Anticholinesterase, Antioxidant and Nitric Oxide Scavenging Activity of the Aqueous Extract of Some Medicinal Plants

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Aims: Enhancement of cholinergic activity and reduction of oxidative stress by scavenging free radicals such as nitric oxide are well recognized therapeutic approaches in several pathological conditions. We evaluated the anticholinesterase, antioxidant and nitric oxide scavenging activity of the aqueous extracts of *Ocimum basilicum, Curcuma longa* and *Solanum nigrum*.
Study design: Experimental
Place and duration of study: Delhi Institute of Pharmaceutical Sciences & Research, Delhi University, New Delhi, India between January 2008 and December 2008.
Methodology: The aqueous extracts of the rhizome of *Curcuma longa*, berries of *Solanum nigrum* and seeds of *Ocimum basilicum* were authenticated by HPTLC fingerprinting. The anticholinesterase activity of these extracts was estimated spectrophotometrically as described by Ellman in 1961 and IC50 was calculated. Total antioxidant capacity of extracts was also estimated spectrophotometrically based on the reduction of molybdenum (Mo) (VI) to Mo(V) by the sample and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH. Ascorbic acid was used as standard. Estimation of nitric oxide scavenging activity of extracts was based on the diazotization reaction.
Results: The anticholinesterase activity (IC50) was observed at the concentrations of 2.73 ± 0.09, 3.38 ± 0.05 and 3.88 ± 0.11 gram/l for *Solanum nigrum, Curcuma longa*, and *Ocimum basilicum* respectively. At these concentrations, maximum antioxidant capacity equivalent to 4.36 ± 0.14 mM of ascorbic acid was shown by *Curcuma longa*, followed by *Solanum nigrum*, and *Ocimum basilicum*. *Curcuma longa* showed the maximum nitric oxide scavenging activity equivalent to 29.78 ± 1.28 mM of sodium nitrite followed by *Solanum nigrum* and *Ocimum basilicum*.
Conclusion: Plant derived pharmacological agents may provide an attractive therapeutic option in future for several pathological conditions especially the neurodegenerative diseases due to their anticholinesterase, antioxidant and nitric oxide scavenging properties.

Keywords: Anticholinesterase, Antioxidant, Nitric oxide scavenging activity, Aqueous plant extracts

1. INTRODUCTION

Neurodegenerative disorders such as Alzheimer's and Parkinson's diseases are characterized by reduced cholinergic activity in brain [1]. The enzyme cholinesterase which exists as acetylcholinesterase (AchE) and butrylcholinestearse (BchE) causes hydrolysis of acetylcholine and its inhibition, therefore, plays a key role in enhancing cholinergic activity.

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Besides reduced cholinergic activity, oxidative stress has also been recognized as a key factor in the pathogenesis of neurodegenerative disorders like Alzheimer's disease [2]. Oxidation is an integral process in the cellular metabolism and oxidation. The free radicals produced in the process have unpaired electron [3-6]. Oxygen and nitrogen atoms with free unpaired electron are highly reactive and produce cellular injury by causing membrane lipid peroxidation and damage to enzymes and DNA [7].

Accordingly, the current therapeutic options in neurodegenerative disorders primarily involve the drugs that can increase the cholinergic activity as well as are antioxidants. A variety of anticholinesterases and antioxidants have been used, however, search for more effective and safer agents continues [8-10].

Several agents from plant origin have previously been investigated for their AchE inhibitory and antioxidant properties. In the present study we evaluated the AchE inhibitory, total antioxidant and NO scavenging activity of the aqueous extracts of the seeds of *Ocimum basilicum* (OB), rhizomes of *Curcuma longa* (CL) and berries of *Solanum nigrum* (SN).

OB is an annual and perrenial herb and shrub that belongs to the family Lamiaceae. It is also known as common basil or the sweet basil. In addition to the essential oils, it is rich in flavonoids and anthocyanins. The different types of OB extracts have been shown to possess antioxidant properties [11]. The essentials oils from OB have also been shown to possess AchE inhibitory activity [12]. CL, a perennial herb, is a member of the Zingiberaceae (ginger) family. Curcuminoids from CL have been shown to possess memory enhancing activities in in vitro and in vivo models [13]. Curcumin from CL has also been shown to possess nitric oxide (NO) scavenging and antioxidant properties [14, 15]. SN belongs to the family Solanaceae and consists of glycoalkaloids, glycoproteins and saponins. Its green berries and leaves contain glycoalkaloids like solanine and solasodine. The principal alkaloid, solanine, has anticholinesterase action that is attributed to its aglycone solanidine and the fruit extract has been shown to possess antioxidant properties [16]. As stated, various components from various parts of these 3 plants have been evaluated for AchE inhibitory, total antioxidant and NO scavenging properties. In the current study, for the first time, we evaluated these activities of the aqueous extracts of OB seeds, CL rhizome and SN berries.

