Tropical Journal of Pharmaceutical Research February 2011; 10 (1): 47-54

Available online at http://www.tjpr.org

Research Article

Anethum Graveolens Linn (Umbelliferae) Extract Attenuates Stress-induced Urinary Biochemical Changes and Improves Cognition in Scopolamine-induced Amnesic Rats

Sushruta Koppula* and Dong Kug Choi

College of Biomedical and Health Sciences, Department of Biotechnology, Konkuk University, Chungju, Republic of Korea.

Abstract

Purpose: *Anethum graveolens Linn. (Umbelliferae, A. graveolens) is a widely used spice with a long history of traditional medicinal use for the treatment of various ailments. The present study examines the anti-stress and cognition-improving effects of A. graveolens extract in a rat model.*

Methods*: Urinary vanillylmandelic acid (VMA) and ascorbic acid were estimated as biomarkers for evaluating antistress activity in rats. Conditioned avoidance response using Cook's pole climbing apparatus in normal and scopolamine-induced amnestic rats was used to assess cognitive-improving activities. Thiobarbituric acid reactive substances (TBARS) assay was used to evaluate antioxidant activity.*

Results*: Daily administration of A. graveolens at doses of 100, 200 and 300 mg/kg body weight 1 h prior to induction of stress inhibited stress-induced urinary biochemical changes in a dose-dependent manner without altering the levels in normal control groups. Changes in cognition (as determined by the acquisition), retention and recovery in rats were dose-dependent. The extract also produced significant lipid peroxidation inhibition in both rat liver and brain, compared to a reference standard antioxidant, ascorbic acid.*

Conclusion*: The aqueous extract of A. graveolens exhibited significant anti-stress, antioxidant and memory enhancing activities. The study provides a scientific basis for the traditional use of the plant as a culinary spice in foods.*

Keywords: *A. graveolens; Stress; Lipid peroxidation; Antioxidant; Cognition.*

Received: 31 August 2010 *Revised accepted: 9 November 2010*

Corresponding author: *Email: koppula@kku.ac.kr; Tel: +82-43-840-3609; Fax: +82-43852-3616.*

INTRODUCTION

Stress is known to induce alterations in various physiological responses, leading to a pathological state. Stress causes disturbance in the body's normal physiological equilibrium and results in threatened homeostasis [1]. Every human today faces stressful situations in day-to-day life and overstress has been postulated to be involved in the pathogenesis of a variety of diseases, such as depression and anxiety, immunosupression, endocrine disorder including diabetes mellitus, male sexual dysfunction, cognitive dysfunction, peptic ulcers, hypertension and ulcerative colitis [2]. There is increasing evidence that severe stress affects cognitive functions and leads to the pathogenesis of various neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and aging [3,4]. There is also available evidence regarding the role of free radicals in the pathogenesis of Alzheimer's disease, diabetes, cancer and aging [5, 6].

Spices, widely recognized as food additives have been used traditionally to prevent and treat various diseases. Of various spices, *Anethum graveolens* Linn. (Umbelliferae), is a popular aromatic herb in Indian, African, Chinese, Cuban, Iranian and Mexican cuisines. As a folk remedy, *A. graveolens* is used for some gastrointestinal ailments such as flatulence, indigestion, stomach ache and colic [7]. The fruit has an antispasmodic effect on the smooth muscles of the gastrointestinal tract [8]. With regard to central nervous system (CNS), *A. graveolens* has been used to alleviate tiredness from disturbed nights and strengthen brain. The aerial parts of the plant are often cooked with fish to add flavor to it and to stimulate the brain [9]. The major components of *A. graveolens* are flavonoids, phenolic compounds and essential oil [10,11].

Experimental studies have shown that *A. graveolens* possess antimicrobial, antispasmodic, antisecretory and mucosal protective effects. The anti-hypercholesterolaemic and anti-hyperlipidaemic activities of the crude extract have also been reported previously. Recent reports reveal that *A. graveolens* possesses anticancer, anti-diabetic, antioxidant, anti-secretory, antispasmodic, cardioprotective, insecticidal and diuretic activities [8,12]. Although traditional usage of *A. graveolens* claims beneficial actions on CNS, its cognition improving and stress relieving activities have not been investigated scientifically. The present study was designed to evaluate the anti-stress effects of the aqueous extract of *A. graveolens* in forced swimming-induced stressed rats as well as its effects on cognition in normal and scopolamine-induced amnesic rats.

