Journal of Phytology 2010, 2(8): 103-109
Phytophysiology
ISSN: 2075-6240
Available Online: www.journal-phytology.com

REGULAR ARTICLE

STABILITY STUDIES OF CRUDE PLANT MATERIAL OF *BACOPA MONNIERI* AND ITS EFFECT ON FREE RADICAL SCAVENGING ACTIVITY

Pratibha Srivastava[1*], Hema N. Raut[1], Hemalata M. Puntambekar[1], Anuradha S. Upadhye[2] and Anagha C. Desai[3]

[1]*Chemistry Group, Animal Science Division, Agharkar Research Institute, G. G. Agarkar Road, Pune-411004, Maharashtra, India*

[2]*Botany group, Plant Science Division, Agharkar Research Institute, G. G. Agarkar Road, Pune-411004, Maharashtra, India*

[3]*Drug Analysis, Indian Drug Research Association & Laboratory, 561/B Shivajinagar, Pune-411005, Maharashtra, India*

SUMMARY

Stability study of the crude plant material of *Bacopa monnieri* has been carried out as per ICH guidelines and its effect on free radical scavenging activity was studied by DPPH, ABTS radical scavenging assays and Deoxyribose degradation assay. The dried whole plant material of *B. monnieri* was kept in stability chambers at 40°C and 75% relative humidity in case of accelerated studies and at 30°C and 65% relative humidity for long term studies. Real time studies samples were stored at room temperature. Samples were taken out at periodic intervals, extracted in methanol to observe the HPTLC profiles and to study free radical scavenging activity. Results of antioxidant assays indicated that the samples of long term studies showed better antioxidant activity than the samples of accelerated and real time studies.

Key words: Brahmi; accelerated study; long term study; real time study; free radical scavenging activity

Abbreviations: BM: *Bacopa monnieri*; AS: Accelerated studies; LS: Long term studies; RT Real time studies

Pratibha Srivastava et al. Stability Studies of Crude Plant Material of *Bacopa monnieri* and Its Effect on Free Radical Scavenging Activity. J Phytol 2/8 (2010) 103-109.
*Corresponding Author, Email: psri94@yahoo.com, Tel.: +91-20-25653680 Ext. 351; Fax: +91-20-25651542

1. Introduction

Medicinal plants are used as raw material in the preparation of Ayurvedic formulations (1-2) due to presence of bioactive molecules, which are used for drug discovery (3). Duration of the activity of the drug depends upon the stability of phytomarkers available in the plants. In standard Ayurvedic texts it is mentioned that 'Veerya kalawadhi' is a certain period within which the drug is to be used in the preparation of medicine. Medicinal plants are either cultivated or collected from natural resources. At present very few species are cultivated systematically and majority of them are collected from wild. In this situation the excess material or one which is required after some time is stored in large store houses. Under such conditions stability and potency of the plant materials must be checked before using the crude drugs in the formulations.

Bacopa monnieri commonly known as 'Brahmi' belongs to family Scrophulariaceae, has been used in various Ayurvedic formulations. It is an important component of *medhyarasayana*, a class of plant drug used (4-5) for promoting mental health and intellect (*medhya*), to provide relief to patients with anxiety or epileptic disorders(6). The plant has also been used in India and Pakistan as a cardiac tonic, a digestive aid, and to improve respiratory function in case of bronchoconstriction (7). Research on anxiety, bronchitis, asthma,

irritable bowel syndrome and gastric ulcers supports the medicinal uses of Brahmi in Ayurvedic treatments. *B. monnieri* also possesses anxiolytic, antidepressant (8-9) and antioxidant effect (10-12). These activities are known to be beneficial for the treatment of tumor as psychological (13-15) and free radical factors (16) which have been implicated in the genesis of tumor. Standardized extract of *B. monnieri* (11) rich in active constituents referred to as saponins, mainly bacoside A (17), have been used as a clinical drug for memory and intellect improvement. Subsequent studies indicated that the cognition facilitating effect is due to the two active saponins, bacoside A and B, present in the ethanol extract (18). These active principles apart from facilitating learning and memory in normal rats inhibit the amnesic effects of scopolamine, electroshock and immobilization stress (19).