2. MATERIAL AND METHODS

2.1 Plant Extracts

Dried aqueous plant extracts from seeds of OB, rhizome of CL and berries of SN were provided by Promed Exports Private Ltd, India, and all extracts were authenticated by HPTLC finger printing.

2.2 Evaluation of AchE Inhibitory Activity

The AChE inhibitory activity of three extracts was measured according to the method developed by Ellman et al., in 1961 [17]. All estimations were done in triplicates. The concentrations of the tested extracts that inhibited the hydrolysis of substrate by 50% (IC50) were estimated for all three extracts by a linear regression analysis between the percentage inhibition and the extract concentrations by using the Microsoft Excel program.

2.2.1 Principle of Reaction

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Acetylcholine iodide is used as the substrate. When acted upon by the AChE, acetylcholine iodide, the substrate, breaks down to thiocholine and acetate. Thiocholine is allowed to react with dithiobisnitrobenzoate (DTNB) and this reaction results in the development of a yellow colour. The changes in the intensity of yellow colour over a period of time, which can be estimated using a UV spectrophotometer, represent the activity of AChE.

2.2.2 Preparation of Enzyme and Solutions

Plasma from the venous blood of human volunteers was used as a source of enzyme AChE. Acetylcholine iodide 0.1mM and DTNB 0.3mM solution was prepared in phosphate buffer with pH 7.4. Physostigmine 1mM was prepared in distilled water and was used as a reference standard. The aqueous plant extracts were dissolved in distilled water so as to get the desired concentrations.

2.2.3 Assay

Fifty microliters of plasma was added to the assay tubes containing 3 ml of phosphate buffer and tubes were then incubated for 5 min at 37ºC. After incubation, 50 µl of extract sample or reference standard was added. 50 µl of distilled water was added instead of sample for blank. Solutions were again incubated for 5 min at 37ºC with intermittent shaking. DTNB solution, 100 µl, was now added to the tubes. This was followed by quick addition of 100 µl of acetylcholine iodide. The intensity of color change was measured with spectrophotometer at 412 nm at kinetic mode. Readings were taken at an interval of 15 sec for a total of 3 min.

2.2.4 Calculation of Enzyme Activity

The rate of color change per min was calculated for each reading. The rates were then averaged within each three min run. The rate of reaction was calculated according to the following formula:

$$\text{Activity (mol/min/l)} = \frac{\text{Change in absorbance} \times \text{Assay volume} \times 1000}{\text{Absorption coefficient} \times \text{Light path} \times \text{Sample volume}}$$

Assay volume = 3.3 ml; Absorption coefficient = 1.36 × 10-4/M/cm; Sample volume = 0.05 ml; Light path = 1cm

As no enzyme inhibition is taking place in blank the enzyme activity of blank was taken as 100%. By comparing with blank, percent enzyme activity and percent inhibition of enzyme activity of the extracts were calculated.

2.3 Evaluation of Antioxidant Activity

The evaluation of total antioxidant capacity was based on the method described by Prieto *et al*., in 1999 [18]. Each extract was used at the concentration equivalent to IC50 for AchE inhibition. All estimations were done in triplicates.

2.3.1 Principle of Reaction

This phosphomolybdenum method is now commonly used in extensive screenings of samples of very different origins and composition in search for powerful antioxidants. The assay is based on the reduction of Mo(VI) to Mo(V) by the sample and the subsequent formation of a green phosphate/Mo(V) complex at acidic pH. The method was optimized and characterized with respect to linearity interval, repetitivity, reproducibility, and molar

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absorption coefficients for the quantitation of several antioxidants by Prieto *et al*., in 1999 [18].

