yoga and meditation to anti-stress drugs, particularly tranquilizers, antiadrenergic agents or even psychelic agents and vitamin. Some of these drugs certainly diminish the distress of excessive emotional liability, but others are totally ineffective or even harmful to any kind of stress situation (Selye, 1976)

Due to the nonspecific nature of the stress pathogenesis, a separate class of therapeutic agents was evolved known as "adaptogens". Indian system of medicine documents several herbs, which are categorized as rasayanas. The properties ascribed to rasayanas in Ayurveda are remarkably similar to those of adaptogens (Panossian, 2003). However, supplementation with various macro and micronutrients and herbal preparations has been evaluated for their adaptogenic activity during exposure to a stressful environment (Kumar R et al., 1996, 1995, 2000, 2002). *Withania somnifera* (WS) is popularly known as *Ashwagandha* or winter cherry (Andallu & Radhika, 2000). The practioners of the traditional system of medicine in India regard *W. somnifera* as the "indian ginseng" (Singh et al., 2001). Many pharmacological studies have been carried out to describe multiple biological properties of WS (Mishra et al., 2000; Bhattacharya et al., 1997[a]; Bhattacharya & Muruganandam., 2003; Ahmad et al., 2005; Naidu et al., 2003; Panda & Kar., 1998; Mohanthy et al., 2004; Kulkarni & Dhir., 2008). The active constituents of the plant (withaferin A, sitoindosides VII-X) are reported to have an antioxidant activity which contributes to the reported antistress, immunomodulatory, cognition facilitating, antiinflammatory and antiageing properties (Bhattacharya et al., 1997b). Besides, WS preparations are reported to modulate GABAnergic or cholinergic neurotransmission, accounting for various CNS related disorders (Tohda et al., 2005). Thus WS is extensively used for relieving stress in patients, thus acting an

adapotogen. Due such diversified and multidimentional activites of WS and its phytochemicals, standardized extract of WS was used as standard drug in our study.

A variety of stress situations have been employed to investigate the consequences of stress and to evaluate anti-stress agents and the lack of consistency of stress protocols and their biological consequences is astounding. Immobilization has been the ideal choice for the induction of stress responses in animals and more specifically, for the investigation of drug effects, on typical stress-related gastrointestinal, neuroendocrine, and immunological pathology. Immobilization model used in our study found to cause long term desensitization of HPA response which affected both peripheral and central components of the HPA axis (Wagner., 1994). The distinct advantage of using immobilization as a stressor lies in the fact that it produces both physical as well as inescapable psychological stress (Krupavaram et al., 2007).

Genobiotic medicinal plants of orchidaceae family are found to be more important therapeutically because of their diversified chemical nature. These members contain phenanthrene, bibenzene, flavone, sterol, terpenes, alkaloids (Guan, 2005). Although *Habenaria* species are used in traditional formulations, scientific validations regarding their therapeutic utility and chemical composition is at scarce. In this investigation, the effect of ethyl acetate (EAHI) and ethanol (EtHI) fractions of *Habenaria intermedia* D. Don., Orchidaceae, was evaluated using acute and chronic immobilization stress. AS exposure in our study has elevated level of glucose compared to CS. The hyperglycemic response in AS was due to release of glucocorticoids, as a result of HPA axis stimulation to compensate initial demand of energy (Deepak et al., 2003). The acute demand of glucose was fulfilled by the increase in glucogenolysis from liver during AS. During CS, this available source depletes. Thus, it utilizes fat as a secondary substrate and gluconeogenesis starts in response to corticosterone and, or due to the compensation of the energy demand during chronic conditions is from non-carbohydrate origin which are slow and rate limiting. Pretreatment with the EAHI 100 and 200 mg/kg, EtHI 100 mg/kg, EtHI 200 mg/kg and WS 100 mg/kg in AS, while during CS, EAHI 200 mg/kg, EtHI 100 mg/kg, EtHI 200 mg/kg and WS 100 mg/kg significantly decreased the circulating glucose level thus it seems to have a direct action on peripheral metabolism.

