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Original Article

Ashwagandha Root Extract Inhibits Acetylcholine Esterase, Protein Modification and Ameliorates H_2O_2-Induced Oxidative Stress in Rat Lymphocytes

Ajay Pal[1], Kandikattu Hemanth Kumar[2], Bharat Bhushan[*1], Vinod Saharan[3]

ABSTRACT

Background: Herbs have long been the basis of medical treatment but the practice of herbalism is not strictly based on evidences gathered using scientific methods. Ashwagandha (*Withania somnifera* L.) is a traditional medicinal herb used in the treatment of various ailments. **Objective**: In current study we have evaluated the acetylcholine esterase (AChE) inhibitory and antioxidant potential of Ashwagandha root extract (ARE) in *in-vitro* and *ex-vivo* models to give a scientific base to its use in herbal medicine. **Methodology**: Simplest extraction e.g. maceration method was performed for preparation of extracts using methanol as solvent. Both *in vitro* and *in vivo* experimental trial were executed to evaluate the efficacy of root extract. **Result and Discussion**: ARE substantially scavenged free radicals and effectively prevented protein degradation as well as modification as studied by SDS-PAGE and Western blotting. Pre-treatment with ARE protected rat lymphocytes against H_2O_2-induced oxidative damage. H_2O_2 treatment resulted in DNA fragmentation and significantly decreased the activities of key antioxidant enzymes namely superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase while decreased and increased the content of glutathione and malondialdehyde, respectively. ARE pre-treatment almost reversed these changes indicating its efficiency to suppress hydrogen peroxide-induced oxidative stress. **Conclusion**: The study provides the scientific basis of pleiotropic functions of Ashwagandha.

Key words: Acetylcholine esterase, Rat lymphocytes, Antioxidants, Ashwagandha, Protein carbonylation, DNA damage.

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INTRODUCTION

Reactive oxygen species (ROS) like superoxide anion, hydroxyl radicals, hydrogen peroxide, singlet oxygen etc. are routinely formed in the animal body as a result of normal metabolic activity[1] and/or as by-products of various oxidative reactions including metabolism of xenobiotics.[2] These species are detoxified by endogenous enzymatic and non-enzymatic antioxidative defences so as to preserve the optimal cellular functions. Weakening of these detoxifying mechanisms leads to an accumulation of ROS thereby resulting in pathological conditions. The oxidative stress caused by accumulated free radicals may contribute to degradation of cellular components like protein oxidation, DNA damage, lipid peroxidation, enzyme inactivation, irreversible cellular dysfunction and ultimately cell death if the pro-oxidant-antioxidant balance is not restored.[3,4]

This oxidative stress has been found to be closely related to many disorders such as cancer, atherosclerosis, diabetes, liver cirrhosis and Alzheimer's disease (AD).[5,6] The AD is the most common type of dementia in modern societies affecting more than 20 million people world-wide. This disease is characterized by the loss of cholinergic innervation, reduction of choline acetyltransferase (ChAT) and enhanced acetylcholine esterase (AChE) activity. AChE terminates the interaction between neurotransmitter acetylcholine (ACh) and the corresponding receptor protein (acetylcholine receptor, nAChR), which is the basis of the intercellular communication in brain. Pharmacological and analytical data from human tissue and body fluids have implicated oxidation products of fatty acids in the pathogenesis of AD.[7] Antioxidants can act by scavenging the reactive products of lipid peroxidation and may be useful in prevention and treatment of AD. The modulation of AChE is presently the most accepted and recognized therapeutic marker for development of cognitive enhancers.[8] Currently used AChE inhibitors, like tacrine, produce side effects such as hepatotoxicity.[9] So, the use of natural bioactive compounds, like antioxidants, finds their possible application in the prevention and treatment of AD.

Dastmalchia and his associates have summarized that interest in the discovery of natural antioxidants has risen exponentially, principally for three reasons:

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(i) the overwhelming epidemiological and clinical evidence suggesting an inverse relationship between the consumption of fruits and vegetables and the risk of developing chronic disease like can cer; (ii) concerns regarding the safety of the chronic consumption of synthetic compounds traditionally used as preservatives in foods and beverages; and (iii) the public's belief that phytochemicals are inherently safer than synthetic chemicals. The principal candidates in this exploratory process have always been herbs, spices, and medicinal plants.[10]

The Indian medicinal plant *Withania somnifera* (L. dunal) (family *Solanaceae*), commonly known as Ashwagandha, is widely used in herbal medicines for stress, arthritis, inflammations, conjunctivitis and tuberculosis. Its active principles, sitoindisides VII-X and withaferin-A, have been shown to exhibit significant antistress and antioxidant effects.[11,12] The medicinal properties of Ashwagandha are attributed mostly to its antioxidant compounds but the detailed report on its antioxidant array is lacking. The information on the antioxidant potential and individual polyphenolic compounds of Ashwagandha root extracts has earlier been reported by us.[12-14] In continuation, an attempt has been made to delineate the AChE inhibitory, protein modification preventive and H_2O_2-induced oxidative stress ameliorating properties of the Ashwagandha root extract.

MATERIAL AND METHODS

Chemicals and reagents

Acetylcholine esterase, DTNB (5,5-dithiobis(2-nitrobenzoic acid)), ascorbic acid, AAPH (2,2-azobis(2-methylpropio-namidine) dihydrochloride), SIN-1 (3-morpholinosydnonimine), ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), FC (Folin-Ciocalteu) reagent were of Hi-media and Merck brands (Bangalore, India). All other chemicals were of analytical grade and obtained from Rankem (Bangalore, India).

Preparation of sample extract

The methanolic extract of Ashwagandha, used in present study, was prepared as described in our earlier reports.[13,14]

Evaluation of AChE inhibitory activity

AChE inhibitory activity was measured by slightly modifying the spectrophotometric method developed by Ellman and his associates.[15] Electric eel AChE was used, while S-acetylthiocholine iodide was employed as substrate of the reaction. DTNB [5,5'-dithio-bis(2-nitrobenzoic) acid] was used for the measurement of the AChE activity. Briefly, 1.25 mL of 100 mM sodium phosphate buffer (pH 8.0), 50 µL of Ashwagandha root extract (ARE) of different concentrations (1 to 5 mg), 5 µL of appropriately diluted AChE and 20 µL of acetylthiocholine iodide (75 mM) were mixed and incubated for 15 min at room temperature. The hydrolysis of substrate was monitored spectrophotometric ally by the estimation of yellow 5-thio-2-nitrobenzoate anion, after adding 100 µL of 10 mM DTNB at a wavelength of 412 nm. The per cent of inhibition (% I) was calculated as following:

$$\%\,I = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where, $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the test sample. The experiments were carried out in triplicate and the IC_{50} (concentration of sample required to inhibit 50% of enzyme activity) was calculated by linear regression analysis.