2.3.2 Assay

An aliquot of 0.1 ml of sample solution containing the aqueous extracts, in the same concentrations as for AchE inhibitory activity, was combined with 1 ml of reagent solution containing 0.6M sulfuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate. The solution was incubated in a water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. A typical blank solution contained 1ml of reagent solution and the equal volume of water as used for the sample. Incubation was done under the same conditions as the rest of the samples. Ascorbic acid, a water-soluble antioxidant, was used as standard and calibration curve was obtained using various concentrations of ascorbic acid. The antioxidant capacity was expressed as the equivalent of mM of ascorbic acid.

2.4 Evaluation of NO scavenging activity

Evaluation of NO scavenging activity was based on the method described by Griess in 1879 [19]. All estimations were done in triplicates.

2.4.1 Principle of Reaction

NO in oxygen-containing aqueous solution has a short half-life due to its rapid oxidation. It has been reported that NO in aqueous solution containing oxygen is oxidized primarily to nitrite (NO_2^-) with little or no formation of nitrate (NO_3^-) [20]. So, the NO formation is assessed by measuring NO_2^-. The assay relies on a diazotization reaction. The reaction utilizes sulfanilamide and N-1-napthylethylenediamine dihydrochloride (NED) under acidic conditions. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates NO [21, 14], which interacts with oxygen to produce nitrite ions that can be estimated by use of Greiss reagent. Scavengers of NO compete with oxygen leading to reduced production of nitrite ions [22].

2.4.2 Assay

Sodium nitroprusside **(**5 mM inPBS at pH 7.4) 100 µl solution was mixed with 750 µl of different concentrations of sodium nitrite (10 – 70 mM in water) or the equal volume of extracts and incubated at 25°C for 150 min. After incubation 200 µl of Griess reagent, containing 1% (w v^{-1}) sulphanilamide, 0.1% (w v^{-1}) NED and 2.5% (v v^{-1}) phosphoric acid, was added and the absorbance of the coloured compound formed due to diazotization of nitrite with sulfanilamide and subsequent coupling with NED was read at 546 nm. The linear standard curve was obtained by plotting the mean absorbance for each standard concentration against the sodium nitrite concentration. The standard curve was used to calculate the sodium nitrite (mM) equivalent activity in the test sample.

3. RESULTS AND DISCUSSION

3.1 AchE Inhibitory Activity

The method was first validated and absorbances were measured in the absence or presence of different concentrations of inhibitor, physostigmine, at concentrations ranging from 0.5 – 1.5 mmol. The linearity of method was established**.** In the presence of physostigmine, a potent anticholinesterase inhibitor, significant inhibition of AchE was observed. Physostigmine in a concentration of 1mM resulted in a 95.25% inhibition of AChE activity.

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Among the extracts 50% of the AchE inhibitory activity (IC_{50}) was observed at the concentrations of 2.73 ± 0.09, 3.38 ± 0.05 and 3.88 ± 0.11 g/l for SN, CL and OB respectively. A combination of all three extracts at above concentrations showed 72.25% AchE inhibition.

3.2 Total Antioxidant Capacity

The phosphomolybdenum assay was performed using ascorbic acid in the concentration range of 1 to 5 mM (Fig 1). The antioxidant capacity of the three aqueous extracts was estimated and expressed as equivalents of mM of ascorbic acid. Among the three extracts the maximum antioxidant capacity was shown by CL, which was equivalent to 4.36 ± 0.14 mM of ascorbic acid followed by SN and OB with a mean value of 2.12 ±0.11 and 1.88 ± 0.18 mM of ascorbic acid respectively. The antioxidant capacity of CL was significantly higher as compared to other extracts (P<0.001). The antioxidant capacity of combination consisting of OB, CL and SN was equivalent to 5.95 ± 0.32 mM of ascorbic acid. The antioxidant capacity of herbal combination was significantly higher than each extract (P<0.05 vs CL and P<0.001 vs OB or SN) (Fig 2).

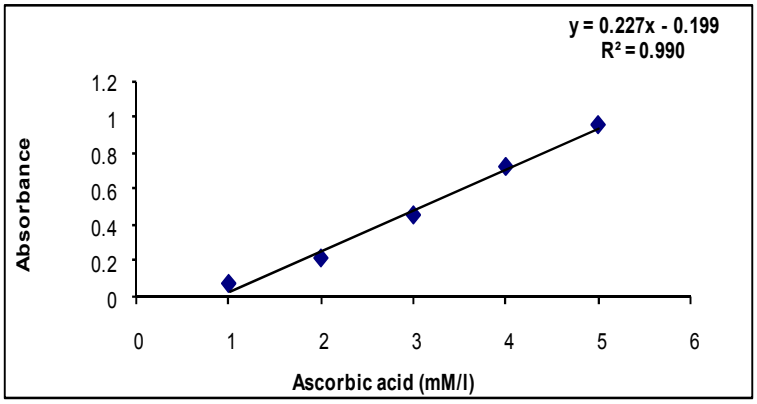

Fig 1: Standard curve for ascorbic acid in phosphomolybdenum assay.