The alcoholic extract of Brahmi has been used in various pharmaceutical and pharmacological studies (20-22). Several products of Brahmi are marketed as drugs and food supplements, but, there is still a lack of information on stability of the crude material and its active components. Although Phrompittayarat *et al*. (23) reported the stability studies of saponins from *B. monnieri* dried ethanolic extract and Bhattacharya *et al*. (11) reported antioxidant activity of ethanolic extract, but to the best of our knowledge there is no report on stability studies of crude plant material of *B. monnieri* and their effect on free radical scavenging activity. Very well established pharmacological properties of *B. monnieri* encouraged us to know that how long these activities remain in the crude material of the plant. Therefore, in the present study we have made an effort to establish the effect of stability of crude material of *B. monnieri* and its consequences on free radical scavenging activity.

2. Methods

Chemicals and reagents

Ammonium persulphate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were procured from Fluka-Sigma Aldrich (USA). Ascorbic acid was procured from Molychem, India. Bacopaside I was procured from Natural remedies, Bangalore. All other chemicals unless otherwise mentioned were procured from Merck (Germany).Schimadzu UV-2501 PC spectrophotometer (Tokyo, Japan) was used for colorimetric analyses. A Camag (Muttenz, Switzerland) HPTLC system with HPTLC CAMAG CAT 4 software and CAMAG SCANNER 3 was used to develop HPTLC profiles. Thermolab stability chambers were used for stability studies.

Plant material

Whole plant of *Bacopa monnieri* was collected from Western Ghat in January 2010. It was authenticated by Dr. A. C.Desai of IDRA&L, Pune. Voucher specimen is deposited in IDRA&L vide voucher no. 02/2009-2010. It was cleaned, shade dried and processed for further studies.

Stability studies of crude plant drugs

Accelerated and long term studies were performed according to the International Committee Harmonization guidelines (24). Thermolab stability chambers were used for stability studies. The conditions maintained were 30 ^{o}C and 65% humidity for long term studies (LS) and 40 ^{o}C and 75% humidity for accelerated studies (AS). Samples were also kept at ambient temperature for real time studies (RT). Plant material was stored in fixed quantity for stability studies and it was taken out periodically at the interval of 1,2,3,6 months and at 3, 6 months for accelerated and long term studies respectively. Real time studies samples were also taken out at 3 and 6 months for the analysis.

Extraction

Dried whole plant material of *B. monnieri* (BM) was taken out from stability chambers at periodic intervals. Five gram of each sample was extracted with methanol. Real time study samples (5 g) stored at ambient temperature was also extracted with methanol at periodic intervals. The methanolic extracts were evaporated to dryness under reduced pressure.

HPTLC profiles of methanolic extracts of *B. monnieri*

A Camag (Muttenz, Switzerland) HPTLC system including a Linomat IV sample applicator, a Camag twin-trough plate development chamber, Camag TLC Scanner 3 and Cats integration software was used. Pre-coated aluminium HPTLC plates 10 ×10 cm with 0.2 mm layers of silica gel 60 F_{254} (Merck), were used. Bacopaside I was used as a standard phytomarker and a stock solution (1mg mL^{-1}) of standard was prepared. Test solutions and standard were applied by means of a Linomat IV sample applicator to the plates about 1 cm above the edge using a bandwidth of 8 mm and distance between the tracks of 5 mm. The chromatogram was developed up to 80 mm under chamber saturation conditions with n BuOH: AcOH: H_2O (4:1:5) as the mobile phase in a Camag twin trough chamber. The plates were then dried with an air dryer and scanned with a Camag TLC scanner 3 using a detector wavelength of 580 nm.

Antioxidant Activity

B. monnieri extracts of concentration (10 mg mL^{-1}) in methanol were screened for antioxidant activity.

DPPH assay

DPPH radical scavenging activity was assessed according to the method of Shimada *et al.* (25). BM extracts (50 µl, 10 mg mL^{-1}) in methanol were mixed with 1 ml of 0.05 M acetate buffer, 0.95 ml methanol and 500 µl of 0.5 mM solution of DPPH in methanol. Blank contained 50 µl of the BM extracts of same concentrations without DPPH, while the control was without test sample. The mixture was shaken immediately after addition of DPPH and allowed to stand at room temperature in dark. Decrease in absorbance at 517 nm was measured at 0 min and after every 30 min until the reaction reached a plateau. Samples were analyzed in triplicates. The inhibitory percentage of DPPH was calculated (26) as per the formula: Scavenging effect (%) = $[(A_0-(A-A_b))/A_0] \times 100$; where, A_0 = Absorbance of control; A = Absorbance of sample and A_b = Absorbance of blank.

