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Anticholinesterase and Antioxidant Effects of Traditional Herbal Medicines used in the Management of Neurodegenerative Diseases in Mauritius

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ABSTRACT

Bioactive plant constituents from traditional herbal medicines can simultaneously protect neurons against oxidative stress and act as cholinesterase inhibitors, two key factors involved in the pathogenesis of neurodegenerative diseases. This study thus aimed at investigating the antioxidant and anticholinesterase activities of five herbal medicines, including 3 polyherbal formulas (F1, F2, F4) and 2 single-herb formulas (F3 and F5), currently used in Mauritius. Antioxidant activities were determined by the reducing potential, scavenging and chelating properties while the pro-oxidant effects was characterized by the copper-phenanthroline assay. While all extracts exhibited antioxidant activity, different extent of such property was observed in each assay. F3 containing *Gingko biloba* L. demonstrated a higher ferric reducing antioxidant potential (1654 ± 37.8 µmol Fe (II) equivalent/g dry weight) compared to the other extracts. The elixir F2 consisting of *Gingko biloba* L., *Hypericum perforatum* L. and *Salvia miltiorrhiza* Bunge was a potent scavenger of hypochlorous acid and hydroxyl radical and was a strong iron (II) chelator. All extracts inhibited acetylcholinesterase enzyme in a dose-dependent manner, which correlated strongly with total phenolics ($r=0.894$, $p<0.01$) and total proanthocyanidins ($r=0.937$, $p<0.01$). These findings suggested that activities of the locally available herbal drugs used to slow the progression of neurodegenerative disorder might be partly ascribed to their antioxidant and anticholinesterase activity.

KEY WORDS: ***Cholinesterase inhibitors; Oxidative stress; Pro-oxidant activities; Traditional herbal medicine; Neurodegenerative diseases***

INTRODUCTION

Life expectancy has significantly increased in the last century. As a consequence, a number of age-related diseases, primarily neurodegenerative diseases (ND), have emerged[1]. The high incidence of neurodegenerative disorders primarily Alzheimer's disease (AD) and Parkinson's disease has been linked to the devastating nature of the condition and the unsuccessful treatment options despite various advances in understanding the disease and treatment with conventional medicines.

In Mauritius, the elderly population consists of 13% of the total population compared to 9.1% in 2000[2] and it is estimated that between 4000 and 7000 persons suffer from AD[3]. AD has been characterized by progressive decline of cognitive function and memory, which subsequently impact on the quality of life[4]. Currently, there are no successful treatments that can stop or reverse the progression of the above-mentioned disease. Drugs currently used to slow down the deterioration of AD include cholinesterase inhibitors and agonists of N-methyl-D-aspartate receptors, but none of these therapies have shown promising result and their use have been limited due to undesirable side effects namely nausea, vomiting, headache and diarrhoea[5].

Conventional treatment consists in relieving symptoms associated with ND, but due to side effects associated with these medicines, interest has been directed towards traditional herbal medicines (TMs) as a potential treatment. A number of herbal medicines have been used to complement the conventional drug therapies in the management of neurodegenerative diseases for centuries[6]. Many active pharmacological compounds from herbal medicines have been identified to be useful in treatment of ND. Some plants, such as *Salvia miltiorrhiza* Bunge and *Ginkgo biloba* L. have been reported to possess anti cholinergic activities[7]. In recent years, scientists have isolated some compounds, which could improve mental function with fewer side effects than conventional drugs[8]. In view of their reduced cost, perceived effectiveness of plants, movement towards self-medication and improved quality and efficacy, several herbal medicines have been formulated for use against ND. Similarly, a range of TMs has been available for the management of ND in Mauritius since decades. However, only limited data on their phytochemistry and biological activities are available to the scientific community.

Oxidative stress has been believed to participate in normal ageing as well as in neurodegenerative processes[9]. Although the brain accounts for less than 2% of the body weight, it uses about 20% of oxygen from respiration[9]. This high oxygen demand accounts for the brain being susceptible to damage by reactive oxygen species (ROS)[9]. Furthermore, due to its abundant lipid content and a relative deficit in antioxidant systems compared to other tissues the brain has been reported as particularly more prone to oxidative damage[10]. Antioxidants have thus been advocated to protect the body defence system against ROS. Many constituents of herbal extracts have inherent antioxidant properties, therefore restoring oxidative balance may be one of the underlying mechanisms by which medicinal plants can protect against ageing and cognitive decline. The antioxidant activity of plants might be due to the presence of polyphenolic compounds, such as phenolic acids and flavonoids[6,11]. It can be speculated that since increased activity of acetylcholinesterase (AChE) enzyme have been involved in ND, these herbal medicines could exhibit their therapeutic action via anticholinesterase effect. This study thus aimed at investigating the anticholinesterase and antioxidant efficacies of selected traditional herbal medicines used against neurodegenerative diseases.

MATERIALS AND METHODS

Chemicals and equipment

Folin-ciocalteau reagent, gallic acid and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were purchased from SD Fine-Chem limited (India) and aluminium chloride from Techno Pharmchem (India)., Quercetin and cyanidin chloride were of HPLC grade, 1, 10-

phenanthroline hydrate, calf thymus DNA and deoxyribose were obtained from Sigma Chemicals (UK). All other chemicals used were of analytical grade. All the spectrophotometric data were obtained using a GENESYS 10S UV-Vis spectrophotometer, Thermo Scientific, USA.

Herbal medicines under study

Five locally used herbal products were investigated. The herbal medicines used in the study have been indicated in **Table 1**. F1 was purchased from a traditional medicinal plant seller at Rose-Hill market and was locally prepared. F2, an elixir (a liquid pharmaceutical preparation) was obtained from Elixirs Herbals Ltd, Port Louis, F3 and F4 were purchased from an herbal medicinal shop in Port Louis while F5 was obtained from Fabric India, Trianon. The compositions of the latter were indicated on the labels.

The extract for each formulation was prepared according to the procedure on the product packaging or as suggested by the herbal practitioner.

Qualitative determination of secondary metabolites in the herbal medicines

The presence of selected phytochemicals, namely alkaloids, saponins, phenolic compounds, anthraquinones and steroids were measured qualitatively by a method adapted and modified from *Ref*[12-14] (**Table 2**).

Total phenolic content

The total phenolic content of the extracts was estimated using the Folin-Ciocalteu method adapted from *Ref*[15]. An aqueous aliquot (0.25 ml) of the extract was added to 3.75 ml of distilled water in a test tube, followed by 0.25 ml of Folin-Ciocalteu reagent. After 3 min, 0.75 ml of 20% sodium carbonate was added. Tube contents were vortexed and heated at 40 °C for 40 min. The optical density was read at 685nm against a blank standard. Gallic acid was used as standard (stock concentration 200 µg/ ml) and the results were expressed as mg gallic acid equivalent (GAE) /g dried weight (DW) of herbal formulation and mg GAE/ml for the elixirs.