EXPERIMENTAL

Chemicals

Vanillylmandelic acid (VMA) and scopolamine butylbromide (SBB) were purchased form Sigma-Aldrich (St Louis, USA) while ascorbic acid was obtained from Loba Chemie (Mumbai). All other reagents used were of analytical grade.

Preparation of extract

A. graveolens fruits were obtained from Chemiloids, Vijayawada, India. The plant was identified by a taxonomist, Dr K Hemadri of Regional Research Institute, India, and a voucher specimen (no. UCPS-H0619) was kept in the herbarium of College of Pharmaceutical Sciences, Andhra University, India. The fruit material (1 kg) was dried, powdered and extracted with boiling water (5 L) for 30 min by Soxhlet method. The filtrate was evaporated at < 70 ^{0}C in a vacuum dryer to give a final yield of 85.5 g. The extract was re-dissolved in distilled water as and when necessary.

Animals

Wistar rats of either sex, obtained from Ghosh Enterprises, Kolkata, were used in the

study. All animal experiments were approved by the Institutional Animal Ethics Committee (regd. no. 516/01/A/CPCSEA) and followed the guidelines of both the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and the International Guidelines for Handling of Laboratory Animals [13]. They were housed five per cage at a temperature of 22 ± 2 °C with 12h/12h light/ dark cycle. The rats were acclimatized to laboratory conditions for 7 days, and fed with standard pellet diet (Rayan's Biotechnologies Pvt Ltd, Hyderabad, India) and water *ad libitum*.

Evaluation of antistress activity

Rats of both sexes weighing between 150 and 200 g were weight-matched and divided into four groups (I, II, III, and IV), each containing five animals in metabolic cages that were designed to facilitate the collection of urine. Twenty four hour urine samples from each group were collected into two different beakers using an inverted "Y" tube fixed at the bottom of each metabolic cage. One end of the tube received and moved urine into a beaker containing 5 ml of 10 % oxalic acid and then passed on for spectrophotometric determination of ascorbic acid at 550 nm using a previous method [14]. The other end of the tube received and moved urine into a beaker containing 0.5 ml of 6M hydrochloric acid for determination of vanillylmandellic acid (VMA) spectrophotometrically at 360 nm [15].

The experimental protocol was divided into four phases. In the first phase of the experiment, 24 h urine samples were collected in all the 4 groups and subjected to analysis for both VMA and ascorbic acid separately and the values were recorded for 5 consecutive days. In the second phase, the animals in each group were individually subjected to fresh water swimming stress [16]. In this method, the rats were forced to swim until they were exhausted (usually after 3 – 4 min) in a cylindrical vessel of height 60 cm and diameter 45 cm containing water at room temperature (28 °C). Water depth was always maintained at 40 cm. The 24 h urinary levels of VMA and ascorbic acid under these stressed conditions were determined again, as outlined above, daily for five consecutive days. The third phase of the experiment consisted of administration of *A. graveolens* extract to the animals following recovery to normal condition (usually in three to four days). Groups II, III and IV were administered orally with *A. graveolens* (dissolved in distilled water) at daily doses of 100, 200 and 300 mg/kg body weight respectively for five consecutive days, while group I served as control and received only distilled water. Twenty four hour urine samples were collected and the levels of both VMA and ascorbic acid were determined. The final phase of the experiment consisted of evaluating the influence of *A. graveolens* extract on stress-induced changes in the animals after a recovery period of one week. Groups II, III and IV were administered *A. graveolens* by oral gavage at daily doses of 100, 200 and 300 mg/kg, respectively, one hour prior to the daily induction of stress for five consecutive days. Group I served as control and received only distilled water. Urine samples (24 h) were collected and analyzed for VMA and ascorbic acid for five consecutive days to assess the influence of the extract on stress-induced biochemical changes.

Evaluation of memory-enhancing activity

The memory-enhancing activity of *A. graveolens* was evaluated by conditioned avoidance response (CAR) technique in rats using Cook's pole climbing apparatus [17]. The rats were divided into 4 groups of 5 animals each. Groups II, III and IV were administered orally with 100, 200 and 300 mg/kg, respectively of *A. graveolens* (dissolved in distilled water) while animals in group I served as control and received only distilled water. After 90 minutes, all the animals were subjected to a training schedule individually by placing them inside the

Perspex chamber of the apparatus. After an acclimatization period of 5 min in the chamber, a buzzer was given followed by a shock through the grid floor. The rat had to jump on to the pole (shock free zone) to avoid foot shock. Jumping on the pole functionally terminates the shock and this was classified as an escape while such jumping prior to the onset of the shock was considered as avoidance. The session was terminated after completion of 60 trials with an interval of 20-30 seconds between trials. This procedure was repeated at 24 h intervals until all groups reached 95 to 99 % avoidance. Following attainment of complete training for a particular group, the animals were treated with a single dose of scopolamine butyl bromide (1 mg/kg, i.p.) to induce amnesia, 30 min before the next day dosing with the extract. The training schedule was continued further with the daily doses of the extract and vehicle until the rats returned to normal level from scopolamine-induced amnesia.