ABTS radical cation decolorisation assay

ABTS assay (27) was used to evaluate the ABTS radical scavenging ability of the test sample. ABTS radical cations were generated by reacting ABTS solution (7 mM, 3 ml) in water with ammonium persulphate (2.45 mM, 15 ml) in water. The mixture was allowed to stand in dark at room temperature for 16 h before use. BM extract (250 µl, 10 mg mL^{-1}) in methanol was added to 150 µl of ABTS solution and final volume was made up to 500 µl with methanol. Control was prepared by adding methanol (350 µl) to ABTS solution (150 µl) while blank was without ABTS solution. All the samples were analyzed in triplicates. Absorbance was read at 745 nm and the percentage inhibition was calculated using the formula: % Inhibition = $[(A_0-(A-A_b))/A_0] \times 100$; where, A_0 = absorbance of control; A = absorbance of test solution and A_b= absorbance of blank.

Deoxyribose degradation assay

Scavenging of hydroxyl radical was measured by employing the method of Halliwell *et al.* (28) with slight modifications. Stock solutions of EDTA (1 mM), $FeCl_3$ (10 mM), ascorbic acid (1 mM), H_2O_2 (10 mM) and deoxyribose (10 mM) were prepared in distilled water. Reaction mixture was prepared by adding 0.05 ml of EDTA, 0.005 ml of $FeCl_3$, 0.05 ml of H_2O_2, 0.18 ml of deoxyribose, BM extracts (500 µl, 10 mg mL-1), phosphate buffer (50 mM, pH 7.4, 165 µl) and ascorbic acid (50 µl). The mixture was incubated at 37° C for 1 h. The incubated mixture (250 µl) was mixed with 250 µl of 10% TCA, 250 µl of 2% TBA in 0.025 M NaOH and heated at 80 ^oC for 1 h to develop pink chromogen, which was measured at 532 nm. Control was without test sample and blank was without H_2O_2. The inhibition effect on hydroxyl radicals was calculated as follows: % Hydroxyl radical scavenging capacity = (1-As/Ac) ×100; where Ac = Absorbance of control and As = Absorbance of sample.

3. Results

HPTLC profiles of the crude extracts of *B. monnieri* indicated that there was a continuous decrease in absorbance value of Bacopaside I at 580 nm in case of accelerated

study samples indicating the reduction in Bacopaside I content. The samples kept for long term studies also showed reduction in Bacopaside I quantity but the reduction of Bacopaside I content was less than the samples kept for real time studies and accelerated studies (Figure 1).

Figure 1a. HPTLC profile of *Bacopa monnieri*, AS and RT samples

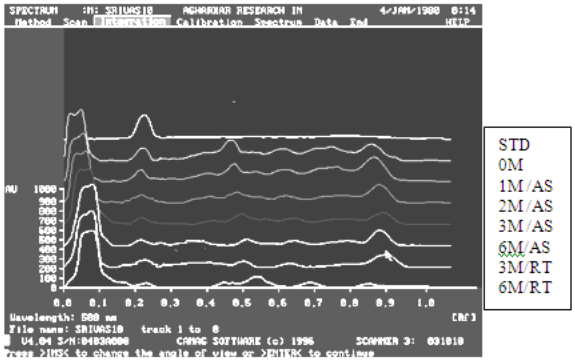
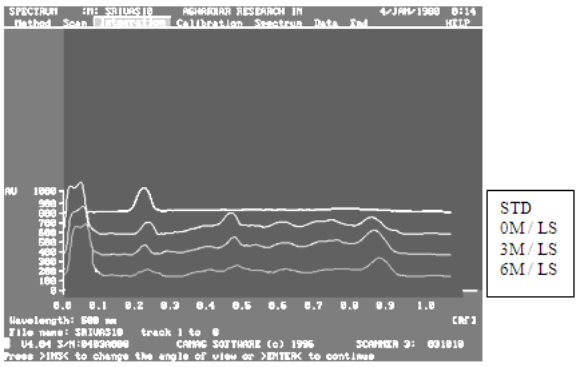


Figure 1b. HPTLC profile of *Bacopa monnieri*, LS samples



DPPH assay

BM samples showed less absorption at 517 nm as compared to control. The crude extract of BM at zero time showed 93.68 % activity by scavenging DPPH radical (Figure 2). In case of AS samples the activity reduced to 73.18 % after one month and it was 30.2 % after 3 months and at 6th month the activity reduced to 25.81%, whereas in LS samples the activity reduced to 53.7 % in 3 months and it was 36.44% after 6 months (Table-1). Similarly the samples kept for real time studies showed reduction in activity up to 27.73 % after 3 months time and the activity reduced to 18.84 % after 6 months (Table-1).