Total flavonoid content

The aluminium chloride method was used for the determination of the total flavonoid content of the extracts[16]. 1.5 ml of the methanolic extract was added to an equal volume of 2% $AlCl_3.6H_2O$. The mixture was incubated for 10 minutes at ambient temperature. The absorbance of the solution was read at 440 nm. Quercetin was used as standard (stock concentration 100 µg/ ml). The results were expressed as mg quercetin equivalent (QE)/ g DW of herbal formulation and mg QE/ml for the elixirs.

Total proanthocyanidin content

A modified acid/butanol assay was used to quantify the total proanthocyanidin content[17]. 0.25 ml aliquot of the methanolic extract was added to 3 ml of a 95% solution of n-butanol/HCl (95:5 v/v) followed by 0.1 ml of a solution of $NH_4Fe(SO_4)_2.12H_2O$ in 2 M HCl. The tubes were incubated for 40 min at 95 ^{o} C. The optical density of the cooled extract was read at 550 nm. The standard used was cyanidin chloride (stock concentration 100 µg/ml). Total proanthocyanidin content was expressed as mg cyanidin chloride equivalent (CCE) /g DW herbal formulation and mg CCE/ml for the elixirs.

Ferric reducing antioxidant power assay

The method of Benzie and Strain was used to assess the reducing power of the extracts[18]. Fresh FRAP reagent was prepared from 10 mM 2, 4, 6-TPTZ and 20 mM $FeCl_3$ in 0.25 M acetate buffer, pH 3.6. 100 µl of plant sample was added to 300 µl water followed by 3 ml FRAP reagent. The mixture was incubated at room

temperature for 4 minutes and the absorbance was read at 593 nm. The calculations were made with respect to calibration curve of ferrous sulphate (stock concentration 100 µM) and expressed in mmol Fe (II)/g dry weight (DW) herbal formulation and mmol Fe (II)/ml for the elixirs.

Table 1: Formulations under study, their composition, indication for use and method of administration

Formulation	Indication for use	Description	Composition of each formulation	Prescribed method of administration	Availability
F1	Alzheimer's disease	A mixture of two air dried leaves	*Verbena officinalis* L. leaves, *Rosmarinus officinalis* L. leaves	Taken as an infusion	Local
F2	Alzheimer's disease	WH/II: A herbal elixir	*Ginkgo biloba* L. leaves, *Hypericum perforatum* L. stems, buds, leaves and *Salvia miltiorrhiza* Bunge roots	Diluted before being orally taken	Imported
F3	Improvement of brain function and memory, Alzheimer's disease	Ginkola: A Chinese Traditional Medicine consisting of capsules	*Ginkgo biloba* L. leaves (40mg *Ginkgo biloba* L. extract powder per capsule)	Swallowed with water	Imported
F4	Neurasthenia, vertigo and dizziness, lumbago and poor memory	Healthy Brain Pills: A Chinese Traditional Medicine consisting of pills	Dry ripe seed of *Ziziphus jujube* Mill. var. spinosa Bunge, dried roots of *Angelica sinensis*(Oliv.) Diels, *Dioscorea oppositaThunb.* , *Cistanche deserticola* Y. C. Ma, *Lycium barbarum* L., *Schisandra chinensis* (Turcz.) Baill., ripe fruit of *Alpinia oxyphylla*Miq., *Succinum* ,*Bambusa arundinacea* Retz., *Dens Draconis*, *Anemone altaica* Fisch. Ex C.A. Mey., dried rhizome of *Gastrodiae lata* Bl., *Radix Salvia Miltiorrhizae* Bunge, dried root of *Panax ginseng* C.A. Meyer , *Semen Biota orientalis* L.	Swallowed with water	Imported
F5	Mental stress, depression, lack of concentration, memory decline, negative emotion, leg oedema due to venous insufficiency Alzheimer's disease and paralysis, herpes simplex	Brahmi: An Ayurvedic Medicine consisting of capsules	*Centella asiatica* L. leaves	Swallowed with water	Imported

Table 2: Qualitative analysis of the five herbal medicines

Phytochemical	Test
1. Alkaloids	A few drops of Dragendorff reagent were added to 1 ml of the test solutions and the formation of a reddish brown precipitate confirmed the presence of alkaloids[13].
2. Saponins	5 ml of 60°Cwater was added to the test solutions and shaken well for 2 - 5 minutes. Frothing activity and the presence of a persistent foam indicated the presence of saponins[14].
3. Phenolic compounds	A few drops of 10% ethanolic ferric chloride were added to 1 ml of the test solutions and a dark blue color indicated the presence of phenolic compounds[12].
4. Anthraquinones	2 ml of the test solution was shaken with 4 ml of hexane. The upper lipophilic layer was separated and treated with 4 ml of dilute ammonia. A pink/reddish colour in the lower layer indicated the presence of anthraquinones[12].
5. Steroids	1 ml of the sample was treated with three drops of acetic anhydride and one drop of concentrated sulfuric acid. A bluish green color indicated the presence of steroids[12].

Scavenging of preformed hypochlorous acid

The ability of the samples to scavenge hypochlorous acid (HOCl) was assessed and the method used involved chlorination of the amino acid taurine[19]. The reaction mixture contained taurine (10 mM), HOCl (1 mM), plant extract (variable concentrations: F1 – 1.46-8.78 mg/ml; F2 – 0.0024-0.048 ml/ml; F3 – 0.4-8 mg/ml; F4 – 1.8-36 mg/ml; F5 – 3.5-70 mg/ml), phosphate buffer saline (pH 7.4) in a final volume of 1 ml. The solution was mixed thoroughly and incubated for 10 minutes at ambient temperature followed by the addition of 10 µl of potassium iodide (2 mM). A yellow coloration was developed and the absorbance was read at 350 nm. Ascorbic acid was used as positive control (concentration range of 0 to 0.5 mg/ml). Percentage scavenging activity was calculated using *equation 1*. The analyses were made in triplicates and the results were expressed as concentration of formulation required to produce 50% scavenging activity (IC_{50}).

$$\%\ Scavenging\ activity\ = 100 \times \frac{Abs_{control} - Abs_{sample}}{Abs_{control}}$$

(Equation 1)

Iron (II) chelating activity assay

The assay was carried out according to *Ref*[19]. 200 µl of different concentrations of extracts (variable concentrations: F1 – 0.351-8.78 mg/ml; F2 – 0.00192-0.048 ml/ml; F3 – 0.32-8 mg/ml; F4 – 0.36-36 mg/ml; F5 – 2.8-70 mg/mL) were added to 50 µl of $FeCl_2.4H_2O$ (0.5 mM). 750 µl of distilled water was added to the tubes and allowed to stand for 5 minutes at ambient temperature. 50 µl of ferrozine (2.5 mM) was then added and absorbance read at 562 nm. The positive control used was EDTA (concentration range: 5-100 µM). Percentage chelation was calculated and the results were expressed as IC_{50} values in mg DW/ml for the herbal formulation and ml herbal product/ml for the elixir.