AS and CS raised the serum cholesterol level through the enhanced activity of hypothalamo-hypophyseal axis resulting in increased liberation of catecholamine and corticosteroids. The effect of AS and CS stress on serum triacylglyceride has been shown to be variable probably due to mobilization of fats from adipose tissue by catecholamine. Treatment with EAHI 200 mg/kg, EtHI 100 mg/kg, EtHI 200 mg/kg and WS 100 mg/kg ameliorated the elevated levels of cholesterol as well as triacylglyceride levels in both AS and CS. The suppression of stress induced triacylglyceride level may be due to the suppression of stress induced lipolysis.

The AS-induced significant increase in ALT, AST, and CK might be the outcome of AS induced secretion of corticosterone from cortex, epinephrine from medulla, and epinephrine from sympathetic nerve terminals to provide substrate for energy metabolism and the assurance of availability of ATP demand in the muscles, CNS, and organ of demand. In contrast to ALT, which is found primarily in liver, AST is present in many tissues, including the heart, kidney, brain, and skeletal muscles. Stress hormones also increase CK activity during stress. The CK system is important in stabilizing the ATP levels and energy metabolism of the myocardium and other skeletal muscles of rats during stress. Perturbations of CK activity during extensive stress may result in ischemia due to the non-availability of ATP. A maximum increase in CK activity was observed after AS exposure when compared to CS. A reduced CK activity in CS as compared to AS is due to partial habituation. Pretreatment with EAHI 200 mg/kg, EtHI 100 mg/kg and 200 mg/kg, WS 100 mg/kg revert the AS induced levels of AST, ALT and CK in blood. In CS, EtHI 100 mg/kg and 200 mg/kg, WS 100 mg/kg were found to be effective. Reduction in ALT and AST by the test fractions may be due to direct action on the peripheral metabolism and of CK may be due to decrease in energy demand.

Stressful events activate autonomic and endocrine responses (Singh et al., 2005) responsible for gastric ulceration. In our study AS and CS induced ulceration in stomach with comparable intensity in both the models. Gastric damage induced by CS and AS has been reduced by EAHI 200 mg/kg, EtHI 100&200 mg/kg and WS 100 mg/kg by decreasing mean ulcer index indicating their protective effects on gastric mucosa during stressful conditions.

Stress-induced adrenal hypertrophy found both in AS and CS was the result of activation of the HPA axis, which is highly responsive to stress and is one of the principal mechanism by which an organism mobilizes its defense against stress events (McEwen et al., 2000) The prolonged activation of HPA axis resulted in an increase in the adrenal hypertrophy in CS as compared to AS. During stress, nerve terminals accelerate recruitment of lymphocytes to blood from spleen, which is a major storage pool of lymphocytes. This result in the squeezing of the spleen causing reduction in weight observed in AS and CS exposures. The atrophy of thymus found during AS and CS exposure may be due to apoptosis and necrosis in immature T and B cells resulting in the decline of thymus weight. Our study showed that pretreatment with EAHI

200 mg/kg, EtHI 100 mg/kg, EtHI 200 mg/kg and WS 100 mg/kg significantly reduced the increased adrenal weight and increased the decreased weight of thymus in AS and CS, where as pretreatment with EAHI 100 and 200 mg/kg, EtHI 100 and 200 mg/kg, and WS 100 mg/kg significantly reverse the stress induced atrophy of spleen.

Forced swimming stress makes the individual immobile after an initial period of vigorous activity. This resembles a state of mental depression. The adrenal glands contain relatively large amount of ascorbic acid and cortisol which are markedly reduced by stress and causes hypertrophy (McEwen et al., 2000; Dhir et al., 2006). Our results showed that pretreatment with EAHI 200 mg/kg, EtHI 100 and 200 mg/kg, WS 100 mg increased labor efficiency and increase of swimming performance, Moreover, prevented the depletion of ascorbic acid, cortisol and hypertrophy of adrenal glands indicating that, drug having steroid sparing effects.

Phytochemical investigations lead to the isolation of scopoletin and gallic acid as potential active components. This is the first report for the presence of these components in the tubers of *H. intermedia*. Gallic acid (3,4,5-trihydroxybenzoic acid), is an organic acid found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and many medicinal plants(Reynolds et al 1991). In pharmaceutical industries it is used as a starting material for the synthesis of the psychedelic alkaloid mescaline (Tsao, 1951). Wide ranges of biological activities like antioxidant (Li, et al. 2005), cytotoxic (Sakaguchi et al., 1999) activity against cancer cell lines have been reported for gallic acid and its derivatives. Some ointments to treat psoriasis and external haemorrhoids contain gallic acid (Ogihara et al., 1999).