Determination of antioxidant capacity using ABTS$^+$ (2, 2'-azinobis 3-ethylbenzothiazoline-6-sulfonate) assay

The method used was the ABTS$^+$ (cation radical) decolorization assay which is based on the ability of an antioxidant compound to quench the ABTS$^+$ free radical.[16] A stock solution of ABTS$^+$ radical cation was prepared by mixing ABTS and potassium persulfate solution at 7 mM and 2.45 mM final concentration, respectively. The mixture was maintained in the dark at room temperature for 12–16 h before use. For experimental purpose, the ABTS$^+$ stock solution was diluted with phosphate-buffered saline (*p*H 7.4) to an absorbance of 0.70 at 734 nm. Different amounts of ARE (50 to 250 µg) was added to ABTS$^+$ working solution (2 mL). Absorbance was measured by means of a UV–visible spectrophotometer at 734 nm after 20 min of mixing and percent inhibition was calculated. The results were calculated in terms of IC_{50}.

Protective effect of ARE on protein modification

Protein oxidation

Protein oxidation was assayed as described previously by Mayo *et al*. with minor modifications.[17] Degradation of BSA (5 µg) in PBS was initiated by AAPH (20 mM) and inhibited by different concentrations of ARE (50 to 150 µg). After incubation for 2 h at 37 °C, 0.02% BHT was added to the reaction mixture to prevent the further formation of peroxyl radical. The samples were then assayed with normal SDS–PAGE.[18]

Protein nitration

In this experiment, the method of Ippoushi *et al*. was employed with slight modifications.[19] Briefly, BSA (30 µg) was incubated in the absence (control) and presence of 3-morpholinosydnonimine (SIN-1) (final concentration 37.5 mM) with (50, 100 150 µg) or without addition of ARE. After 3 h incubation at room temperature, the samples were run on 10% SDS-PAGE and transferred to nitrocellulose membrane (Millipore, MA, USA). The membrane was blocked overnight at 4°C with 5% (v/v) non-fat dry milk in Tris-buffered saline with Tween-20 (TBS-T) (10 mM Tris–HCl, 150 mM NaCl and 0.1% Tween-20, *p*H 7.5). Further the membrane was incubated for 3 h with 3-nitrotyrosine antibody (3-NT, N5538, Sigma, St. Louis, MO, USA) and washed with TBS-T buffer followed by incubation with goat anti-mouse peroxidase-labelled secondary antibody (Dako, Denmark) and washing with TBS-T buffer. Finally the immunoreactivity was detected by chemiluminescent peroxidase substrate (Sigma, USA).

Inhibitory activity of ARE on protein carbonyl (PCO) formation

The efficacy of ARE to prevent PCO formation was carried out according to the slightly modified method of Wang *et al*. where BSA was oxidized by a Fenton-type reaction.[20] The reaction mixture (1.2 mL) containing extract, potassium phosphate buffer (20 mM, *p*H 7.4), BSA (4 mg), $FeCl_3$ (50 µM), H_2O_2 (1 mM) and ascorbic acid (100 µM) were incubated for 60 min at 37 °C. For determination of PCO content in samples, 1 mL of 10 mM DNPH (2, 4-Dinitrophenylhydrazine) in 2 N HCl was added to the reaction mixture. Samples were incubated for 60 min at room temperature. Then, 1 mL of 10 % TCA was added to reaction mixture and centrifuged at 3,000 x g for 10 min. The protein pellet was washed thrice with 2 mL of ethanol/ethyl acetate (1:1, v/v) and dissolved in 1 mL of guanidine hydrochloride (6 M, *p*H 2.3) and incubated for 10 min at 37 °C. The absorbance of the sample was read at 370 nm.

The data were expressed in terms of percentage inhibition, calculated from a control measurement of the reaction mixture without extract.

Amelioration of H_2O_2-induced stress in rat lymphocytes

Isolation of rat lymphocytes

Lymphocytes were isolated from fresh whole blood using HiSep™ LSM (HiMedia). Briefly, anti-coagulated blood was diluted with an equal

volume of PBS, gently overlaid on HiSep™ LSM and centrifuged at 400g for 15 min at 4°C. Lymphocytes were separated as a pink layer at the top of the HiSep™ LSM. Cells were washed twice with PBS and centrifuged at 400×g for 15 min at 4°C. Cells were rinsed with PBS and diluted to ~6×10^6 cells/mL.

Evaluation of cytotoxicity of ARE

Cell number and viability (Trypan blue exclusion) were determined using a Haemocytometer before treatment. Rat lymphocytes were incubated at a density of ~5×10^5/mL and viability was over 95%. Cell suspensions were incubated with different concentrations of ARE (the final concentration was 10-50 µg/mL) for 30 min at 37 °C in a dark incubator together with untreated control samples. Samples were then centrifuged at 1000×g, the lymphocytes were resuspended in RPMI 1640 and 0.4% trypan blue, and viable and dead cell were scored.

Grouping of lymphocyte

The lymphocytes were divided into 3 groups:

Group I: Treated with PBS and served as control.

Group II: Treated with 100 µM H_2O_2.

Group III: Pre-treated with ARE followed by 100 µM H_2O_2.

The cells were pre-treated with ARE at a concentration of 50 µg/mL for 2 h at 37°C, together with control group, which contained PBS alone. These samples were then centrifuged at 1,000×g for 5 min at 4°C. The ARE treated cells were re-suspended in PBS containing 100 µM H_2O_2 for 30 min at 4°C in dark condition. The samples were then centrifuged at 1,000×g for 5 min at 4°C. After H_2O_2 treatment, cells were centrifuged and washed twice with PBS at 1,000×g for 5 min at 4°C. The cells were then lysed in RIPA buffer.

Biochemical assays

Superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione reductase (GR) activities were assessed using commercially available kits as per manufacture instructions (RANDOX, Canada). Catalase activity was assayed by noting the decrease in absorbance at 240 nm caused by decomposition of H_2O_2.[21] Concentration of malondialdehyde was estimated using thiobarbituric acid, forming a red-coloured compound absorbing at 535 nm.[22] Glutathione content was estimated using Ellaman reagent.[24] Protein content was estimated using the method of Lowry *et al.*[24]

Determination of DNA damage by single cell gel electrophoresis (SCGE)

The integrity of DNA under H_2O_2 induced oxidative stress was evaluated by SCGE method with minor modifications.[25] Briefly, ~2.5×10^4 lymphocytes from each group (in 85 µL of 0.75% LMP agarose) were spread on a base layer of 100 µL of 1% NMP agarose placed on frosted microscopic slides and covered with a cover slip. Once the agarose set, slides were covered with another 100 µL of 0.75% LMP agar and to lyse the cells and nuclear membranes, slides were immersed in freshly prepared cold lysis solution buffer (2.5 mol/L NaCl, 0.1 mol/L Na_2EDTA, 10 mmol/L Tris, *p*H 10.0 and 1% Triton X-100) for 90 min. After lysis the slides were transferred to an electrophoresis buffer (0.3 mol/L NaOH, 1 mmol/L Na_2EDTA, *p*H > 13.0) for unwinding (40 min, 4°C) and then subjected to electrophoresis at 25 V (current adjusted to 0.3 A) for 30 min at 4°C. After electrophoresis, the slides were neutralized with Tris–HCl (0.4 mol/L, *p*H 7.5) twice for 10 min and stained with ethidium bromide (EtBr, 20 µg/mL). Finally the DNA damage was evaluated with fluorescence microscope (Olympus microscope, Germany) by measuring the percentage of fluorescence in tail using RS image software and the results were expressed as per cent inhibition of tail length.