Deoxyribose assay

The hydroxyl radical scavenging potential of the extracts was determined using the deoxyribose assay[19]. Hydroxyl radicals were generated in the reaction mixture

containing ascorbate, H_2O_2 and Fe^{3+}-EDTA at pH 7.4 (*equations 2* and *3*). The ascorbic acid increased the rate of OH- generation significantly by reducing iron and maintaining a supply of Fe^{3+}-EDTA.

$$Fe^{3+} - EDTA + ascorbate \rightarrow Fe^{2+} - EDTA + Oxidized\ ascorbate \quad \textit{(Equation 2)}$$

$$Fe^{2+} - EDTA + H_2O_2 \rightarrow Fe^{3+} - EDTA + OH \quad \textit{(Equation 3)}$$

The reaction mixture was in a final volume of 1mL and contained the following reagents: 200µl of 100mM KH_2PO_4-KOH, 200µl of 0.5mM $FeCl_3$, 100µl of 1mM EDTA, 100µl extract (variable concentrations: F1 – 0.439-8.78 mg/ml; F2 – 0.0024-0.048 ml/ml; F3 – 0.4-8 mg/ml; F4 – 1.8-36 mg/ml; F5 – 3.5-70 mg/ml), 200µl of 15mM deoxyribose, 100µl of 10mM H_2O_2 and 100µl of 1mM ascorbic acid. Reaction mixtures were incubated for 1hr at 37°C.

After the incubation period, 1ml of 1% (w/v) thiobarbituric acid (TBA) was added to the mixture, followed by 1ml of 2.8% (w/v) trichloroacetic acid. The solutions were heated in a water-bath at 80°C for 20 minutes to develop the pink coloured MDA-$(TBA)_2$ complex. Due to turbidity encountered, the MDA-$(TBA)_2$ chromogen was extracted into 3ml butan-1-ol and its absorbance was measured at 532nm. The results were being regarded as indications of propensity to scavenge the OH· by virtue of their ability to inhibit deoxyribose oxidation. The scavenging potential of the extracts was calculated using *equation 1* and the results were expressed as 50% inhibition of deoxyribose oxidation in mg DW/ml of herbal medicine and as ml herbal product for elixir F2.

Copper-phenanthroline assay

The herbal formulations were tested for their pro-oxidant ability. The final volume of the reaction mixture (1.2ml) contained the following reagents: 100µl of 1.8mM 1, 10-phenanthroline hydrate (dissolved in a few drops of ethanol before addition of water), 100µl of 1.2mM copper (II) chloride, 100ul calf thymus DNA (2.75mg/mL), 100µl of 120mM KH_2PO_4-KOH buffer at pH 7.4, 200µl distilled water and 600µl plant extracts (of different concentrations: F1 – 0.549-8.78 mg/mL; F2 – 0.003-0.048 ml/ml; F3 – 0.5-8 mg/ml; F4 – 2.25-36 mg/ml; F5 – 4.38-70 mg/ml). Reaction mixtures were incubated at 37°C for 1 hour. 100µl of 0.1M EDTA was then added to stop the reaction, followed by addition of 1ml of 1% (w/v) TBA and 1ml of 25% (v/v) HCl. After incubation at 80°C for 15 minutes, the absorbance was measured at 532nm. Results were expressed as the absorbance at 532nm.

Acetyl cholinesterase inhibition assay

The acetyl cholinesterase inhibition assay was based on the use of acetylthiocholine iodide (ATCI) as the substrate analogue[20]. 250µl of the extract (variable concentrations: F1 – 0.549-8.78 mg/ml; F2 – 0.003-0.048 ml/ml; F3 – 0.5-8 mg/ml; F4 – 2.25-36 mg/ml; F5 – 4.38-70 mg/ml) in phosphate buffer (200mM, pH 7.7) was added to 80µl of dithionitrobenzoic acid (DTNB) (9.9mg of DTNB and 3.75mg sodium bicarbonate dissolved in 25ml phosphate buffer, pH 7.7), and 10µl of 2U/mL AChE enzyme. After 5 minutes incubation at 25°C, 15µl of substrate (21.7mg ATCI in 10ml of phosphate buffer) was added. The mixture was again incubated at 25°C for 5 minutes. Absorbance was measured at 412nm. The percentage of inhibition was calculated (*equation 4*) and the results were expressed in terms of IC_{50}. Galantamine was used as a positive control.

$$\%\ Inhibition = 100 \times \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \quad \textit{(Equation 4)}$$

Statistical analysis

All values were expressed as the mean ± standard deviation and were analyzed by one-way of variance (ANOVA) using SPSS version 20 software. Differences were separated out by using the least significant difference (LSD) test at a 0.05 probability level. Pearson's correlation coefficient was used at the 0.01 level (2-tailed).

RESULTS

The five herbal formulations were assessed for the major classes of phytochemicals as well as their antioxidant capacity using different mechanism-based antioxidant assays. The extracts were also tested for their pro-oxidant activity using the copper-phenanthroline assay. The inhibition of AChE enzyme by the five herbal medicines was also evaluated.

Qualitative analysis of the herbal medicines

The extracts were subjected to qualitative analysis to detect the presence of different chemical groups of secondary metabolites, namely, alkaloids, saponins, phenolic compounds, anthraquinones and steroids (**Table 3**). Saponins and phenolic compounds were found to be present in all five formulations while alkaloids were present in F3 only.

Analysis of phenolic content

The amount of total phenolics measured by the Folin-Ciocalteu method present showed highest amount of total phenolics in F3 (51.5 ± 0.038 mg GAE/g DW). The amount of total phenolics was found in a hierachical order of F3 > F1 > F2 > F4 > F5 (**Table 4**). The phenolic content of each extract was significantly different from each other ($p<0.05$).

A relatively low amount of proanthocyanidin was found to be present in the five formulations. The total proanthocyanidin content varied from 0.152 to 3.90 mg CCE/g DW (**Table 4**). No significant difference was found between the formulations F3, F4 and F5 ($p>0.05$).

The total flavonoid content measured was found highest in F1 (1.85 ± 0.12mg QE/g DW) while the lowest content was found with F4 (0.722 ± 0.011 mg QE/g DW) (Table 4). No significant difference was found between flavonoid content of the formulations F3, F4 and F5 ($p>0.05$).

Antioxidant activities

Ferric reducing antioxidant power assay: The reducing potential of the extracts was determined using the FRAP assay. Among the five extracts, F3 showed the most potent reducing potential. The FRAP values ranged between 81.6 ± 0.78 and 1654 ± 38 µmol Fe (II) equivalent (**Figure 1**) with no statistical difference found between FRAP values of formulations F4 and F5 ($p>0.05$).

