Effect of Berberine and Ipriflavone Mixture against Scopolamine-Induced Alzheimer-Like Disease

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ABSTRACT

Some herbal medications like berberine and ipriflavone have a potential effect on Alzheimer. We studied the effect of berberine/ipriflavone mixture on the memory deficit and cognitive dysfunction which are induced by scopolamine and causing Alzheimer-like disease. 56 male Wistar rats were randomly subdivided into seven groups; including, two control groups (one received saline and the other one received polyethylene glycol), induced group, and four different treated groups (berberine, ipriflavone, berberine/ipriflavone mixture, and donepezil). Induced group and treated groups were received 2 mg/kg scopolamine intraperitoneally, once a day for 4 weeks. Berberine or ipriflavone orally administered (50 mg/kg dissolved in 20% polyethylene glycol). Group of berberine/ipriflavone mixture was orally administered 25 mg/kg each dissolved in 20% polyethylene glycol. Donepezil group was orally administered 2.25 mg/kg dissolved in saline. All treatments were continued daily for 4 weeks; afterward, the Morris water maze (MWM) test was initiated. Rats were euthanized 24 hours after probe trial for retention test; then brain tissues were collected from all groups for determination of pro-oxidants, antioxidants, an inflammatory marker, acetylcholine esterase activity, Alzheimer disease markers, and energy profile. Treatment with berberine/ipriflavone mixture improved scopolamine-induced cognitive dysfunctions in the MWM test and was significantly related to the increment in anti-oxidant levels, anti-inflammatory activity, cholinergic function, and brain energy profile. The current study revealed the relation between glucose-6-phosphate dehydrogenase and Alzheimer disease progression. Also, this mixture showed a substantial protective effect against the cognitive impairment induced by scopolamine.

Key Words: *Berberine, Ipriflavone, Alzheimer, G6PD, Scopolamine, Acetylcholinesterase.*

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease, which leads to the progressive loss of mental-behavioral function and ability to learn. The entire cause remains unidentified, and there is a lack of effective therapy that can stop over the disease progression [1]. AD is related to the elevated levels of Amyloid-β (Aβ) and the hyperphosphorylated Tau protein, along with alterations of the central cholinergic system [2]. Moreover, there is some evidence pointing to the role of oxidative stress, inflammation, and cell cycle regulatory defect in its pathogenesis [3]. Recent studies have also underlined the role of Aβ, which causes direct injury to neurons, along with enhanced neuroinflammation, gliosis, astrocytosis, and finally neuronal loss [4]. Also, AD leads to hyperactivation of glucose-6-phosphate dehydrogenase (G6PD), which leads to energy disruption. The defective metabolism of glucose leads to the metabolic defect of amyloid precursor protein (APP) and the defective clearance of amyloid-β in addition to a hyperphosphorylated Tau that causes the breakdown of microtubular aggregation and synaptic deficiency, which leads to AD progression. In addition, AD causes

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cholinergic dysfunctions that arise in the uncontrolled central nervous system of aged subjects. Memory weaknesses can be induced by stalling cholinergic systems in young subjects. On the contrary, under certain conditions, cholinergic stimulation can improve memory in aged subjects [5]. Currently approved therapies have been shown to delay the progression of AD. Unfortunately, people taking these therapies don't respond to the treatment [3]. Accordingly, more effort is required for the discovery of new modifying therapeutic agents to prevent the progression of the disease and to repair the synaptic and neuronal loss.

Scopolamine (SCO) was considered as an inducer for AD in rats due to its action as muscarinic acetylcholine (Ach) receptor blocker. SCO induces defect of the cholinergic neuronal pathway and memory dysfunctions in the CNS, resulting in severe deficiencies in learning, acquisition, and short-term retention of spatial memory functions [6].