Statistical analysis

All the data were expressed as mean±standard deviation of three experiments. IC_{50} values were calculated using linear regression equation. Statistical analysis was performed using Student's *t*-test and the *p*-values less than 0.05 were considered as significantly different.

RESULTS

AChE inhibitory activity

In the present study, we observed that ARE inhibited the AChE activity in a dose-dependent manner with a R^2 value of 0.97. The IC_{50} value which is inversely related to the activity, as calculated from linear regression equation, was found to be 4.44 mg/mL (Table 1).

Radical scavenging competence of ARE

This blue-green radical discoloration assay is widely used to assess the antioxidant activity of plant extracts. The ARE exhibited $ABTS^+$ radical-scavenging activities to different extents in a concentration dependent manner (Table 2). The R^2 value between concentration and per cent inhibition was 0.96 and the IC_{50} value, calculated using linear regression equation, was found to be 127.49 µg/mL.

Protective effect of ARE on protein modification

Protein oxidation

In the present study, protection against protein degradation damage was determined by the oxidation of BSA initiated by AAPH. After incubation with ARE, the samples were assayed with normal SDS–PAGE. The results (Figure1) demonstrate that ARE exhibited significant protective effect against oxidation of BSA which was augmented by increasing concentrations of extract. At a concentration of 150 µg, the extract showed almost complete inhibition of BSA degradation.

Inhibitory activity of ARE on protein nitration

In the present study, SIN-1 was used to induce protein nitration and the extent of nitration was detected by immune-reactivity with 3-NT antibody. Pre-treatment of BSA with 50, 100 and 150 µg of ARE dose dependently inhibited the protein nitration, while no immunoreactivity was observed in BSA alone group which served as a control (Figure 2).

Inhibitory activity of ARE on PCO formation

Protein oxidation was determined in terms of PCO formation. As shown in Table 3, ARE exhibit dose-dependent inhibitory effects on PCO formation by 15.4, 39.3, 50.6, 70.4 and 74.1% at the extract concentrations of 100, 200, 300, 400 and 500 µg/mL, respectively. The IC_{50} calculated using linear regression equation of $R^2 = 0.97$ was found to be 303.61 µg/mL.

Amelioration of H_2O_2-induced stress in rat lymphocytes

Cytotoxicity of ARE

The cytotoxicity of ARE towards rat lymphocytes, induced at room temperature for 30 min, is shown in Table 4. As a whole, the cell viability was greater than 95% at the concentrations tested, indicating no cytotoxicity toward selected lymphocytes.

Biochemical assays

Results of the experiment describe the onset of oxidative stress in rat lymphocytes by the treatment of H_2O_2 as evidenced by enhanced TBARS/MDA. A significant decrease in the activities of all the five antioxidant enzymes namely SOD, CAT, GPx, GR and G6PDH was observed in H_2O_2-treated (group II) lymphocytes as compared to normal control group (group I) (Figure 3 and its sub figure). But, the activities of antioxidant enzymes were significantly restored almost to their normal levels in ARE pre-treated lymphocytes (group III). Similarly, concentration of

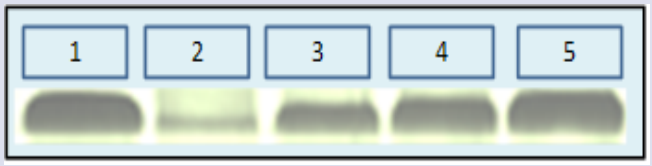


Figure 1: Gel electrophoresis of BSA protein after treatment with AAPH in the presence of different concentrations of extract.

1 = BSA control 2 = BSA treated with AAPH (20mM) 3 = BSA treated with AAPH and ARE (50 µg) 4 = BSA treated with AAPH and ARE (100 µg) 5 = BSA treated with AAPH and ARE (150 µg)

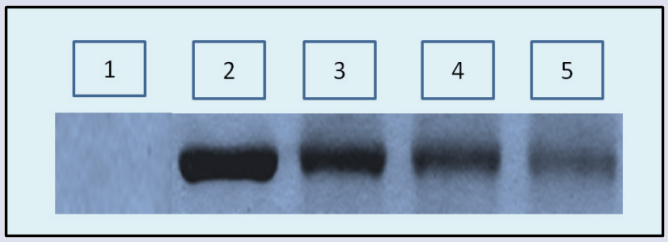


Figure 2: Protective effect of ARS on SIN-1 induced protein nitration biomarker 3-NT analyzed by Western blotting.

1 = BSA control 2 = BSA treated with SIN-1 3 = BSA treated with SIN-1 and ARE (50 µg) 4 = BSA treated with SIN-1 and ARE (100 µg) 5 = BSA treated with SIN-1 and ARE (150 µg).

Table 1: AChE inhibition activity of Ashwagandha root extract (ARE)

Extract (mg/ml)	Percent inhibition	Linear regression equation (correlation coefficient)	IC_{50} (mg/ml)
1	4.58 ± 0.3		
2	15.6 ± 1.2		
3	28.5 ± 1.9	y = 12.462x - 5.3076 (R^2 = 0.9728)	4.44
4	47.5 ± 2.6		
5	58.9 ± 2.3		

Table 2: ABTS$^+$ radical scavenging activity Ashwagandha root extract (ARE)

Extract (µg/ml)	Percent inhibition	Linear regression equation (correlation coefficient)	IC_{50} (µg/ml)
50	24.4±0.4		
100	47.6±1.6		
150	64.2±2.2	y = 0.3344x + 7.3667 (R^2 = 0.9576)	127.49
200	76.1±3.6		
250	82.7±2.6		

Table 3: Inhibition of protein carbonyl formation through Ashwagandha root extract (ARE)