Scavenging of hypochlorous acid: All the extracts showed a dose-dependent scavenging activity against HOCl acid and the IC50 values were calculated from the dose-dependent curves. The lowest IC50 value was observed for F2 and the highest for F4 (**Table 5**). Statistically significant difference was found among IC50 values of the extracts ($p<0.05$). However, F3 was a very effective scavenger of HOCl acid as compared to ascorbic acid at a concentration of 0.0066 mg/ml ($p>0.05$).

Deoxyribose assay: The ability of the extracts to inhibit deoxyribose damage by hydroxyl radical was assessed. The extracts except F1 conferred protection to the sugar moiety in a dose-dependent manner. F1 showed a minimum inhibition of deoxyribose oxidation at the highest concentration tested (31%) while F2 showed a maximum inhibition of 77%. The calculated IC50 values (**Table 5**), extrapolated from the dose-dependent curves, and decreased in the following order: F5 > F4 > F3 > F2. No significant

differences were found between the IC_{50} values of F3 and gallic acid ($p>0.05$).

Iron (II) chelating activity: The ability of the extracts to chelate iron (II) was evaluated. A dose-dependent activity was observed and the calculated IC_{50} values have been indicated in Table 5. No statistically significant difference was observed between IC_{50} values of F1, F3 and F5 ($p>0.05$) and between F4 and EDTA ($p>0.05$), a potent iron chelator, indicating the effectiveness of F4 as iron (II) ions chelators. F2 was also a potent iron (II) chelator (**Table 5**).

Table 3: Qualitative analysis of the five herbal medicines

Phytochemical	F1	F2	F3	F4	F5
Alkaloids	-	-	+	-	-
Saponins	+	+ +	+ + +	+ +	+
Phenolic compounds	+	+ + +	+ + +	+ +	+
Anthraquinones	-	-	-	-	-
Steroids	-	-	-	-	-

+: present; + +: strongly present; + + +: very strongly present; -: absent

Table 4: Total phenolic content, proanthocyanidin content and flavonoid content of the five herbal formulations under study

Extract	Total Phenolic content[1]	Total Proanthocyanidin content[2]	Total Flavonoid content[3]
F1	16.3 ± 0.37 [a]	3.90 ± 1.22 [a]	1.85 ± 0.12 [a]
F2	7.51 ± 0.029	0.328 ± 0.062	1.12 ± 0.030
F3	51.5 ± 0.038 [b]	1.17 ±0.21 [b]	0.778 ± 0.058 [b]
F4	6.41 ± 0.052 [c]	0.380 ±0.093 [b]	0.722 ± 0.011 [b]
F5	4.24 ± 0.19 [d]	0.152 ±0.030 [b]	0.822 ± 0.13 [b]

Common superscript within columns showed no significant difference between respective values of extracts; LSD at 95% confidence interval. Values were expressed as mean ± standard deviation (n=3).

[1]Results expressed mg GAE/g DW, except for F2, which was expressed as mg GAE/ml of herbal product.

[2]Values expressed in units mg CCE/g DW, except for F2 which was expressed mg CCE/ml of herbal product.

[3]Results expressed in units mg QE/g DW, except for F2 which was expressed as mg QE/ml of herbal product.

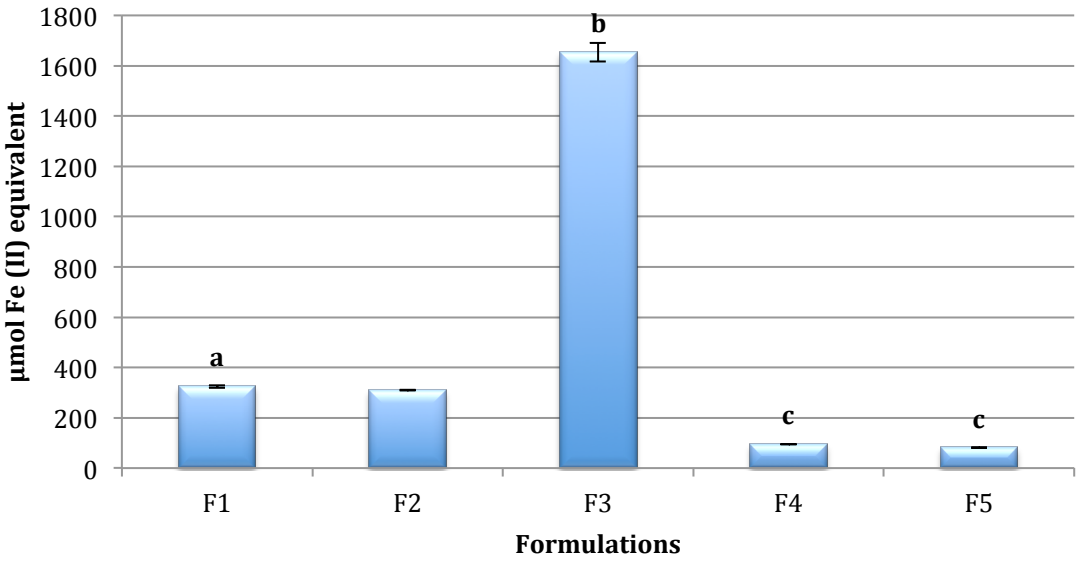

Figure 1: FRAP analysis of the five formulations expressed in terms µmol Fe(II)/g herbal material. Common superscript between columns showed no significant difference between FRAP values of extracts; LSD at 95% confidence interval. Values were expressed as mean ± standard deviation (n=3). Results expressed in µmol Fe (II) equivalent/g DW of herbal medicine, except for F2, which was expressed as µmol Fe (II) equivalent/ml of herbal medicine.

Table 5: IC50 values for scavenging of HOCl, deoxyribose assay, Fe (II) chelating activity and anti-cholinesterase activity

	Scavenging of HOCl	Deoxyribose assay	Fe (II) chelating activity	Anti-cholinesterase activity
F1	0.44 ± 0.012 [a]	N.D	0.69 ± 0.401 [a]	N.D
F2	0.00021 ± 0.000	0.00060 ± 0.000	0.0026 ± 0.000	0.013 ± 0.004
F3	0.027 ± 0.002 [b]	0.024 ± 0.002 [a]	0.66 ± 0.336 [a]	0.45 ± 0.131 [a]
F4	0.53 ± 0.038 [c]	0.58 ± 0.064 [b]	0.24 ± 0.006 [b]	14.83 ± 2.066 [b]
F5	0.49 ± 0.036 [d]	0.66 ± 0.02 [c]	0.99 ± 0.201 [a]	27.81 ± 1.574 [c]
Positive control	0.0066 ± 0.000 [b]	0.0041 ± 0.000 [a]	0.00093 ± 0.000 [b]	0.0019 ± 0.000 [a]

Common superscript within columns showed no significant difference between the values; LSD at 95% confidence interval. Values were expressed as mean IC_{50} ± standard deviation (n=3); Data were expressed as mg DW/ml except for F2, which were expressed as ml herbal product/ml.