Inhibition of lipid peroxidation in rat liver and brain (TBARS assay)

Rats weighing 150 - 200 g were sacrificed by spinal traction and the whole brain and liver of each were isolated. The pooled brain and liver were homogenized seperately in four volumes of 40 mM Tris-HCl buffer (pH 7.0) using a tissue homogenizer. The antioxidant activity of *A. graveolens* was determined as its capacity to inhibit lipid peroxidation in homogenates of the liver and brain [18]. Briefly, the reaction mixture (0.5 mL) containing rat liver homogenate (0.1 mL), KCl (30 mM), ascorbic acid (0.06 mM), and ferrous iron (0.16mM) and various concentrations of *A. graveolens* were incubated for 1 h at 37 °C. At the end of the incubation period, 0.4 mL of the reaction mixture was treated with 0.2 mL of sodium dodecyl sulphate (8.1%), 1.5 mL of thiobarbituric acid (0.8 %), and 1.5 mL of acetic acid (20 %, pH 3.5). The total volume was then made up to 4 mL with distilled water and mixture incubated in an oil bath at 100 ^{o}C for 1 h. On cooling, 1 mL of distilled water and 5 mL of butanol-pyridine mixture (15:1 v/v) were added. Following vigorous shaking, the tubes were centrifuged and the absorbance of the organic layer containing the chromophore was read at 532 nm. Percent inhibition of lipid peroxidation by the extract was determined by comparing the absorbance values of the control and test samples. The 50 % inhibition values were derived from a plot quantity (µg) *vs* optical density.

Statistical analysis

The results are expressed as mean ± S.E.M. Statistical analysis was carried out using Student's paired *t*-test. In all the cases, $p < 0.05$ was considered statistically significant.

RESULTS

Inhibition of stress-induced urinary biochemical changes

As shown in Figs 1 and 2, induction of forced swim stress in the animals produced a significant ($p < 0.05$) increase in VMA level from 223.6 ± 11.4 (basal level) to 396.1 ± 15.1 µg/kg/24 h and a decrease in ascorbic acid excretion levels from 141.1 ± 17.0 (basal levels) to 66.7 ± 9.3 µg/kg/24 h, respectively. Both parameters returned to their normal basal levels in three to four days after withdrawal of stress. Daily administration of the extract alone to the animals produced no change in the excretion of VMA and ascorbic acid in relation to normal basal levels. Daily administration of the extract one hour prior to the induction of stress inhibited increase in urinary VMA levels in a dose-dependent manner (Fig 1). In contrast, daily administration of the extract one hour prior to the induction of stress inhibited the decrease in ascorbic acid excretion (Fig 2). The inhibition of the increase in VMA levels and decrease in ascorbic acid levels was significant at all dose levels ($p < 0.05$).

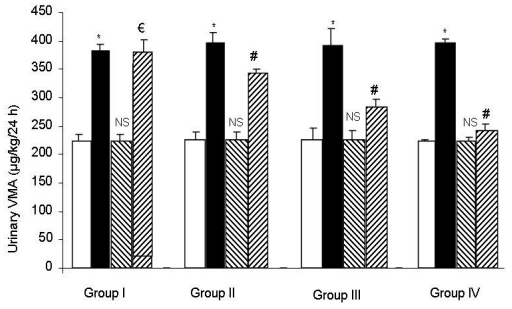

Fig 1: Influence of *A. graveolens* extract on the 24 h urinary levels of VMA in normal and stress induced rats **(**mean ± SEM, n=5). * $p < 0.001$ compared to normal condition of the corresponding groups; # $p < 0.05$, compared with stressed condition of the corresponding groups; NS no significant difference from normal condition of the corresponding groups; € no significant difference from stressed condition. Significance was determined using Student's *t*-test, $p < 0.05$ was considered statistically significant