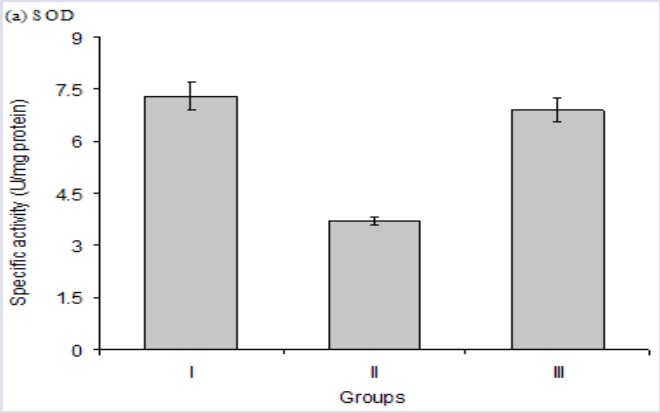
Extract (mg/ml)	Percent inhibition	Linear regression equation (correlation coefficient)	IC_{50} (µg/ml)
100	15.4±0.5		
200	39.3±1.7		
300	50.6±2.9	y = 0.1562x + 2.5762 (R^2 = 0.9732)	303.61
400	70.4±2.6		
500	74.1±3.3		

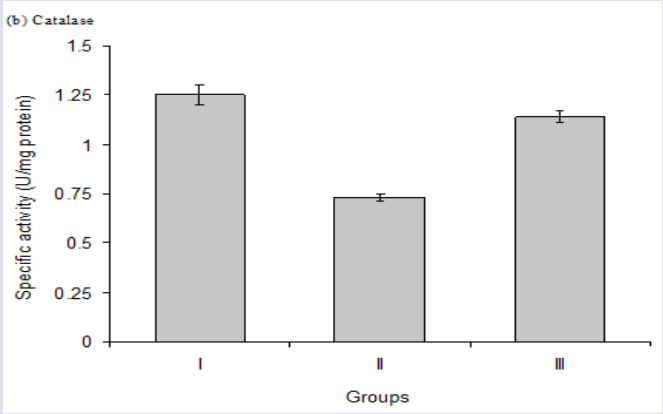
Table 4: Cytotoxicity of Ashwagandha root extract (ARE) towards rat lymphocytes

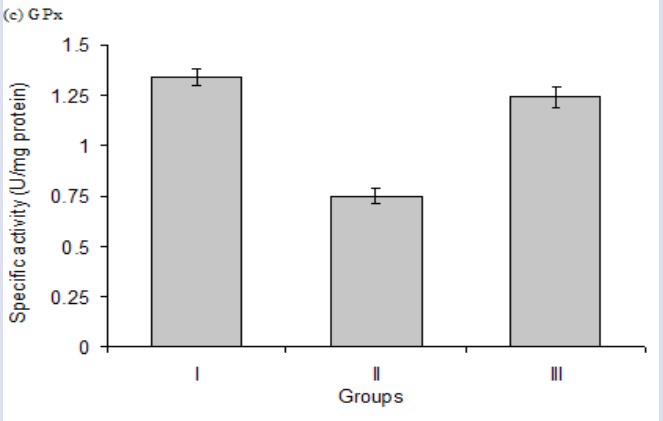
Concentration (µg)	Viability (%)
10	99.1±1.2
20	98.9±1.0
30	99.0±0.9
40	98.7±1.1
50	98.8±1.2

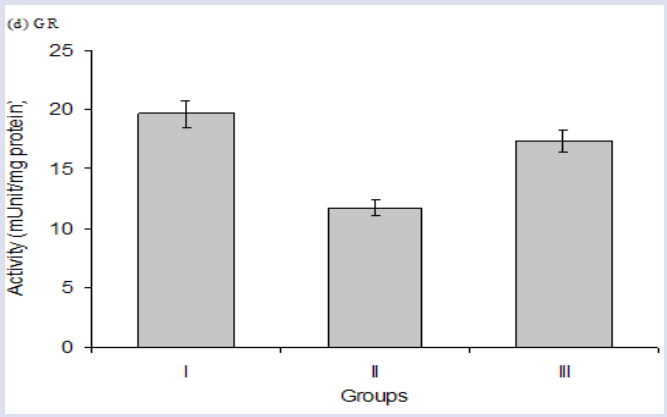
The viability = [nonstained cells/ (stained+nonstained cells)]x100%.

The high viability % indicated high live cells. Results are means ± SD for triplicate experiments.









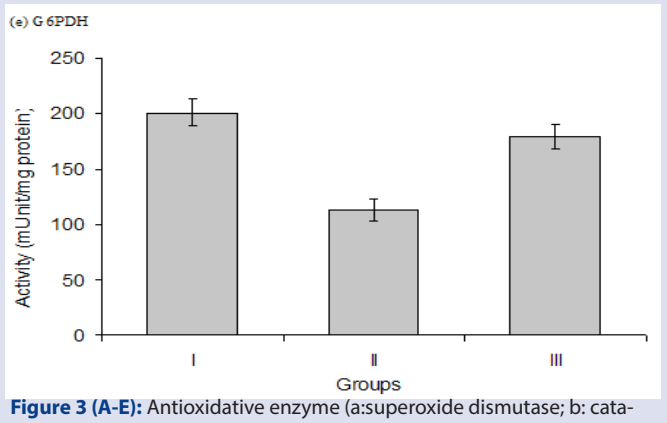
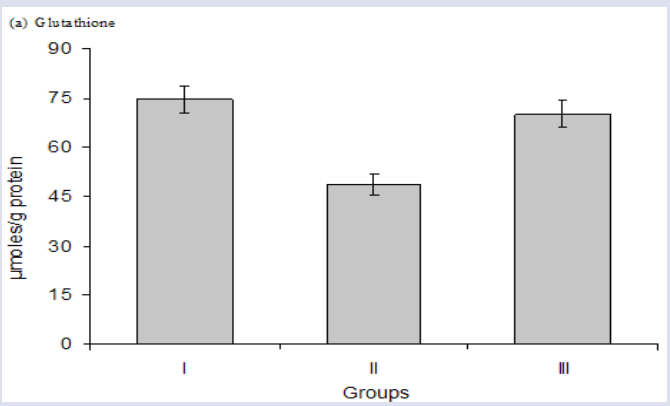


Figure 3 (A-E): Antioxidative enzyme (a:superoxide dismutase; b: catalase; c: glutathione peroxidase; d: glutathione reductase) and glucose-6-P-dehydrogenase activities under the oxidative stress.