Pro-oxidant activity

The copper-phenanthroline assay was used to evaluate the pro-oxidant activity of the formulation. **Table 6** showed the extent of DNA damage compared to ascorbic acid for the range of concentration tested, a concentration range, which was indicative of the normal dosage of the herbal drug. The prooxidant activity of ascorbic acid (0.18 µg/ml) was used as arbitrary 100% for estimating the percentage proxidant activity. The concentration range tested for F1, F4 and F5 did not promote DNA damage compared to ascorbic acid, which was used as positive control. However, F2 and F3 at concentration above 0.0065 ml herbal product/ml and 0.55 mg DW/ml respectively showed some pro-oxidant effects compared to the control.

Table 6: Extent of DNA damage by traditional herbal medicine compared to ascorbic acid as measured by the copper-phenanthroline assay

	Concentration	% Prooxidant activity
Ascorbic acid	**(µg/ml)**	
	0.18	100
F1	**(mg/ml)**	
	0.10	22.3
	0.40	21.2
	0.60	27.4
	0.80	37.4
	1.00	48.6
	1.20	59.8
	1.40	75.4
	1.60	86.0
F3	0.09	14.5
	0.18	30.7
	0.36	44.7
	0.55	99.8
	0.73	138.0
	0.91	180.4
	1.09	236.3
	1.27	261.5
F4	0.41	27.3
	0.82	20.7
	2.45	11.2
	3.27	14.0
	4.09	15.6
	4.91	15.1
	5.73	16.2
	6.55	49.7
F5	0.80	15.1
	1.59	20.7
	3.18	15.6
	4.77	21.2
	6.36	36.3
	7.95	26.3
	12.73	18.4

Anticholinesterase activity

This study examined the anticholinesterase activity of the five herbal medicines. The activity was found to be dose-dependent (**Figure 2**). The maximum inhibition of 80% at a dose of 2.60 mg DW/ml was exhibited by F3 and a minimum inhibition by F1 (47%). F2 was a potent AChE inhibitor as indicated by the IC50 value (Table 5). No significant difference was observed between F3 and galantamine, a known AChE inhibitor ($p>0.05$).

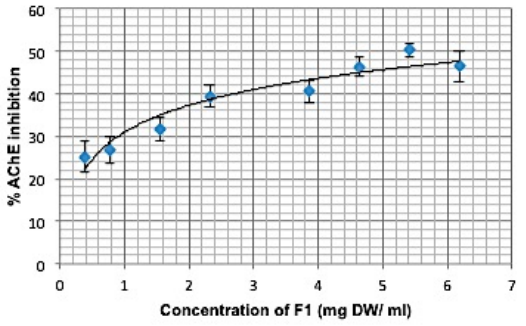

Figure 1a

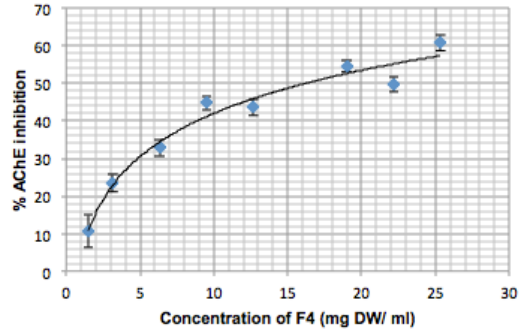

Figure 2b

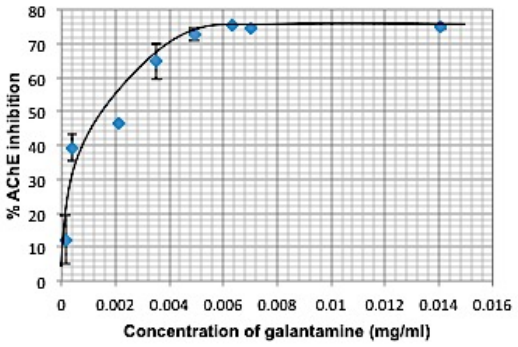

Figure 2c

Figure 2: Dose-dependent curves for AChE inhibition for F1, F4 and galantamine

Values were expressed as mean ± standard deviation (n=3) (a) Dose-dependent curve for F1. (b) Dose-dependent curve for F4. (c) Dose-dependent curve for galantamine

DISCUSSION

The surge of interest in plants as promising source for treatment of clinical disorders has markedly increased over the past decades and a wide range of plant extracts have been reported for their neuroprotective action[21]. Oxidative stress and impaired cholinergic neurotransmission have played an important role in the pathogenesis of several ND[9,22]. Thus, restoring the balance of antioxidants and enhancing cholinergic neurotransmission might be possible mechanisms of action of herbal remedies. In this study, five traditional herbal medicines, available locally but out of which four were imported, were tested for their antioxidant as well as anticholinesterase efficacies.

Different mechanisms contribute to the prevention of oxidative processes in the body amongst which scavenging of biologically relevant reactive oxygen and nitrogen species and inhibition of Fenton reactions are prominent mechanisms[23]. Natural compounds such as polyphenolics have been reported as major contributors to the antioxidant activity[24]. The ability for phenolics to scavenge free radicals might be due to the presence of numerous hydroxyl groups, to double bond conjugation and resonance effects[25,26]. The antioxidant activity might be attributed to the redox properties of the hydroxyl groups, which enable them to act as singlet oxygen quenchers, hydrogen donators and reducing agents, as well as metal chelators. The presence of hydroxyl and carboxyl groups in phenolics has enabled them to bind to iron as well as inactivate iron ions by chelation or suppression of the superoxide-driven Fenton reactions. The antioxidant activity could also result via inhibition of some enzymes involved in radical generation, such as lipoxygenases and xanthine oxidase[27,28]. In the present study, four different *in vitro* assays were performed to determine the antioxidant efficacies of the five available traditional herbal medicines on the island.

In the presence of a reducing antioxidant, Fe (III)-TPTZ was reduced to Fe (II)-TPTZ at low pH[18] and F3 which was composed of *Gingko biloba* L. leaves had the highest reducing potential[29]. Neutrophil enzyme myeloperoxidase oxidised Cl^- ions at sites of inflammation, resulting in the production of HOCl acid. The latter can in turn inactivate catalase[30]. The HOCl acid scavenging potential of the herbal medicines indicated that all the samples tested possessed the ability to scavenge HOCl acid in a dose dependent manner. F2 and F3 were found to be potent scavengers with important scavenging capacities towards this biologically relevant ROS suggesting the potential of these herbal medicines.