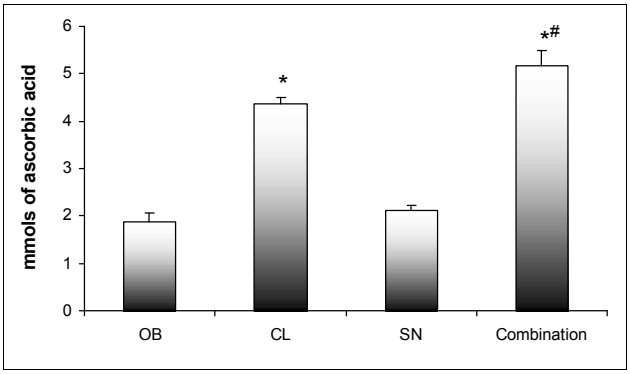

Fig 2: Total antioxidant capacity of three extracts and their combination.
*P<0.001 versus OB and SN; #P<0.05 versus CL

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3.3 NO Scavenging Activity

The NO scavenging activity of three extracts and their combinations was estimated in a diazotization reaction. The calibration curve for sodium nitrite (10-70 mM/l) was used to calculate the NO scavenging activity of test drugs which was expressed as equivalent to mM of sodium nitrite (Fig 3). Among the three aqueous extracts, the CL showed the maximum NO scavenging activity, which was equivalent to 29.78 ± 1.28 mM of sodium nitrite. The NO scavenging activity of SN and OB was equivalent to 11.71 ± 1.84 and 11.34 ± 2.30 mM of sodium nitrite respectively. The NO scavenging activity of CL was significantly higher than that of two other extracts (P<0.01). The NO scavenging activity of the combination of three extracts was equivalent to 39.83 ± 1.82 mM of sodium nitrite and this was significantly higher than that of each extract (P<0.001vs OB & SN, P<0.05 vs CL). (Fig 4)

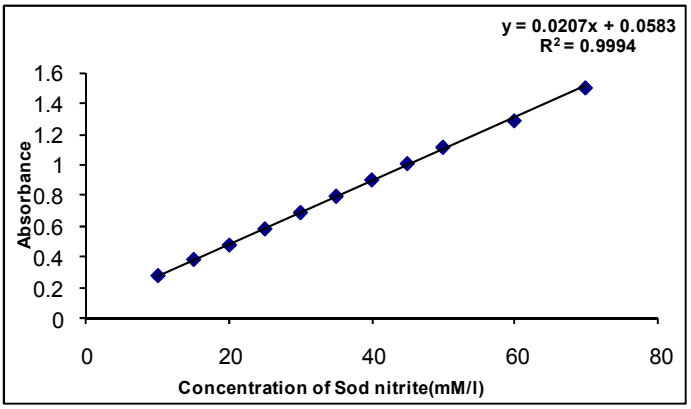

Fig 3: Diazotization reaction using different concentrations of sodium nitrite

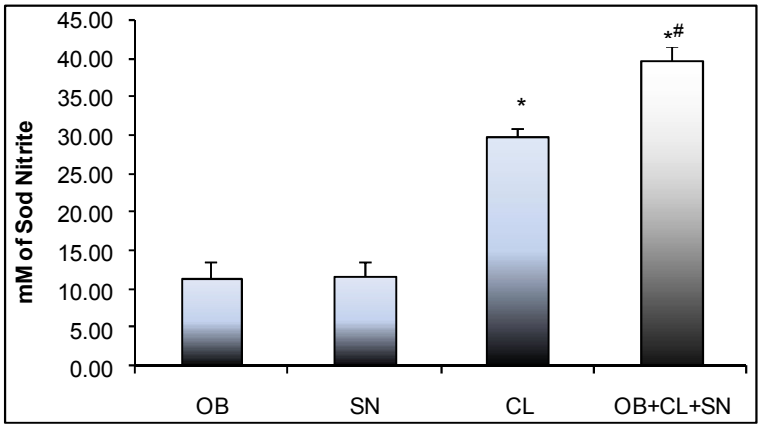

Fig 4: Nitric oxide scavenging activity of three plant extracts and their combination
*P<0.001 versus OB or SN; #P<0.05 versus CL