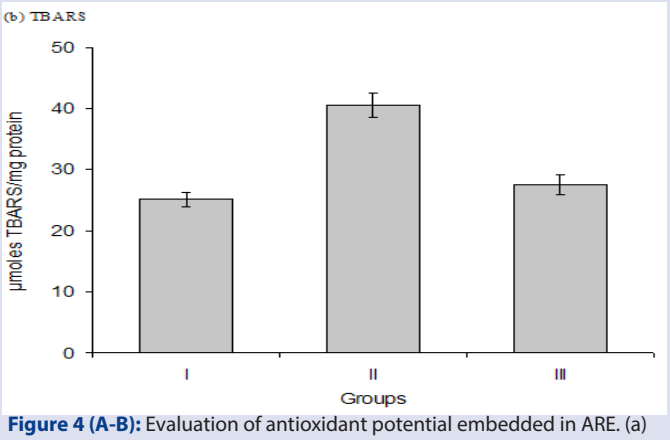
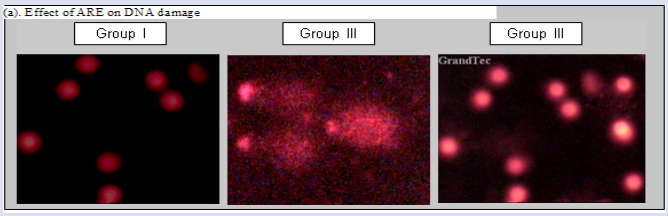


Figure 4 (A-B): Evaluation of antioxidant potential embedded in ARE. (a) glutathione; (b) TBARS: Thiobarbituric acid reactive substances.



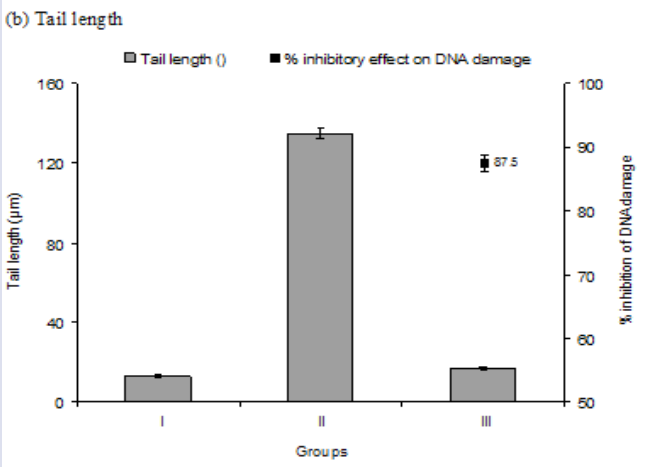


Figure 5 (A-B): Effect of ARE (Ashwagandha root extract) on DNA through fluorescence microscopy and quantitative estimation of chromosomal tail length damage.

glutathione showed a significant decrease after H_2O_2 treatment in comparison to normal group (Figure 4a). However, pre-treatment with ARE caused a significant increase in the concentration of glutathione and it was almost restored to normal values. When compared with the normal group, there was a significant increase in the concentration of MDA in the H_2O_2 treated group while a substantial decrease in MDA concentration was brought by pre-treatment of ARE (Figure 4b).

Effect of ARE on DNA damage

The effect of ARE on DNA damage in each group of lymphocytes was investigated and the results were expressed in terms of tail length. The value of tail length in H_2O_2 challenged lymphocyte was 135 µm. However, the DNA damage in rat lymphocytes induced by H_2O_2 was decreased by ARE supplementation to an extent of ~88 % (Figure 5). The values of tail lengths in group I and III were not significantly different ($P > 0.05$) indicating that ARE almost completely prevented the DNA damage in rat lymphocytes.

DISCUSSION

Many groups of independent researchers have studied the plausible beneficial effects of dietary antioxidants in altering, reversing and/or forestalling the negative effects of oxidative stress.[26-28] The antioxidants defence system of an organism comprising of different antioxidant enzymes and metabolites plays an important role in cellular defense against oxidative abuse. Natural antioxidants present in herbs are responsible for inhibiting and/or preventing the deleterious consequences of oxidative stress. Herbs contain free radical scavengers like polyphenols, flavonoids and phenolic compounds. Reports have shown that there is

lesser likelihood of drug and herb interaction when subjects are concomitantly administered with CY3PA substrates.[29] This suggests that herbal formulation have their own effect without counteracting with other drugs. Apart from it, the sub-effective doses of *withania somnifera* enhance the anticataleptic action of L-DOPA and this adjunctive therapy reduces the doses and the adverse effects of dopamine precursors in Parkinson disease.[30] The novel herbomineral test formulation based on Ashwagandha might act as an effective anti-inflammatory and immunomodulatory product, and this can be used as a complementary and alternative treatment for the prevention of various types of inflammatory and auto-immune disorders.[31] In continuation with our previous work [13,14] we have examined *ex-vivo* model of stressed rat lymphocytes and observed AChE inhibitory and pro-antioxidant activities of ARE.

According to Atta-ur-Rahman and Choudhary,[32] the use of antioxidants may slow the progression of Alzheimer's disease (AD) and minimize neuronal degeneration. Currently there is no cure for AD. To date, the most promising target for the symptomatic treatment and slowing of AD progression is cholinesterase inhibitors.[33] In AD, the destruction of cholinergic neurons causes the depletion of the neurotransmitter, acetylcholine (ACh). By inhibiting, AChE, the enzyme which catalyses breakdown of ACh, the levels of this transmitter can be elevated and function improved. The compounds that exhibit AChE inhibitory activity are also related to radical-scavenging activity.[34] The potentiation of cholinergic activity through the inhibition of AChE is one of the major strategies of pharmacotherapy for AD disease.Its inhibitors like tacrine, donepezil, rivastigmine, and galanthamine are generally used in treating neurodegenerative disorders but because of the side effects associated with these drugs, it is worthwhile to explore the herbal remedies as an alternative and safe medicine to the existing pharmacological agents. In order to verify these approaches, we have evaluated the AChE inhibitory activity of ARE and found it to substantially inhibit AChE activity which is in line with an earlier study by Mukherjee *et al*. who reported the AChE inhibitory activity of various plant extracts.[35] *Amorpha fruticosa* fruit having acetylcholinesterase inhibitory activity, 48.86 ± 0.55% (2 mg/mL) could be useful in therapy of free radical pathologies and neurodegenerative disorders.[36] The results of the present work showed that ARE can effectively be used to increase the level of ACh.

Antioxidants are closely related to their multifaceted biofunctionalities like reduction of chronic diseases by inhibiting mutagenesis and carcinogenesis; inhibition of pathogenic bacterial growth etc.[37] Thus, antioxidant capacity is widely used as a parameter for medicinal bioactive components. The $ABTS^+$ method has widely been employed for measuring the relative radical scavenging activity of hydrogen donating and chain breaking antioxidants in many plant extracts.[38-39] $ABTS^+$ radicals are more reactive than DPPH radicals and unlike the reactions with DPPH radical which involve H atom transfer, the reactions with $ABTS^+$ radicals involve electron transfer process. Phenolic compounds have been ascribed as an important contributor to the antioxidant activity of various plant extracts.[26] In our study, the ARE was found effective in $ABTS^+$ radical scavenging assay in a concentration dependent manner. This ability of ARE to scavenge $ABTS^+$ radical may contribute to its significant antioxidant potential.