Hydroxyl radicals produced *in vivo* are highly reactive and can react with a variety of molecules found in living cells, such as amino acids, sugars, lipids and nucleotides. Thus, the ability of the extracts to inhibit deoxyribose damage was evaluated using the deoxyribose assay. F2 was a potent scavenger of hydroxyl radicals with 77% inhibition of deoxyribose oxidation at the highest dose tested. It was also demonstrated that *Ginkgo biloba* L. extract

had high level of hydroxyl radical-scavenging activity[31].

Another important mechanism of antioxidant action involves the chelation of iron (II) ions which serves as catalyst in Fenton reactions. All extracts were effective iron (II) chelators. They demonstrated a dose-dependent activity, with the maximum chelating activity measured using F5 (94%). F2, which was composed of *Salvia miltiorrhiza* Bunge*, Hypericum perforatum*L. and *Ginkgo biloba* L., had a low calculated IC_{50} value (**Table 5**), similarly it was reported that *Hypericum perforatum* L. extract did not possess metal-chelating activity[32]. Thus, the metal chelating activity of F2 might thus be due to the presence of *Ginkgo biloba* L.or *Salvia miltiorrhiza* Bunge that could act in a synergistic or additive way.

All the five herbal medicines studied possessed antioxidant properties, which might be largely responsible for their use in the treatment of ND. The results showed that phenolic compounds contributed largely to the antioxidant behavior of the herbal medicines. A very high significant correlation was obtained between the FRAP values ($r=0.995$, $p<0.01$) and the HOCl scavenging ($r=0.983$, $p<0.01$) with the total phenolics content. A significantly strong relationship was also observed between IC_{50} for inhibition of deoxyribose damage and phenolic content ($r=-0.991$, $p<0.01$) as well as with proanthocyanidin content ($r=-0.954$, $p<0.01$).

F1, F2 and F4 contained more than one plant material while F3 and F5 were single plant formulations (**Table 1**). *Verbena officinalis* L. *and Rosmarinus officinalis* L.present in F1, have been reported to exert their antioxidant action particularly through radical scavenging properties[33,34]. The activity of *Rosmarinus officinalis*L. has been mainly attributed to the presence of rosmarinic acid[33]. This study demonstrated that a mixture of *Rosmarinus officinalis* L. and *Verbena officinalis* L. in F1 exhibited antioxidant activities through a multi-mechanism approach. However, F1 was not an effective scavenger of hydroxyl radical. In addition, it has been reported that *Verbena officinalis* L. caused attenuation of Aβ- triggered DEVD- and VDVAD-cleavage[35] and Aβ was known to be responsible for the production of ROS[23].

F2 contained mainly *Salvia miltiorrhiza* Bunge, *Hypericum perforatum* L. and *Gingko biloba* L. Although the literature reports the antioxidant activity of *S. miltiorrhiza* Bunge*, H. perforatum* L. and *G. biloba* L.*,* no studies have indicated the efficiency of the polyherbal formulation including the three-plant material together. Tanshinone IIA from *S. miltiorrhiza* Bunge was found to inhibit nitric oxide production. Additionally, danshensu, tanshinone, salvianolic acid A and salvianolic acid B, again identified in *S. miltiorrhiza* Bunge, have demonstrated anti-lipoperoxidant activity through scavenging of superoxide anion radical[36]. Other antioxidant mechanisms through which *H. perforatum* L. exerted its action were through 2,2-diphenyl-1-picrylhydrazyl scavenging activity, nitric oxide inhibition and FRAP assay. Previous work carried out with *G. biloba* L. also demonstrated antioxidant activity, which might be ascribed to its free radical scavenging action. It has also been shown to activate gene or protein expression of endogenous antioxidants, such as glutathione and manganese superoxide dismutase[37,38]. In addition, the activity of antioxidant enzymes, in particular, catalase and superoxide dismutase was significantly improved following treatment with *G. biloba* L. extract [39].

F2 and F3 were found to be very good scavengers of HOCl and hydroxyl radical, with F3 being as potent as the positive control. The antioxidant activity of F2 was more pronounced than observed with F3 which contained only *Gingko biloba* L. which can be suggestive of additive or synergistic

interactions occurring in F2. The synergistic effect observed with F2 might be due to the different phytochemicals present in the mixture.

F4, a multi-herb formulation nevertheless was not more effective than F2 except for the ferric reducing potential. *Radix Angelicae sinensis* (Oliv.) Diels, which was present in F4, has been reported to decrease malondialdehyde levels and increase activity of superoxide dismutase[40,41] while roots of *Panax ginseng* C.A. Meyer, containing saponins, could reduce free radical damage and increase glutathione peroxidase and superoxide dismutase activities[42]. F5 contained *Centellaasiatica* L. which has previously been reported to inhibit the activity of ROS and decrease lipid peroxidation[43,44].

Despite the antioxidant property of phytophenolics, it has been reported that they could also act as pro-oxidants under certain conditions, such as in systems where redox-active metals were present or at high doses. In the presence of oxygen, ROS and other organic radicals could be formed through the redox cycling of phenolics by transition metals such as copper and iron. These ROS could cause damage to biological molecules, for example lipids, proteins and DNA[45,46]. The copper-phenanthroline assay was thus used to assess the pro-oxidant activity of the herbal extracts. The copper-phenanthroline complex in the assay induced DNA strand breakage and herbal extracts with pro-oxidant activity would further accentuate the damage to DNA. A normal dose concentration of the herbal medicine ranging between 8 and 70 mg/ml indicated that F1, F4 and F5 did not promote DNA damage (**Table 6**). The extent of damage was lower than that of ascorbic acid used as reference. However, pro-oxidant activity was observed at dose concentration above 0.0065ml/ml and 0.55 mg/ml for F2 and F3 respectively, where their extents of damage were higher than the arbitrary value for ascorbic acid. Ascorbate could generate hydroxyl radical through its ability to redox-recycle and sustain the supply of Fe^{2+}, but no clear evidence has been shown *in vivo*[11]. Thus, despite the pro-oxidant activity which was observed with F2 and F3, there could be pharmacokinetic changes in particular phase I and II biotransformation that could render the phytoconstituents harmless. However, further clinical studies are warranted to justify this claim. Moreover, the antioxidant activities of these herbal medicines might compensate for their pro-oxidant activity, thus cancelling their ability to cause oxidation *in vivo*. More in depth analyses are however required to determine such an effect since the prooxidant effect may also nullify the antioxidant activities of both F2 and F3.

Another approach to the treatment of ND has been the improvement of brain cholinergic activity by inhibition of AChE, resulting in hydrolysis of acetylcholine and termination of nerve impulse transmission at cholinergic synapses. Several drugs, such as tacrine, galantamine and donepezil, were proven to be effective AChE inhibitors and delayed disease progression, but several side effects were involved with their use. Thus, research is ongoing for the search of potential natural products, which could act as AChE inhibitors, with fewer side effects. A variety of plants has been found to possess AChE inhibitory activity. Phytochemicals, which might be responsible for this action, include alkaloids, ursolic acid, lignans, flavonoids, terpenoids and coumarins[47,48].