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Present study demonstrated AchE inhibitory, antioxidant and nitric oxide scavenging activity of the aqueous extracts of three medicinal plants. Enhancement of cholinergic activity by prolonging the availability of acetylcholine in synaptic clefts is a well recognized therapeutic approach in several pathological conditions especially the neurodegenerative diseases. Inhibition of AchE and butrylcholinesterase (BchE) provides the basis of such therapeutic options. Inhibition of AchE has been shown to enhance cholinergic transmission in the brain and additionally it has been observed that AchE inhibition reduces aggregation of β-amyloid and formation of neurotoxic fibrils in Alzheimer's disease [23]. Inhibition of BchE in cases with BchE polymorphism having reduced BchE activity has also been shown to slow down the progression of Alzheimer's disease [24]. Thus, AchE and BchE inhibitors have been recognized as remarkable alternatives [25]. As the Ellman reaction measured both AchE and BchE activity, the extracts evaluated in this study were found to have significant AchE and BchE inhibitory activity. Oxidative stress as an underlying pathophysiological process is also well recognized in these neurodegenerative disease processes. ROS are responsible for the damage of cellular bio-molecules such as proteins, enzymes, nucleic acids, lipids and carbohydrates and may adversely affect immune functions [26]. Antioxidants and nitric oxide scavengers, therefore, play a key role by preventing the cellular damage either by scavenging them or by reducing their production. Currently used anticholinesterase drugs used to treat Alzheimer's disease such as tacrine, donepezil, galantamine and heptylphysostigmine cause several adverse effects such as hepatotoxicity. Additionally these drugs have short duration of action, low bioavailability, peripheral cholinergic adverse effects and narrow therapeutic window. Therefore, investigations of newer drugs that possess both AchE inhibitory and antioxidant properties and are safe are extremely important not only for treatment Alzheimer's disease but also for prevention of the neuronal cell damage from ROS.

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Historically, active components from plants have provided important sources of new drugs. Since, neurodegenerative diseases such as Alzheimer's have become a public health burden and the currently available drugs have undesirable side-effects, new treatment options based on medicinal plants may be useful therapeutic options.

4. CONCLUSION

The aqueous extracts of the *Curcuma longa* rhizome, *Solanum nigrum* berries and *Ocimum basilicum* seeds showed significant anticholinesterase, antioxidant and nitric oxide scavenging properties. New treatment options based on these plant extracts may provide an attractive therapeutic option in future.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support by Department of Science and Technology, Government of India, for carrying out this work.

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COMPETING INTERESTS

All authors declare that no competing interests exist.

AUTHORS' CONTRIBUTIONS

Renu Agarwal, designed the study, performed the experiment and statistical analysis, wrote the protocol, and wrote the first draft of the manuscript.
SK Gupta designed the study and protocol and participated in manuscript revision and final approval.
Puneet Agarwal participated in writing protocol and manuscript
Sushma Srivastava participated in study design, conducting the experiment and manuscript preparation.
Renad Alyautdin participated in manuscript revision and final approval.
All authors approved the final version of manuscript.