Figure 2. DPPH radical scavenging assay of *B. monnieri* at 517 nm

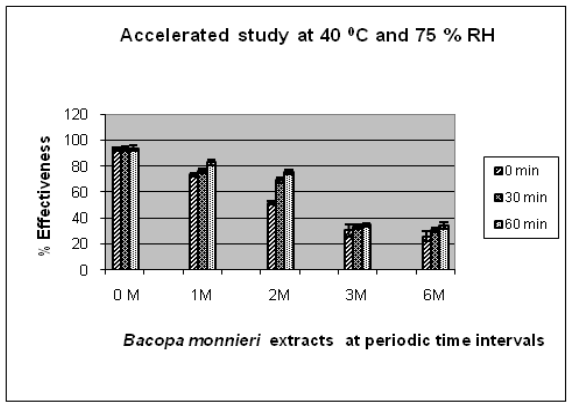


Table 1: Results of free radical scavenging activity of AS, LS, and RT samples of *B. monnieri*

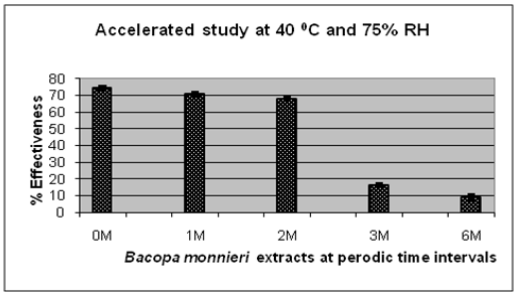
Conditions	Duration in Months	DPPH assay % Inhibition	ABTS assay %Effectiveness	Deoxyribose degradation Assay % Effectiveness
AS sample of BM	3	30.2	16.41	46.35
LS sample of BM	3	53.77	46.53	84.27
RT sample of BM	3	27.73	41.54	47.47
AS sample of BM	6	25.81	8.90	38.82
LS sample of BM	6	36.44	39.21	69.21
RT sample of BM	6	18.84	16.54	40.58

ABTS assay

Crude extract of BM at zero time intervals showed 74.7 % inhibition of ABTS radical cation (Figure 3). In AS samples the activity reduced gradually from 70.77 % in one month to 16.41 % in 3 months and at 6th

month the activity reduced to 8.9 %, whereas in LS samples the activity reduced to 46.53 % after 3 months and it was 39.21 % after 6 months (Table-1). The activity of the samples kept for real time studies also reduced up to 41.54 % after 3 months time and it was 16.54 % after 6 months (Table-1).

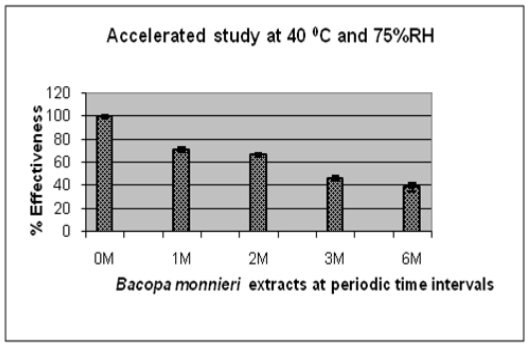
Figure 3. ABTS radical cation decolorization assay of *B. monnieri* at 745 nm



Deoxyribose degradation assay

The results of deoxyribose degradation assay for crude extract of BM showed 99.16 % inhibition of H_2O_2 radical at zero time (Figure 4). The activity reduced in gradual manner from 70.77 % in one month to 46.35 % in 3 months and at 6th month the activity was reduced to 38.82 % in case of AS samples, whereas for LS samples the activity reduced to 84.27 % in 3 months and it was 69.21% after 6 months (Table-1). The activity of the samples of real time studies reduced up to 47.47 % after 3 months time and it was 40.58 % after 6 months (Table-1).