It is also a well known fact that the progressive accumulation of macromolecular oxidative damage is the root cause of senescence-associated cellular changes. Oxidation of cellular proteins has been described under many pathological conditions. AAPH, a water–soluble initiator, decomposes at physiological temperature and produces alkyl radicals followed by fast reaction with oxygen to give alkyl peroxyl radicals to initiate protein oxidation.[40] The free radical provoked oxidative protein damages have been demonstrated to play a significant role in aging and several pathological events.[41] Radical mediated damages to proteins might be initiated by electron leakage, metal-ion dependent reactions, and auto-oxidation of lipids and sugars.[42] In our study it was found that ARE has the capability to almost block the AAPH induced protein degradation.

Nitric oxide (NO) or reactive nitrogen species (RNS) have been found to alter the structural and functional behavior of many cellular components particularly proteins. In biological system, NO is synthesized by nitric oxide synthase (NOS) as a by-product during conversion of L-arginine to L-citrulline. The NO-mediated peroxynitrite formation results in protein nitration and its effects have also been implicated in the pathophysiology of neurodegenerative disorders.[43] Peroxynitrite is not only a powerful oxidant but also a strong nitrating agent.[44] Protein tyrosine residues are especially susceptible to peroxynitrite-mediated nitration reactions producing 3-nitrotyrosine.[45] To test the protective effect of ARE against peroxynitrite-induced nitration of tyrosine, we selected BSA as a model target. Nitration reaction of BSA was carried out in the presence of various dosages of ARE and peroxynitrite-induced nitration of tyrosine residues in BSA was examined by Western blotting using anti-3-nitrotyrosine antibody. Exposure of BSA to *in-situ* generated peroxynitrite resulted in nitrotyrosine immune-reactivity. The intensity of the SDS-PAGE gel band derived from 3-nitrotyrosine residues in BSA decreased with increasing amounts of ARE confirming the protection of BSA by ARE against peroxynitrite induced tyrosine nitration. The present investigation shows the potential antioxidant activity of ARE which can therefore play a key role in arresting the chain of free radical reactions initiated by excess generation of NO. Our results are in line with the recently published report published for NO scavenging activities of *Lycopodium* species.[47]

Proteins have also been identified to be damaged by free radicals directly and to be the targets of secondary modifications by aldehydic products of lipid peroxidation or ascorbate auto-oxidation resulting in PCO formation. The assessment of PCO is a widely used marker for oxidative protein modification and it is reported to be a sensitive and early marker of oxidative stress. Our study shows that ARE is fully equipped to inhibit PCO formation. The inhibitory effect of extracts might operate by scavenging the peroxyl radical generated in the reaction mixture.

H_2O_2 has been found to induce oxidative stress in isolated rat lymphocytes as shown by decreased activities of antioxidant enzymes (Figure 3 and subfigure). The levels of reduced glutathione and MDA approached the normal levels in ARE pre-treated H_2O_2 exposed lymphocytes. The restoration of MDA to almost normal values by ARE may be due to an enhancement of antioxidant enzymes, such as SOD, catalase, GPx, GR and G6PDH.

ROS, such as superoxide anions and H_2O_2, and RNS are produced throughout cells during normal aerobic metabolism. The intracellular concentration of ROS depends both on their production as well as removal by various antioxidants. A major component of the antioxidant system in mammalian cells consists of three enzymes, namely SOD, CAT and GPx. These enzymes work in concert to detoxify superoxide anion and H_2O_2 in cells. Our results indicated that pre-treatment with ARE caused an increase in the activities of antioxidant enzymes in H_2O_2-challenged lymphocytes. The ameliorating effect of ARE may be ascribed to its protective phytochemicals like total phenolic and flavonoids contents.[12-14] Earlier reports also advocate that flavonoids exert a stimulatory action on transcription and gene expression of certain antioxidant enzymes.[47] GPx catalyses the reduction of peroxides using reduced glutathione and converting into oxidized glutathione. Hence, increased activities of the enzymes, GPx and GR, were correlated with increased glutathione levels.

The comet assay also called as 'single-cell gel electrophoresis assay', is a sensitive method for detecting DNA strand breaks. Therefore, an increasing number of laboratories have begun to use this extremely versatile assay

to detect DNA damage. In present work, this assay was used to determine the effect of ARE on DNA damage in selected lymphocyte exposed to H_2O_2 and protection of DNA damage by ARE clearly indicates its prophylactic potential at molecular level. As proved earlier by us,[13,14] ARE exhibited a scavenging effect on H_2O_2 which might have contributed to inhibition of DNA damage in rat lymphocytes. Our results corroborate with previous work where Aherne and O'Brien showed that pre-treatment with quercetin and rutin had reduced DNA damage induced in Caco-2 and Hep G2 cells by a subsequent exposure to H_2O_2.[48] Glei *et al.* reported that β-carotene reduces H_2O_2-induced genetic damage in human lymphocytes when pre-treated with β-carotene.[49] Another report by Lean *et al.* suggested that dietary flavonols protect diabetic human lymphocytes against oxidative damage to DNA.[50] Docosahexaenoic acid was also reported to protect human lymphocytes against H_2O_2-induced oxidative stress.[51]

CONCLUSION

The current study indicates that ARE has AChE inhibitory activity and is equally effective in preventing the oxidative stress induced by free radicals. The extract showed substantial eukaryotic DNA damage protection induced by H_2O_2 and also exhibited a significant protection against protein oxidation and nitration induced by AAPH and SIN-1. Therefore ARE can be used as a source of antioxidant for dietary supplementation to alleviate oxidative stress induced cellular injuries.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interests regarding the publication of this paper.

ABBREVIATION USED

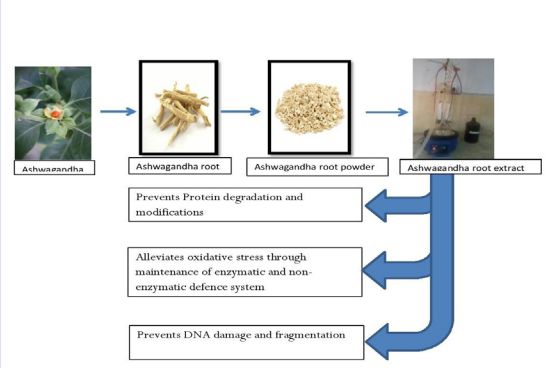
ARE: Ashwagandha root extract; **AChE:** Acetylcholine esterase; **PCO:** Protein carbonylation; **TBARS:** Thiobarbituric acid reactive substances; **MDA:** Malondialdehyde.