REFERENCES

1. Nunes-Tavares N, Santos LE, Stutz B, Brito-Moreira J, Klein WL, Ferreira ST, de Mello FG. Inhibition of choline acetyltransferase as a mechanism for cholinergic dysfunction induced by amyloid-β peptide oligomers. J Biol Chem. 2012;287(23):19377-85.
2. Lee SH, Kim KR, Ryu SY, Son S, Hong HS, Mook-Jung I, Lee SH, Ho WK. Impaired short-term plasticity in mossy fiber synapses caused by mitochondrial dysfunction of dentate granule cells is the earliest synaptic deficit in a mouse model of Alzheimer's disease. J Neurosci. 2012;33(17):5953-63.
3. Finkel T, Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. Nature. 2000;408: 239-47.
4. Halliwell B. The antioxidant paradox. Lancet. 2000;355:1179-80.
5. Pietta P. Flavonoids as antioxidant. J Nat Prod. 2000;63:1035-42.
6. Visioli F, Keaney Jr JF, Halliwell B. Antioxidants and cardiovascular disease; pancreas or tonics for tired sheep. Cardiovasc Res. 2000;47:409-18.
7. Husain SR, Cillard J, Cillard P. Hydroxyl radical scavenging activity of flavonoids. Phytochemistry. 1987;26:2489-97.
8. Colombres M, Sagal JP, Inestrosa NC. An overview of the current and novel drugs for Alzheimer's disease with particular reference to anti-cholinesterase compounds. Curr Pharm Des. 2004;10(25):3121-30.
9. Subhashini N, Nagarajan G, Kavimani S. In vitro antioxidant and anticholinesterase activities of Garcinia combogia. Int J Pharm Pharm sci. 2011;3(3):129-32.
10. Ahirwar S, Tembhre M, Gour S, Namdeo A. Anticholinesterase Efficacy of Bacopa monnieri against the Brain Regions of Rat- A novel approach to therapy for Alzheimer's disease. . Asian J Exp Sci. 2012;26(1):65-70.
11. Kaurinovic B, Popovic M, Vlaisavljevic S, Trivic S. Antioxidant capacity of Ocimum basilicum L. and Origanum vulgare L. extracts. Molecules. 2011:16(9):7401-14.
12. Orhan, Kartal M, Kan Y, Sener B. Activity of essential oils and individual components against acetyl- and butyrylcholinesterase. Z Naturforsch C. 2008;63(7-8):547-53.
13. Ahmed T, Gilani A H. Inhibitory effect of curcuminoids on acetylcholinesterase activity and attenuation of scopolamine-induced amnesia may explain medicinal use of turmeric in Alzheimer's disease. Pharmacol Biochem Behav. 2009;91(4):554-9.
14. Sreejayan, Rao M N. Nitric oxide scavenging by curcuminoids. J Pharm Pharmacol. 1997;49:105-7.

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15. Huang HC, Xu K, Jiang Z F. Curcumin-Mediated Neuroprotection Against Amyloid-β-Induced Mitochondrial Dysfunction Involves the Inhibition of GSK-3β. J Alzheimers Dis. 2012;32(4):981-96.
16. Perez RM, Perez JA, Garcia LMD, Sossa HM. Neuropharmacological activity of Solanim nigrum fruit. J. Ethnopharmacol. 1998;62(1):43-8.
17. Ellman GL, Callaway E. Erythrocyte cholinesterase-levels in mental patients. Nature. 1961;192:1216.
18. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem. 1999;269(2): 337-41.
19. Griess P. Bemerkungen zu der abhandlung der H.H.Weselsky und Benedikt "Ueber einige azoverbindungen". Chem Ber. 1879;12:426-8.
20. Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE, Byrns RE. Oxidation of Nitric Oxide in Aqueous Solution to Nitrite but not Nitrate: Comparison with Enzymatically Formed Nitric Oxide From L-Arginine. Proc Nat Acad Sci. 199;90:8103-7.
21. Marcocci L, Maquire JJ, Droy-Lefaix MT, Packer L. The nitric oxide-scavanging properties of *Gingko biloba* extract EGb 761. Biochem Biophys Res Commun. 1994;201(2):748-55.
22. Marcocci L, Maquire JJ, Droy-Lefaix MT, Packer L. Antioxidant actions of *Gingko biloba* extract EGb 761. Methods Enzymol. 1996;234:462-75.
23. Hodges L. Alzheimer's centennial legacy: origins, landmarks and the current status of knowledge concerning cognitive aspects. Brain. 2006;129:2811-22.
24. Loizzo M R, Menichini F, Conforti F, Tundis R, Bonesi M, Saab A M, Statti G A, Cindio B, Houghton PJ, Menichini F, Frega N G. Chemical analysis, antioxidant, anti-inflammatory and anticholinesterase activities of Origanum ehrenbergii Boiss and Origanum syriacum L. essential oils. Food Chem. 2009;117:174-80.
25. Orhan, Sener B, Choudhary MI, Khalid A. Acetylcholinesterase and butyrylcholinesterase inhibitory activity of some Turkish medicinal plants. J Ethnopharmacol. 2004;91:57-60.
26. Nilsson J, Stegmark R, Åkesson B. Total antioxidant capacity in different pea (*Pisum sativum*) varieties after blanching and freezing. Food Chemistry. 2004;86(4):501–7.

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