□ = Normal; ■ = Stress; ▨ = Normal + *A. Graveolens*; and ▨ = *A. graveolens* + stress

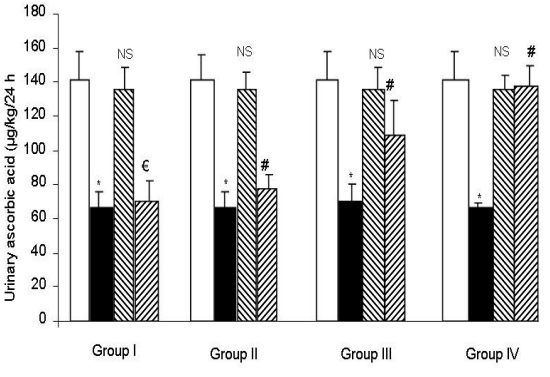

Fig 2: Effect of *A. graveolens* extract on the 24 h urinary levels of ascorbic acid in normal and stress-induced rats **(**mean ± SEM, n=5). * $p < 0.001$, compared to normal condition of the corresponding groups. # $p<0.05$, compared with stressed condition of the corresponding groups; NS no significant difference from normal condition of the corresponding groups; €no significant difference compared to stressed condition. Significance was determined using Student's *t*-test, $p < 0.05$ was considered statistically significant.

□ = Normal; ■ = Stress; ▨ = Normal + *A. Graveolens*; and ▨ = *A. graveolens* + stress

Effect of *A. graveolens* extract in attenuating scopolamine-induced memory loss

The CAR of rats administered the extract increased gradually to 95 % over seven days (Fig 3). The acquisition time (time to achieve 95 % CAR) for the rats administered with the extract was dose- and time-dependent, compared to vehicle-treated (control) group which took 10 days for acquisition. Percent avoidance was always higher in the extract-treated groups compared to the vehicle-treated (control) group. Animals receiving 300 mg/kg body weight of the extract took six days while groups treated with 200 and 100 mg/ kg doses of the extract required eight and nine days, respectively, to reach the point of acquisition. Administration of scopolamine produced amnesia as seen from the reduction in the observed CAR. Amnesia was greater in the control group than in extract-treated groups and was also found to be dose-dependent. However, continued treatment with *A. graveolens* produced higher retention and recovery in a dose-dependent manner than in the vehicle-treated animals. Recovery from scopolamine-induced amnesia in the extract-treated groups took 3 - 4 days, compared to normal (control) group which took over 5 days.

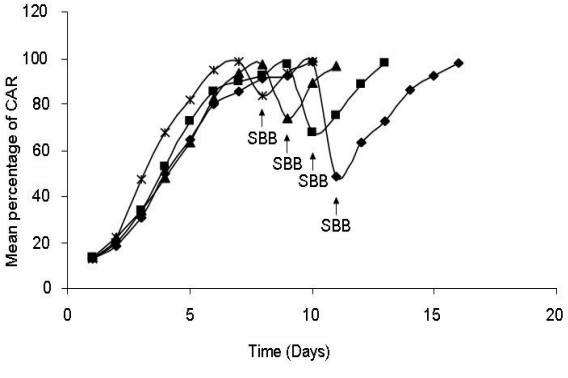

Fig 3: Effect of *A. graveolens* extract on conditioned avoidance response after oral administration in rats. *Note:* ♦ = Control, ■ = 100 mg, ▲ = 200 mg and * = 300 mg

Effect of extract on inhibition of lipid peroxidation of brain/liver homogenates

As shown in Fig 4, generation of lipid peroxidase by Fe^{2+}/ascorbate in rat liver and brain homogenates was inhibited by the extract in a dose-dependent fashion. The extract inhibited lipid peroxides in brain homogenate more than in liver, indicating that it is more effective in brain. The quantity of the extract needed for 50 % inhibition of lipid peroxidation in rat liver homogenate was 3300 µg (Fig. 4A). A similar effect was produced by 5350 µg of ascorbic acid. The quantity of the extract needed for 50 % inhibition of brain lipid peroxidation was 2480 µg; asimilar effect was produced by 4690 µg of ascorbic acid (Fig 4B).