Figure 4. Deoxyribose degradation assay of *B. monnieri* at 532 nm



4. Discussion

Excessive production of free radicals, due to oxidative stress in living being is known to be responsible for various neurodegenerative diseases such as Alzheimer's disease, Parkinsonism and aging (29). Free radicals accumulate in the body because of inefficient defense mechanism of certain free radical scavenging enzymes like SOD, CAT and GPX (30). Supplementation of natural antioxidants is a remedy to fight with oxidative stress but stability of natural antioxidants is equally important and should be considered before use of them as a drug.

Literature studies of *B. monnieri* indicate that most of the biological activities are due to their richness in saponins (11). These saponins are not very stable and their percentage reduces due to environmental conditions (23). Therefore, in the present study we have stressed on the stability studies of *B. monnieri* plant material and evaluated its effect on *in- vitro* free radical scavenging activity. The studies indicate that the fresh plant material shows a very good antioxidant activity in all the assays, but the activity decreases on keeping the plant material at different temperature and humidity conditions. The antioxidant activity has reduced in considerable amount in case of plant material stored for six months under various conditions. The experimental results also indicate that antioxidant activity reduces considerably in case of AS samples in comparison with LS samples of Brahmi plant. The results of real time studies also demonstrate that antioxidant activity of the real time studies sample was less than LS samples of Brahmi. These results also confirm the effect of storage conditions on antioxidant activity of *B. monnieri* and point out that activity decreases considerably on keeping the crude material as compared to fresh sample of *B. monnieri*

5. Conclusion

The present investigation supports the use of fresh Brahmi plant as a natural antioxidant and enlightens on suitable storage conditions of the plant material. It also suggests that samples stored for long term study possess better antioxidant activity than the samples of accelerated and real time studies. Outcome of the study indicates that Brahmi plant material should be preserved at lower temperature under dry conditions in

order to maintain physical, chemical and biological properties.

Funding

This work was supported by National Medicinal Plant Board, New Delhi, India. (R&D/MH-01/2009-10)

Acknowledgment

Authors are thankful to National Medicinal Plant Board (R&D/MH-01/2009-10), New Delhi, India for financial assistance to carry out this research.

References

1. Bagul, M. S., Srinivasa, H., Kanaki, N. S., Rajani, M. 2005. Antiinflammatory activity of two Ayurvedic formulations containing guggul. *Indian J. Pharmacol.* 37: 399-400.
2. Manonmani, S., Vishwanathan, V. P., Subramanian, S., Govindasamy, S. 1995. Biochemical studies on the antiulcerogenic activity of Cauvery 100, an Ayurvedic formulation in experimental ulcers. *Indian J. Pharmacol*. 27: 101-105.
3. Newman, D. J., Cragg, G. M., Snader, K. M. 2000. The influence of natural products upon drug discovery. *Nat. Prod. Rep*. 17: 215–234.
4. Chunekar, K. C. 1960. *Bhav Prakasa Nighantu* (Hindi translation). Chaukhamba Bharati Publications, Varanasi.
5. Satyavati, G. V., Raina, M. K., Sharma, M. 1976. *Medicinal Plants of India* 1: 118-122. ICMR, New Delhi.
6. Chopra, R. N. 1958. *Indigenous drugs of India*. (2nd ed). UN Dhur and Sons: Calcutta, India, 341.
7. Nadkarni, K. M. 1988. *The Indian Materia Medica*: South Asia Books: Columbia, MO, 624-625.
8. Bhattacharya, S. K., Ghosal, S. 1998. Anxiolytic activity of a standardized extract of *Bacopa monniera*: an experimental study. *Phytomedicine* 5: 77-82.
9. Zhou, Y., Shen, Y. H., Zhang, C., Su, J., Liu, R. H., Zhang, W. D. 2007. Triterpene saponins from *Bacopa monnieri* and their antidepressant effects in two mice models. *J. Nat. Prod.* 70: 652-655.
10. Tripathi, Y. B, Chaurasia, S., Tripathi, E., Upadhyay, A., Dubey, G. P. 1996. *Bacopa monniera* Linn. as an antioxidant: mechanism of action. *Ind. J. Exp. Biol.* 34: 523-526.
11. Bhattacharya, S. K., Bhattacharya, A., Kumar, A., Ghosal, S. 2000. Antioxidant activity of *Bacopa monniera* in rat frontal cortex, striatum and hippocampus. *Phytother. Res.* 14: 174-179.
12. Ghosh, T., Maity, T. K., Sengupta, P., Dash, D. K., Bose, A. 2008. Antidiabetic and *in vivo* antioxidant activity of ethanolic extract of *Bacopa monnieri* Linn. Aerial part: A possible mechanism of action. *Iranian J. Pharm. Res.* 7: 61-68.
13. Levy, S., Herberman, R., Lippman, M., d'Angelo, T. 1987. Correlation of stress factors with sustained depression of natural killer cell activity and predicted prognosis in patients with breast cancer. *J. Clin. Oncol.* 5: 348-353.
14. Levy, S. M., Herberman, R. B., Maluish, A. M., Schlien, B., Lippman, M. 1985. Prognostic risk assessment in primary breast cancer by behavioral and immunological parameters. *Health Psychol*. 4: 99-113.
15. Andersen, B. L., Kiecolt-Glaser, J. K., Glaser, R. 1994. A biobehavioral model of cancer stress and disease course. *Am. Psychol*. 49: 389-404.
16. Pawar, R., Gopalakrishnan, C., Bhutani, K. K. 2001. Dammarane triterpene saponin from *Bacopa monniera* as the superoxide inhibitor in polymorphonuclear cells. *Planta Med.* 67: 752-754.
17. Garai, S., Mahato, S. B, Ohtani, K., Yamasaki, K. 1996. Dammarane type triterpenoid saponins from *Bacopa monniera. Phytochemistry* 42: 815-820.
18. Singh, H. K., Dhawan, B. N. 1992. Drugs affecting learning and memory. In *Lectures in Neurobiology* 1: 189-207. ed. by Tandon, P. N.; Bijiani, V.; Wadhwa, S. Wiley Eastern, New Delhi.
19. Dhawan, B. N., Singh, H. K. 1996. Pharmacology of Ayurvedic nootropic *Bacopa monniera. Int. Conve. Biol. Psychiat.* Bombay Abstr No. NR 59,