The formulations used in this study showed cholinesterase inhibitory activity (**Figure 2**). No significant difference was found between the IC_{50} values of F3 and galantamine, a well knownAChE inhibitor. The elixir F2 was also a very effective inhibitor, suggesting that these two herbal formulations had potent activity in

inhibiting AChE enzyme, which is a major target in the treatment of several neurological disorders.

The strongest AChE activity was exhibited by F2 which contained in addition to two other herbs *Hypericumperforatum* L. The latter was reported to be responsible for neurochemical modulation, which might be due to the presence of flavonoids, namely hyperforin and hypericin[49]. It was reported that methanolic extract of *Hypericumperforatum* L. did possess anticholinesterase activity[32]. The inhibitory activity of AChE with *Salvia miltiorrhiza* Bunge was found to be due to the presence of triterpenoids[7]. *Ginkgo biloba* L. present in F2 and F3 has also been shown to have potential activity in reducing AChE activity[50]. Roots of *Angelicaesinensis* (Oliv.) Diels, making up 12% of F4, has been reported to contain Z-Ligustilide, which was able to increase choline acetyl transferase activity and decrease AChE activity[41]. Inhibition of AChE activity might not be the only mechanism by which F4 could act in ND. F4 also contained dried roots of *Panax ginseng* C.A. Meyer that has been shown to possess central cholinomimetic and catecholaminomimetic activity[41].

The active ingredient from F5, *Centella asiatica* L. has been reported for its AChE inhibitory activity. The AChE inhibitory activity might be due to the presence of triterpenes[49]. Apart from AChE inhibition, other mechanisms might be responsible for the potential efficacy of F5 in treating ND, such as neuroprotective effect and sedative effect[42,43].

Among the five formulations, F1 was found to be the less potent in decreasing AChE activity. F1 contained *Rosmarinus officinalis* L. and its anti-AChE activity might be due to the synergistic action between 1,8 cineole and 2-pinene[51]. Significant ($p<0.01$) negative correlation was observed between the IC_{50} for anti-AChE activity and with phenolic content ($r=-0.894$) and proanthocyanidin content ($r=-0.937$). Therefore, the findings suggested that phenolic compounds present, mainly proanthocyanidins, played an important role in inhibiting AChE enzyme.

CONCLUSION

The treatment of ND remains a challenge to modern medicine because no successful treatments have yet been found. Plants have been regarded as promising sources for treatment and various herbal medicines have been available since time immemorial, but limited data have been made available on their phytochemistry and biological activities. The herbal medicines used against ND in Mauritius have been sparsely studied and data from this study has indicated the phenolic richness of the herbal medicines used against ND. The protective effects of the extracts may stem, in part, from the antioxidative property of the bioactive constituents as demonstrated from the activities using a multi-method approach. F2 and F3 also exhibited pro-oxidant activity. However the absorption and bioavailability of the molecules responsible for such effect as well as biotransformation of the latter *in vivo* can confirm such activities within the body. Thus further studies to determine the pharmacokinetics of these herbal formulations are required. These herbal medicines, in addition, could exert their protective effect by their ability to inhibit AChE enzyme. The findings indicated antioxidant and anticholinesterase activity as potential mechanisms of action of the five herbal remedies used in the treatment of ND locally. However, the data cannot be directly extrapolated to human biological environment since pharmacokinetic phenomena have not been taken into consideration and this provides ground for investigating the clinical effects of these herbal drugs.

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REFERENCES

1. World Health Organization. 2014. Available from: http://www.who.int/mediacentre/news/releases/2014/world-health-statistics-2014/en. Accessed 12 August 2015.
2. CSO: Central Statistics Office, Digest of demographic statistics. 2013. Available from: http://statsmauritius.govmu.org/English/StatsbySubj/Documents/Digest/Demographic13.pdf. Accessed 13 January 2014
3. CSO: Central Statistics Office, Observatory on ageing report. 2014. Available from: http://socialsecurity.govmu.org/English/Documents/Health.pdf. Accessed 13 January 2014
4. Parihar MS, Hemnani T. Alzheimer's disease pathogenesis and therapeutic interventions. *J Clin Neurosci*. 2004;11:456-67.
5. Ip PS, Tsim KW, Chan K, Bauer R. Application of complementary and alternative medicine on neurodegenerative disorders: Current status and future prospects. *Evid Based Complement Alternat Med*. 2012;2012:930908.
6. Steele ML, Truong J, Govindaraghavan S, Ooi L, Sucher NJ, Münch G. Cytoprotective properties of traditional Chinese medicinal herbal extracts in hydrogen peroxide challenged human U373 astroglia cells. *Neurochem Int*. 2013;62:522-9.
7. Lin HQ, Ho MT, Lau LS, Wong KK, Shaw PC, Wan DC. Anti-acetylcholinesterase activities of traditional Chinese medicine for treating Alzheimer's disease. Chem Biol Interac. 2008;175:352-4.
8. Wu TY, Chen CP, Jinn TR. Traditional Chinese medicines and Alzheimer's disease. *Taiwan J Obstet Gynecol*. 2011;50:131-5.
9. Iriti, M.; Vitalini, S.; Fico, G.; Faoro, F. Neuroprotective herbs and foods from different traditional medicines and diets. *Molecules*. 2010;15:3517-55.
10. Schulz JB, Lindenau J, Seyfried J, Dichgans J. Glutathione, oxidative stress and neurodegeneration. *Eur J Biochem*. 2000;267:4904-11.
11. Luximon-Ramma A, Bahorun T, Crozier A. Antioxidant actions and phenolic and vitamin C contents of common Mauritian exotic fruits. *J Sci Food Agric*. 2003;83:496-502.
12. Chitravadivu C, Manian S, Kalaichelvi K. Qualitative analysis of selected medicinal plants, Tamilnadu, India. *Middle East J Sci Res*. 2009;4:144–6.
13. Palanisamy P, Jayakumar B, Kumuthavalli MV, Kumar Y, Srinath KR. Preliminary phytochemical evaluation of whole plant extract of Dipteracanthusprostratus Nees. *Int Res J Pharm*. 2012;3:150-3.
14. Yadav RNS, Agarwala M. Phytochemical analysis of some medicinal plants. *J Phytol*. 2011;3:10-4.
15. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdicphosphotungstic acid reagents. *Am J Enol Vitic*. 1965;16:144-58.
16. Lamaison JLC, Carnet A. Teneurs en principaux flavonoides des fleurs de Crataegus monogyna Jacq et de Crataegus laevigata (Poiret D. C) en fonction de la vegetation. *Pharm Acta Helv*. 1990;65:315-20.
17. Porter LJ, Hrstich LN, Chan BG. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochem*. 1986;25:223-320.
18. Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP Assay. *Anal Biochem*. 1996;239:70-6.
19. Neergheen VS, Soobrattee MA, Bahorun T, Aruoma OI. Characterization of the phenolic constituents in Mauritian endemic plants as determinants of their antioxidant activities in vitro. *J Plant Physiol*. 2006;163:787-99.