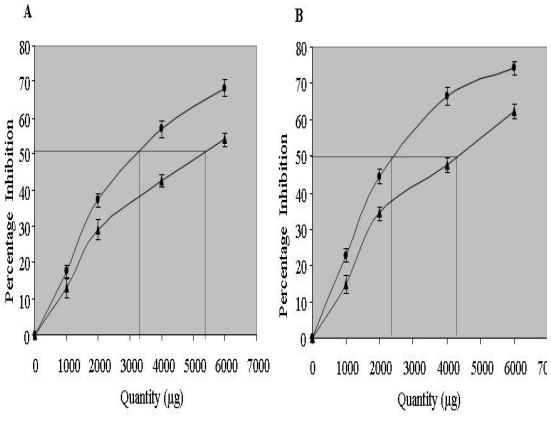

Fig 4: Effect of *A. graveolens* on the *in-vitro* inhibition of lipid peroxidation in liver and brain of rat (n = 6); ● = *A. graveolens* and ▲= ascorbic acid.

DISCUSSION

During stressful conditions noradrenaline is released and metabolized to vanillyl mandelic acid (VMA) peripherally and 3-methoxy 4-hydroxyphenyl glycol (MOPEG) centrally [19]. In the present study, VMA the major metabolite of sympathetic amines, was taken as an indirect biochemical index to represent the increase in peripheral sympathetic activity during stress. The increase in the urinary VMA excretion during stress was used as a non-invasive biochemical marker to study the anti-stress activity of *A. graveolens*.

On the other hand, L-ascorbic acid (vitamin C) is synthesized biologically from D-glucose in rats. Ascorbic acid is present in adrenal glands as a metabolite of glucose in rats and glucaric acid is the corresponding metabolite in humans and primates. Several factors such as age, exposure to environmental situations, stress, dietary, biochemical changes, etc, produce alteration of L-ascorbic acid levels in body fluids. Several studies also indicate that the tissue levels of ascorbic acid decrease on application of stress [20-23].

Ascorbic acid, being a free radical scavenger, is more likely utilized in scavenging the free radicals involved in stress, resulting in its decreased urinary concentration. Also, it plays a role in the biosynthesis of noradrenaline as a cofactor in the conversion of dopamine to noradrenaline [24]. Based on the above studies, ascorbic acid excretion in urine was taken as an indirect biochemical index to indicate the influence of stress on catecholamine synthesis in rats and hence, antistress activity of the *A. graveolens* extract after prior administration of stress induction. Treatment of stressed rats with *A. graveolens* extract reversed stress-induced biochemical changes, i.e., increase in urinary VMA levels and decrease in urinary ascorbic acid levels, in a dose-dependent manner. Previously published reports concluded that the antistress activity of some potential medicinal plants could be attributed to their antioxidant effects [25]. In the present sudy, *A. graveolens* extract showed higher antioxidant activity than that of a known antioxidant, ascorbic acid, in both liver and brain homogenates

It has been reported that scopolamine impairs retrieval memory of rats and such amnesia is associated with elevated MDA and reduced GSH levels [26]. Since oxidative stress has been implicated in the

pathophysiology of dementia, and also scopolamine has been reported to elevate rat brain oxidative stress, scopolamine-induced amnesia in rats could be used as a valid model to screen drugs with potential therapeutic benefit in dementia [26]. Earlier reports also indicated that improvement in cognition through inhibition of central acetylcholine esterase activity and decrease in brain amyloid beta protein deposition is at least, in part, mediated by antioxidant effects [26].

Antistress and antioxidant activities were correlated with the nootropic activity of the extract since the role of stress and free radicals have been implicated in loss of memory, cognitive deficits and also in Alzheimer's disease [27]. The memory process involves acquisition, retention and retrieval, and is measured using conditioned avoidance response (CAR). The acquisition was quicker in the extract-treated rats (100, 200 and 300 mg/kg body weight) than in control, indicating significant antistress effect of the extract. When challenged with scopolamine butylbromide, amnesia was less in the treated groups, showing better retention and recovery than the control group. The extract was shown to attenuate memory loss, probably due to its free radical scavenging mechanisms. Furthermore, the antioxidant activity of the extract, probably by combating oxidative damage, may provide a basis for its traditional use in relieving stress.

CONCLUSION

The present study provides scientific support for the antistress, antioxidant and cognition improving activities of *A. graveolens* extract and lends some credence to traditional claims of its therapeutic benefits in stress and stress-related disorders.

ACKNOWLEDGEMENT

This work was supported by Konkuk University in 2010.