REFERENCES

1. Halliwell B, Gutterid JMC. Reactive species as useful biomolecules. In: (eds.) Free radicals in biology and medicine. Oxford: Clarendon Press. 1989;2:188–218.
2. Stohs SJ. The role of free radicals in toxicity and disease. J Basic Clin Physiol Pharmacol. 1995;6(3–4):205–28 https://doi.org/10.1515/jbcpp.1995.6.3-4.205.
3. Halliwell B. Antioxidants in human health and disease. Ann Rev Nutr. 1996;16(1):33–50 https://doi.org/10.1146/annurev.nutr.16.1.33.https://doi.org/10.1146/annurev.nu.16.070196.000341 PMid:8839918.
4. Morrissey PA, O'Brien NM. Dietary antioxidants in health and disease. Int Dairy J. 1998;8(5-6):463–72https://doi.org/10.1016/S0958-6946(98)00070-3
5. Muramatsu H, Kogawa K, Tanaka M, Okumura K, Koike K, Kuga T. Superoxide dismutase in SAS human tongue carcinoma cell line is a factor defining invasiveness and cell motility. Cancer Res. 1995;55(24):6210–4.PMid:8521415.
6. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol: Modification of low density lipoprotein that increase its artherogenicity. New Eng J Med. 1989;321(17):1196–7.
7. Akhondzadeh S, Abbasi SH. Herbal medicine in the treatment of Alzheimer´s disease. Am J Alzheimers Dis Other Demen. 2006;21:113–118. https://doi.org/10.1177/153331750602100211 PMid:16634467
8. Kim HK, Kim M, Kim S, Kim M, Chung JH. Effects of green tea polyphenol on cognitive and acetylcholinesterase activities. Biosci Biotechnol Biochem. 2004;68(9):1977-9. https://doi.org/10.1271/bbb.68.1977 PMid:15388975.
9. Perry NSL, Bollen C, Perry EK, Ballard C. Salvia for dementia therapy: Review of pharmacological activity and pilot tolerability clinical trial. Pharmacol Biochem Behav. 2003;75(3):651–9.https://doi.org/10.1016/S0091-3057(03)00108-4
10. Dastmalchia K, Damien Dormana HJ, Kosarb M, Hiltunen R. Chemical composition and *in vitro* antioxidant evaluation of a water soluble Moldavian balm (Dracocephalum moldavica L.) extract. LWT Food Sci Technol. 2007;40(2):239–48. https://doi.org/10.1016/j.lwt.2005.09.019
11. Gupta GL, Rana AC. *Withania somnifera* (Ashwagandha): a review. Pharmacog Rev. 2007;1(1):129.
12. Pal A, Bhushan B, Khanum F. (2012) Therapeutic uses of *Withania somnifera* (Ashwagandha). In: (eds.) Recent Progress in Medicinal Plants (RPMP). Phytoconstituents and physiological processes. USA: Stadium Press. 2012;34:97-118.
13. Pal A, Naika M, Khanum F, Bawa AS. *In-vitro* studies on the antioxidant assay profiling of *Withania somnifera* L (Ashwagandha) Dunal root: Part 1. Pharmacog J. 2011;3(20):47-55.https://doi.org/10.5530/pj.2011.20.10
14. Pal A, Naika M, Khanum F, Bawa AS. *In-vitro* studies on the antioxidant assay profiling of root of *Withania somnifera* L. (Ashwagandha) Dunal: Part 2. Agric Consp Scient. 2012;77(2):95-101.
15. Ellman GL, Courtney KD, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961;7(2):88-95.https://doi.org/10.1016/0006-2952(61)90145-9.
16. Re R, Nicoletta P, Anna P, Ananth P, Min Y, Catherine RE. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biol Med. 1999;26(9):1231–7.https://doi.org/10.1016/S0891-5849(98)00315-3
17. Mayo JC, Tan DX, Sainz RM, Natarajan M, Lopez-Burillo S, Reiter RJ. Protection against oxidative protein damage induced by metal-catalyzed reaction or alkylperoxyl radicals: comparative effects of melatonin and other antioxidants. Biochim Biophys Acta. 2003;1620(1):139–50. https://doi.org/10.1016/S0304-4165(02)00527-5
18. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680–5. https://doi.org/10.1038/227680a0 PMid:5432063.
19. Ippoushi K, Azuma K, Ito H, Horie H, Higashio H. [6]-Gingerol inhibits nitric oxide synthesis in activated J774.1 mouse macrophages and prevents peroxynitrite-induced oxidation and nitration reactions. Life Sci. 2003;73(26):3427-37. https://doi.org/10.1016/j.lfs.2003.06.022 PMid:14572883.
20. Wang B, Lin S, Hsiao W, Fan J, Fuh L, Duh P. Protective effects of an aqueous extract of Welsh onion green leaves on oxidative damage of reactive oxygen and nitrogen species. Food Chem. 2006;98(1):149–57.https://doi.org/10.1016/j.foodchem.2005.05.057
21. Cohen G, Dembiec D, Marcus J. Measurement of catalase activity in tissue extracts. Anal Biochem. 1970;34(1):30–38.https://doi.org/10.1016/0003-2697(70)90083-7.
22. John AB, Steven DA. Microsample lipid peroxidation. In: Fleischer S, Packer L, editors. Methods in enzymology. New York: Academic Press. 1978;52:302–10.
23. Ellman GL. Liver glutathione: A colorimetric method for determining low concentrations of mercaptans. Arch Biochem Biophys. 1958;74(2):443-50. https://doi.org/10.1016/0003-9861(58)90014-6.
24. Lowry ON, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem. 1951;193(1):265-75.PMid:14907713
25. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res. 1988;175(1):184–91. https://doi.org/10.1016/0014-4827(88)90265-0
26. Giampieri F, Alvarez-Suarez JM, Battino M. Strawberry and human health: Effects beyond antioxidant activity. J Agric Food Chem. 2014;62(18):3867–76. https://doi.org/10.1021/jf405455n PMid:24450925.
27. Ilango K, Chitra V. Wound healing and anti-oxidant activities of the fruit pulp of Limonia acidissima Linn (Rutaceae) in rats. Trop J Pharma Res. 2010;9(3).
28. Kangralkar VA, Patil SD, Bandivadekar RM, Nandagaon VS. Hepatoprotective activity of Feronia elephantum fruit extract against paracetamol induced hepatic damage in Wistar rats. Int J Pharma Appl. 2010;1:46–9.
29. Savai J, Varghese A, Pandita N. Lack of cytochrome P4503A interaction of methanolic extract of *withania somnifera*, withaferin A, withanolide A and withanoside IV. J Pharma Negative Results. 2013;4(1):26.https://doi.org/10.4103/0976-9234.116766
30. Gupta GL, Rana AC. Synergistic effect of *withania somnifera* dunal and L-DOPA in the inhibition of haloperidol-induced catalepsy in mice. Pharmacog Mag. 2009;5(19):46.
31. Trivedi MK, Mondal SC, Gangwar M, Jana S. Effect of a novel ashwagandha-based herbomineral formulation on pro-inflammatory cytokines expression in mouse splenocyte cells: A potential immunomodulator. Phcog Mag. DOI: 10.4103/0973-1296.197709.https://doi.org/10.4103/0973-1296.197709
32. Atta-ur-Rahman, Choudhary MI. Bioactive natural products as a potential source of new pharmacophores. A theory of memory. Pure Appl Chem. 2001;73(3):555-60.
33. Crowch CM, Okello EJ. Kinetics of acetylcholinesterase inhibitory activities by aqueous extracts of Acacia nilotica (L.) and Rhamnus prinoides (L'Her.). Afr J Pharma Pharmacol. 2009;3(10):469-75.
34. Papandreou MA, Dimakopoulou A, Linardaki ZI, Cordopatis P, Klimis-Zacas D, Margarity M, Lamari FN. Effect of polyphenol-rich wild blueberry extract on cognitive performance of mice, brain antioxidant markers and acetylcholine esterase activity. Behav Brain Res. 2009;198(2):352-8.https://doi.org/10.1016/j.bbr.2008.11.013 PMid:19056430.
35. Mukherjee PK, Kumar V, Mal M, Houghton PJ. Acetylcholinesterase inhibitors from plants. Phytomed. 2007;14(4):289-300. https://doi.org/10.1016/j.phymed.2007.02.002 PMid:17346955.