Berberine (BER), an isoquinoline alkaloid, has neuroprotective effects due to acting as an antioxidant; inhibitor for acetylcholine esterase (AChE), monoamine oxidase (MAO); and butyrylcholine esterase (BChE), and a reducing agent for Aβ level and cholesterol [7]. Lee, et al., 2012, demonstrated that administration of *Phellodendron amurense* (PA) and BER had significant neuroprotective effects against memory dysfunction and neuronal impairment caused by scopolamine in rats [8]. Thus, PA and berberine may be useful agents in preventing cholinergic dysfunction, neuronal impairment, and anti-inflammatory effects.

Ipriflavone (IPR) is a synthetic isoflavone derivative. Previous studies suggested that IPR acts as a non-competitive inhibitor for AChE due to its similarity in structure to donepezil, which is an approved treatment of AD symptoms [9]. IPR was shown to have impressive treatment effect on AD as proved by antagonizing Aβ [10]. Recently, it was shown that ipriflavone demonstrated neuroprotective effects against scopolamine-induced memory dysfunction in rats [11]. According to the individual efficacy of BER or IPR in AD treatment, the present study was designed to evaluate the therapeutic effect of BER/IPR mixture on the improvement of memory and learning deficits in rats injected with SCO. Also, this study was conducted to highlight the biochemical effect of BER/IPR mixture on brain performance and its relation to glucose metabolism and energy expenditure in AD induced rats.

MATERIAL AND METHODS

Animals

Fifty-six male Wistar rats weighing from 150 to 200 g were obtained from the Medical Research Institute, University of Alexandria. The animals were kept in polypropylene cages (four animals per cage with the size of 90×55×55 cm.) covered with metallic grids. They were maintained at proper environmental conditions of temperature and humidity with a 12 hours light-dark cycle. During the experiment, the standard chow diet and drinking water were given *ad libitum* to the rats. The animals were adapted in two weeks before starting the experiment. All animal procedures were done according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Institute of Laboratory Animal Resources 1996).

Chemical compounds

Scopolamine hydrobromide, ipriflavone, and berberine hydrochloride were purchased from Sigma-Aldrich, USA. All other chemicals were analytical-grade commercial products.

Methods

Experimental animal design

For developing memory and learning deficits, rats were injected (ip) by 2 mg/kg BW of scopolamine hydrobromide (SCO), dissolved in physiological saline solution, once a day for 4 weeks [12]. Control animals separately received saline or polyethylene glycol. Treatment was carried out by oral administration of BER and IPR, either individually or in the mixture, prepared in 20% polyethylene glycol [9, 11]. Reference drug "Donepezil", a centrally acting cholinesterase inhibitor, was used as a positive control. Donepezil hydrochloride was obtained as 5 mg tablets. The tablets were crushed, suspended in physiological saline (2 mg/ml) and administered in a dose of 2.25 mg/kg based on the previous studies [13, 14]. The rats were subdivided into seven groups, randomly each containing 8 rats. Two control groups, one received saline (0.5 ml/day, intraperitoneally) only, and the other received polyethylene glycol (20%, 0.5 ml/day, orally); SCO induced group received scopolamine dissolved in saline (2 mg/kg, intraperitoneally), BER treated group received BER (50 mg/kg, orally) dissolved in 20% polyethylene glycol followed by SCO (2 mg/kg, intraperitoneally) after 40 minutes, IPR treated group received IPR (50 mg/kg, orally) dissolved in 20% polyethylene glycol followed by SCO (2 mg/kg, intraperitoneally) after 2 hours, BER/IPR treated group received a mixture of BER/IPR (each 25 mg/kg, orally) dissolved in 20% polyethylene glycol followed by SCO (2 mg/ kg, intraperitoneally) after 40 minutes; and donepezil treated group received donepezil (2.25 mg/kg, orally) followed by SCO (2 mg/kg, intraperitoneally) after 2 hours. All treatment doses were determined according to our pilot experiments as the minimal effective doses. The above-mentioned treatments were continued daily for 4 weeks. At the 24th day after SCO injection, rats performed to practice the MWM experiment. Rats were anesthetized by Isoflurane and

euthanized by decapitation 24 hours after probe trial for retention test in MWM task.