20. Rahman AU, Choudhary MI. Bioactive natural products as a potential source of new phrarmacophores. A theory of memory. *Pure Appl Chem*. 2001;73:555-60.
21. Mata AT, Proenca C, Ferreira AR, Serralheiro MLM, Nogueira JMF, Araujo MEM. Antioxidant and antiacetylcholinesterase activities of five plants used as Portuguese food spices. *Food Chem*. 2007;103:778-86.
22. Mukherjee PK, Kumar V, Mal M, Houghton PJ. Acetylcholinesterase inhibitors from plants. *Phytomedicine*. 2007;14:289-300.
23. Liu T, Jin H, Sun QR, Xu JH, Hu HT. The neuroprotective effects of tanshinone IIA on β-amyloid-induced toxicity in rat cortical neurons. *Neuropharmacology*. 2010;59:595-604.
24. Soobrattee MA, Neergheen VS, Luximon-Ramma A, Aruoma OI, Bahorun T. Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. *Mutat Res*. 2005;579:200-13.
25. Siddique NA, Mujeeb M, Najmi AK, Akram M. Evaluation of antioxidant activity, quantitative estimation of phenols and flavonoids in different parts of Aegle marmelos. *Afri J Plant Sci*. 2010;4:1-5.
26. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol Med*. 1996;20:933-8.
27. Pereira DM, Valentao P, Pereira JA, Andrade PB. Phenolics: From chemistry to biology. *Molecules*. 2009;14:2202-11.
28. Michalak A. Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. *Pol J Environ Stud*. 2006;15:523-30.
29. Song FL, Gan R, Zhang Y, Xiao Q, Kuang L, Li H. Total phenolic contents and antioxidant capacities of selected Chinese medicinal plants. *Int J Mol Sci*. 2010;11:2362-72.
30. Hazra B, Sarkar R, Biswas S, Mandal N. Comparative study of the antioxidant and reactive oxygen species scavenging properties in the extracts of the fruits of Terminalia chebula, Terminalia belerica and Emblica officinalis. *BMC Complement Altern Med*. 2010;10:1-15.
31. Chen J, Zhang T, Jiang B, Mu W, Miao M. Characterization and antioxidant activity of Ginkgo biloba exocarp polysaccharides. *Carbohydr Polym*. 2012; 87:40-5.
32. Altun ML, Yilmaz BS, Orhan IE, Citoglu GS. Assessment of cholinesterase and tyrosinase inhibitory and antioxidant effects of Hypericum perforatum L. (St. John's wort). *Ind Crop Prod*. 2013;43:87-92.
33. Al-Sereitia MR, Abu-Amerb KM, Sena P. Pharmacology of rosemary (*Rosmarinus officinalis* Linn.) and its therapeutic potentials. *Indian J Exp Biol*. 1999;37:124-31.
34. Casanova E, Garcia-Mina JM, Calvo MI. Antioxidant and antifungal activity of *Verbena officinalis* L. leaves. *Plant Foods Hum Nutr*. 2008;63:93-7.
35. Lai S, Yu M, Yuen W, Chang C. Novel neuroprotective effects of the aqueous extracts from *Verbena officinalis* Linn. *Neuropharmacol*. 2006;50 641-50.
36. Wang B. Salvia miltiorrhiza: Chemical and pharmacological review of a medicinal plant. *J Med Plants Res*. 2010; 4:2813-20.
37. Luo Y, Smith JV. Review Studies on molecular mechanism of Ginkgo biloba extract. *Appl Microbiol Biotechnol*. 2004;64:465-72.
38. Christen Y. Ginkgo biloba and neurodegenerative disorders. Front Biosci 2004;9:3091-104.
39. Kaur S, Chhabra R, Nahru B. Ginkgo biloba extract attenuates hippocampal neuronal loss and cognitive dysfunction resulting from trimethyltin in mice. *Phytomedicin*e. 2013;20:178-86.
40. Li S, Yu Y, Li S. Identification of antioxidants in essential oil of Radix AngelicaeSinensis using HPLC coupled with DAD-MS and ABTS-Based assay. *J Agric Food Chem*. 2007;55:3358-62.
41. Kuang X, Du J, Liu, Y, Zhang G, Peng H. Postischemic administration of Z-Ligustilide ameliorates cognitive dysfunction and brain damage induced by permanent forebrain ischemia in rats. *Pharmacol Biochem Behav*. 2008; 88:213-21.
42. Yan H, Li L, Tang XC. Treating senile dementia with traditional Chinese medicine. *J ClinInterv Aging*. 2007;2:201-8.
43. Tiwari S, Gehlot S, Gambhir IS. Centellaasiatica: A concise drug review with

probable clinical uses. *J Stress Physiol Biochem*. 2011;7:38-44.
44. Hashim P, Sidek H, Helan MHM, Sabery A, Palanisamy UD, Ilham M. Triterpene composition and bioactivities of Centella asiatica. *Molecules*. 2011;16:1310-22.
45. Sakihama Y, Cohen FM, Grace SC, Yamasaki H. Plant phenolic antioxidant and prooxidant activities: phenolics-induced oxidative damage mediated by metals in plants. *Toxicol*. 2002;177:67-80.
46. Yordi E, Molina Perez E, Matos MJ, UriarteVillares E..Antioxidant and prooxidant effects of polyphenolic compounds and structure-activity relationship evidence. In: Bouayed, J., Bohn, T. (Eds.), Nutrition, Well-Being and Health. InTech. 2012:24-48.
47. Zhou Y, Li W, Xu L, Chen L. In Salvia miltiorrhiza, phenolic acids possess protective properties against amyloid β-induced cytotoxicity, and tanshinones act as acetylcholinesterase inhibitors. *Environ toxicol Pharm*. 2011;31:443-52.
48. Tavares L, McDougall GJ, Fortalezas S, Stewart D, Ferreira RB, Santos CN. The neuroprotective potential of phenolic-enriched fractions from four Juniperus species found in Portugal. *Food Chem*. 2012;135:562-70.
49. Gomes NG, Campos MG, Orfão JM, Ribeiro CA. Plants with neurobiological activity as potential targets for drug discovery. Prog. Neuropsychopharmacol. *Biol Psychiatry*. 2009;33:1372-89.
50. Das A, Shanker G, Nath C, Pal R, Singh S, Singh H. A comparative study in rodents of standardized extracts of Bacopamonniera and Ginkgo biloba: Anticholinesterase and cognitive enhancing activities. *Pharmacol Biochem Behav*. 2002;73:893-900.
51. Faixova Z, Faix S. Biological effects of rosemary (Rosmarinusofficinalis L.) essential oil (Review). *Folia Veterinaria*. 2008;52:135-9.