36. Zheleva-Dimitrova DZ. Antioxidant and acetylcholinesterase inhibition properties of Amorpha fruticosa L. and Phytolacca americana L. Phcog Mag. 2013;9(34):109. https://doi.org/10.4103/0973-1296.111251 PMid:23772105 PMCid:PMC3680849.
37. Zhu QY, Hackman RM, Ensunsa JL, Holt RR, Keen CL. Antioxidative activities of Olong tea. J Agric Food Chem. 2002;50(23):6929-34. https://doi.org/10.1021/jf0206163 PMid:12405799.
38. Brand-Williams W, Cuvelier ME, Berset C. Use of a free radical method to evaluate antioxidant activity. LWT Food Sci Technol. 1995;28(1):25–30https://doi.org/10.1016/S0023-6438(95)80008-5
39. Netzel M, Strass G, Bitsch I, Konitz R, Christmann M, Bitsch R. Effect of grape processing on selected antioxidant phenolics in red wine. J Food Engineer. 2003;56(2):223-8.https://doi.org/10.1016/S0260-8774(02)00256-X.
40. Cai YJ, Fang JG, Ma LP, Li Y, Liu ZL. Inhibition of free radical induced peroxidation of rat liver microsomes by resveratrol and its analogues. Biochim Biophys Acta. 2003;1637(1):31-8.https://doi.org/10.1016/S0925-4439(02)00174-6
41. Stadtman ER, Levin RL. Protein oxidation. Ann NY Acad Sci. 2000;899:191-208. https://doi.org/10.1111/j.1749-6632.2000.tb06187.x PMid:10863540.
42. Dean RT, Fu S, Stocker R, Davies MJ. Biochemistry and pathology of radical mediated protein oxidation. Biochem J. 1997;324(1):1-18. https://doi.org/10.1042/bj3240001 PMid:9164834 PMCid:PMC1218394.
43. Scabo C, Ischiropoulos H, Radi R. Peroxynitrite: Biochemistry, pathophysiology and development of therapeutics. Nat. Rev. Drug Discovery. 2007;6(8):662-80. https://doi.org/10.1038/nrd2222 PMid:17667957.
44. Radi R, Peluffo G, Alvarez MN, Naviliat M, Cayota A. Unraveling peroxynitrite formation in biological systems. Free Radical Biol Med. 2001;30(5):463-88. https://doi.org/10.1016/S0891-5849(00)00373-7.
45. Pannala A, Razzaq R, Halliwell B, Singh S, Rice-Evans C. Inhibition of peroxynitrite dependent nitration of hydroxycinnamic acids: nitration or electron donation? Free Radical Biol Med. 1998;24(4):594-606.https://doi.org/10.1016/S0891-5849(97)00321-3.
46. Konrath EL, Neves BM, Lunardi PS, Passos C, Simoes-Pires A, Ortega MG, *et al.* Investigation of the and *ex vivo* acetylcholineesterase and antioxidant activites of traditionally used Lycopodium species from South America on alkaloid extracts. J Ethnopharmacol. 2012;139(1):58-67. https://doi.org/10.1016/j.jep.2011.10.042 PMid:22108682.
47. Rohrdanz E, OhleTran-Thi RS, Tran-Thi QH, Kahl R. The phytoestrogen daidzein affects the antioxidant enzyme of rat hepatoma H4IIE cells. J Nutr. 2002;132(3):370-75.PMid:11880557.
48. Aherne SA, O'Brien NM. Protection by the flavonoids myricetin, quercetin, and rutin against hydrogen peroxide-induced DNA damage in Caco-2 and Hep G2 cells. Nutr Cancer. 1999;34(2):160–6.https://doi.org/10.1207/S15327914NC3402_6 PMid:10578483.
49. Glei M, Liegibel UM, Ebert MN, Bohm V, Pool-Zobel BL. β-Carotene reduces bleomycin-induced genetic damage in human lymphocytes. Toxicol Appl Pharmacol. 2002;179(2):65–73. https://doi.org/10.1006/taap.2001.9329 PMid:11884238.
50. Lean ME, Noroozi M, Kelly I, Burns J, Talwar D, Sattar N. Dietary flavonols protect diabetic human lymphocytes against oxidative damage to DNA. Diabetes. 1999;48(1):176–81. https://doi.org/10.2337/diabetes.48.1.176 PMid:9892240
51. Bechoua S, Dubois M, Dominguez Z, Goncalves A, Nemoz G, Lagarde M. Protective effect of docosahexaenoic acid against hydrogen peroxide-induced oxidative stress in human lymphocytes. Biochem Pharmacol. 1999;57(9):1021–30. https://doi.org/10.1016/S0006-2952(99)00012-X

GRAPHICAL ABSTRACT



SUMMARY

- Acetylcholine esterase (AChE) inhibitory potential of Ashwagandha root extract (ARE) was explored.
- ARE effectively prevented protein degradation as well as modification as studied by SDS-PAGE and Western blotting.
- ARE protected rat lymphocytes against H_2O_2-induced oxidative damage.
- H_2O_2 treatment resulted in DNA fragmentation as evidenced by 'Comet' assay and ARE pre-treatment almost reversed these changes indicating its efficiency to suppress hydrogen peroxide-induced oxidative stress.
- Study provides the scientific basis of pleiotropic functions of Ashwagandha.

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