Scopoletin (7-hydroxy-6-methoxy-2H-1-benzopyran-2-one), is a naturally occurring coumarin component found in various medicinal plants with wide range of biological activities. It has been isolated from various parts of the plants like *Solanum lyratum*, tonka bean, lavender, sweet clover grass, strawberries, and cinnamon, or produced synthetically from an amino acid, phenylalanine. Recent studies demonstrated that scopoletin has anticonvulsant (Mishra et. al, 2010), antioxidant (Thuong et al, 2010), antimicrobial (Yang B et al., 2010), hypotensive, (Ojewole et al, 1983) activities. It also found to inhibit hepatic lipid peroxidation and increased the activity of endogenous antioxidants (Kang et al., 1998). The anticancer property of scopoletin is due to the inhibition of proliferation of certain cancer cells by inducing apoptosis (Liu et al., 2001; Manuele et al., 2006). Antidepressant-like effect of Scopoletin in the forced swimming test induced by immobility stress in mice was also evaluated, and it was observed that, the effect is dependent on the serotonergic, noradrenergic and dopaminergic systems (Capra et. al, 2010).

More recent research postulates that adaptogens re-regulate a highly disordered or stress system by regulating biochemical activators like catecholamines, glucocorticoids, cortisol, nitric oxide, serotonin, corticotropic-releasing factor and sex hormones. This broad array of biochemical activators helps explain antioxidant, antidepressant, anxiolytic effects of adaptogenic drugs (Panossian, 2003). It has been shown that exposure to stress situations can stimulate numerous pathways leading to increased production of free radicals. Both immobilization and variable stress are followed by an increase in lipid peroxidation, measured in plasma and in brain structures. A number of Indian medicinal plants like *Ocimum sanctum, Withania somnifera, Vitis vinifera* have been identified for antistress activity and it was concluded that antioxidant activity of these plants was partly responsible for their antistress activity (Bhattacharya et al., 2001; Satyanarayana et al., 2005). The freshwater orchid, *Habenaria repens,* produces the structurally unusual compound, habenariol, which protects this plant against grazing by crayfish and provides one of the few examples of chemical defenses in freshwater plants. Recent studies demonstrated the antioxidant potential of habenariol to inhibit copper-induced lipid peroxidation of human low density lipoprotein (Johnson et al., 1999). Based on these reports and also on claim that *Habenaria intermedia* is a potent nitric oxide scavenger, the free radical scavenging activity against DPPH, hydroxyl radicals and *in vitro* lipid poroxidation assay was performed for EtHI and EAHI. EAHI was found to be a potent free radical scavenger with minimum IC50 values as compared to EtHI. Scopoletin and gallic acid isolated from the title plant as well as from other medicinal plant are well known antioxidant and free radical scavengers (DellaGreca et al., 2009; Thuong et al., 2010; Abreu et al., 2008; Nguyen et al., 2011; Rawat et al., 2011).

Ashtavarga (group of eight medicinal plants) is vital part of Indian traditional formulation like *Chyvanprasha* and two plants from *Habenaria* species like *Riddhi (Habenaria edgeworthii* H.f.), *Vriddhi* (*Habenaria intermedia* D.Don), *Jivaka* (*Malaxis acuminta* D.Don) and *Rishbhaka* (*Microstylis muscifera* Ridley) have been used traditionally. *Ashtavarga* plants are considered as very good *Rasayana* with rejuvenating and health promoting properties, and are known to strengthen the immune system and have immense cell generation capacity. Due to high medicinal value, *Ashtavarga* plants are used in different forms like *Taila* (oil), *Ghritham* (medicated clarified butter), *Churna* (powder) and formulations in traditional medical system. More importantly, some of these formulations are available in Indian market as pharmaceutical products (Dhyani et al., 2010).

This leads to a conclusion that the observed anti-stress activity of *H. intermedia* may be attributed to the presence of scopoletin and gallic acid or their synergistic properties, which may be mediated through antioxidant mechanism. Further, the activity might be due to the prevention of desensitization of both peripheral and central components of the hypothalamic-pituitary-adrenal axis.

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