REFERENCES

1. *Selye HA. Syndrome produced by diverse nocuous agents. Nature 1936; 38: 32-35.*
2. *Elliott GR, Eisdorfer C. Stress and human health. New York: Springer Publishing, 1982; p 19*
3. *Gerard E, Konrad B, Colin L, Jean-Marie M. Prospects for pharmacological intervention in Alzheimer disease. Arch Neurol 2000; 57: 454 – 459.*
4. *Perrig WJ, Perrig P, Stahelin HB. The relation between antioxidants and memory performance in the old and very old. J Am Geriatric Society 1997; 45: 718 – 724.*
5. *Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. Oxford: Clarendon Press, 1985; p 279 – 313.*
6. *Oberley LW. Free radicals and diabetes. Free Radic Biol Med 1988; 5: 113-124.*
7. *Yazdanparast R, Bahramikia S. Evaluation of the effect of Anethum graveolens L. crude extracts on serum lipids and lipoproteins profiles in hypercholesterolaemic rats. DARU 2008; 16: 88-94.*
8. *Kaur GJ, Arora DS. Bioactive potential of Anethum graveolens, Foeniculum vulgare and Trachyspermum ammi belonging to the family Umbelliferae - Current status. J Med Plant Res 2010; 4:87-94.*
9. *McIntyre A. Herbal treatment of children: Western and Ayurvedic perspectives,. Elsivier butterworth Heinemann publishing, 2005; p 21.*
10. *Ishikawa T, Kudo M, Kitajima J. Water-soluble constituents of Dill. Chem Pharm Bull 2002; 50: 501–507.*
11. *Stavri M, Gibbons S. The antimycobacterial constituents of Dill (Anethum graveolens). Phytother. Res 2005; 19: 938-941.*
12. *Sushruta K, Satyanarayana S, Srinivas N, Raja Sekhar J. Evaluation of the blood-glucose reducing effects of aqueous extracts of the selected umbelliferous fruits used in culinary practices. Trop J Pharm Res 2006; 5 : 613-617.*
13. *Derrell C. Guide for the care and use of laboratory animals . Istitute of Laboratory Animal Resources. National Academy Press, Washington DC, USA 1996.*
14. *Roe JH, Kuehter CA. The determination of ascorbic acid in bile blood and urine through 2, 4 dinitrophenylhydrazine derivatives of dehydroascorbic acid. J Biol Chem 1943; 147: 399-408.*
15. *Pisano JJ, Crout JR, Abraham A. Determination of 3-methoxy, 4-hydroxymandellic acids in urine. Clin Chim Acta 1962; 7: 277-284.*
16. *Nagaraja HA, Jaganathan PS. Influence of different types of stress on selected cardiovascular parameters in rats. J Physiol Pharmacol 1999; 43: 296-304.*

17. Cook L, Weidley E. Behavioural effects of some psycho-pharmacological agents. Ann NY Acad Sci 1957; 66: 740-752.
18. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 1979; 95: 351-358.
19. Ion M. Urinary excretion of catecholamines and vanillyl mandelic acid in rats exposed to cold. Acta Physiol Scand 1969; 76: 393-395.
20. Cheng TK, Coon CN, Hamre ML. Effect on environmental stress on the ascorbic acid requirement of laying hens. Pult Sci 1990; 69: 774-780.
21. Abramyan E, Kostanyan A. Vitamin C – An anti-stressor. Ptitsvevodstvo 1990; 4: 27-28.
22. Rivero GC, Orcutt DM. Antioxidant metabolism in water stressed peanut treated with diniconazole. Biotropia 1992; 5: 22-25.
23. Kutlu HR, Forbes JM. Changes in growth and blood parameters in heat stressed broiler chicks in response to dietary ascorbic acid. Liverstock Prod. Sci 1993; 36: 335-350.
24. Goodman GA. The pharmacological basis of therapeutics. (10th ed). New York: McGraw-Hill. 2001; p 1804-1805
25. Sreemantula S, Nammi S, Kolanukonda R, Koppula S, Boini KM. Adaptogenic and nootropic activities of aqueous extract of Vitis vinifera (grape seed): an experimental study in rat model. BMC Complement Alternat Med 2005; 5: 1-8.
26. El-Sherbiny DA, Khalifa AE, Attia AS, Eldenshery El D. Hypericum perforatum extract demonstrates antioxidant properties against elevated rat brain oxidative status induced by amnestic dose of scopolamine. Pharmacol Biochem Behav 2003; 76: 525-533.
27. Esch T, Stefano GB, Fricchione GL, Benson H. The role of stress in neurodegenerative diseases and mental disorders. Neuroendocrinol Lett 2002; 23: 199-208.