20. Peng, L., Zhou, Y., Kong, D.Y., Zhang, W. D. 2010. Antitumor activities of dammarane triterpene saponins from *Bacopa monniera*. *Phtoyther. Res.* 24: 864-868.
21. Singh, H. K., Dhawan, B.N. 1997. Neuropsychopharmacoloical effects of Ayurvedic nootropic *Bacopa monnieri* Linn.(Brahmi). *Ind. J. pharmacol.* 29: 359-365
22. Das, A., Shanker, G., Nath, C., Pal, R., Singh, S., Singh, H. K. 2002. A comparative study in rodents of standardized extracts of *Bacopa monnieri* and *Ginkgo biloba*. Anticholinesterase and cognitive enhancing activities. *Pharmacol. Biochem. Behavior.* 73: 893-900
23. Phrompittayarat, W., Wittaya-areekul, S., Jetiyanon, K., Putalun, W., Tanaka, H., Ingkaninan, K. 2008. Stability studies of Saponins in *Bacopa monnieri* dried ethanolic extracts. *Planta Med.* 74: 1756-1763.
24. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. 2003. Stability testing of new drug substances and products Q1A (2). ICH Harmonized Tripartite Guideline (Step 4 version).
25. Shimada, K., Fujikawa, K., Yahara, K., Nakamura, T. 1992. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *J. Agric. Food. Chem.* 40: 945-948.
26. Shyu, Y. S., Hwang, L. S. 2002. Antioxidative activity of the crude extracts of lignan glycosides from unroasted Burma black sesame meal. *Food Res. Intl.* 35: 357-365.
27. Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free. Rad. Biol. Med.* 26: 1231-1237.
28. Halliwell, B., Gutteridge, J. M, Aruoma, O. I 1987. The deoxyribose method: a simple "test tube" assay for the determination of rate constants for reactions of hydroxyl radicals. *Anal Biochem* 165: 215-219.
29. Glover, V., Sandler, M. 1993. Neurotoxins and monoamine oxidase B inhibitors: possible mechanism for the neuroproductive effect of (-) deprenyl. In Inhibitors of monoamine Oxidase B. *Pharmacology and Clinical Use in Neurodengenerative Disorders*, ed. by I. Szelenyi, 169-181. Birkhauser Verlag, Basel.
30. Harman, D. 1991. The aging process; major risk factor for health and disease. *Proc Natl Acad Sci.* 88: 5360-5363.