Morris Water Maze (MWM)

According to Morris R., 1984; [15], the MWM apparatus consists of a large round black pool (120 cm diameter, 50 cm height), water was poured to a depth of 30 cm. The water temperature was adjusted at 26±2 °C. 4 equally spaced points around the pool edge were designed as N (North), E (East), S (South) and W (West). A black-colored platform (8 cm diameter) was positioned 1cm below the water surface in a fixed position, in the middle of the pool's NE quadrant; the starting point was in the SW quadrant in all the trials. One kg of starch was added to hide the location of the hidden platform by making the water opaque. The MWM test was started on the 24th day after the scopolamine (SCO) administration. Rats were trained to find the hidden platform, located in a fixed location throughout the test. Also, rats were given up to 180 seconds (cut-off time) to find the hidden platform. Latency time of reaching the platform was recorded in the trials. 3 trials/day was done for 5 consecutive days, and on the 6th day, the rats received a 60-seconds probe trial. Staying on the platform for at least 4 seconds before ending the 180 seconds was defined as finding the platform. If the rat could not find the platform in the first trial of the hidden platform test in the assigned time, they were placed on the platform for 20 seconds and assigned a latency of 180 seconds. Water was stirred after each trial to erase olfactory traces of the previous swimming patterns. All of the procedures lasted six consecutive days, and each animal had 3 training trials/day, with 30-40 minutes inter-trial interval. Latency time of the last trial of each session was recorded. A significant decrease in the latency time in comparison to the first session was considered as successful learning. Probe trial tests for memory-retention were performed (to verify the animal's understanding of platform location) at the sixth day after the last training session. The platform was removed and each rat was allowed free swimming for 1 min. The number of crossings over the position of the platform in each quadrant was recorded and counted by replay using a fixed video camera recorder. All sides of the maze were within the camera's view field. Data are presented as mean ± SD.

Specimen preparation

Rats were euthanized 24 hours after probe trial for retention test in MWM task. The brain was quickly removed, washed in cold saline, and dissected to separate the hippocampus and cortex to be used as brain homogenate. One gram of hippocampal and cortical tissues was homogenized with 9 volumes of phosphate buffer, 0.1M, pH=7.4, centrifuged at 3000 rpm. for 15 minutes and the supernatant was preserved to be used as a brain homogenate.

Assessment of oxidative stress in brain tissue

Lipid peroxidation was assessed in hippocampal and cortical tissues by thiobarbituric acid-reactive substances (TBARS) assay, according to Tappel and Zalkin, 1959 [16]. The effect of nitric oxide (NO) in memory and learning was assessed based on the Montgomery and Dymock method, 1961 [17]. The anti-oxidant profile was investigated in brain homogenate by measuring the reduced glutathione (GSH) level based on the method of Jollow et al., 1974 [18]. In addition to measuring the activities of glutathione peroxidase (GPx) and glutathione-S-transferase (GST) according to the methods of Paglia and Valentine, 1967 and Habig *et al*., 1974, respectively [19, 20].

Assay of brain homogenate acetylcholinesterase (AChE) activity

Briefly, 150 µl of phosphate buffer (pH=8.0) was added to the ELISA plate blank well and 130 µl of phosphate buffer (pH=8.0) was added to the ELISA activity wells. Then 10 µl of substrate acetylcholine iodide (ACHI, 75mM) was added to the activity and blank wells and after that, 60 µl of brain homogenate supernatant was added to the activity ELISA wells only. ELISA plate was pre-incubated at 37 $^{\circ}$C for 15 min before the addition of the second substrate (0.32 mM DTNB). 60 µL of DTNB was added to both activity and blank wells. Absorbance was measured at 405 nm every 2 min. The obtained values were analyzed and the blank reading was subtracted from the sample readings [21].

Glucose 6-phosphate dehydrogenase activity

According to Zhu *et al.*, 2011 [22], G6PD activities were evaluated using the microplate assay. Assay solution was freshly made by mixing the following materials: 250 µl of 20 mM $MgCl_2$, 250 µl of 0.5 mM $NADP^+$, 250 µl of 10 mM DCPIP, 250 µl of brain homogenate, and 2.25 ml of 0.2M Tris buffer (pH=8.5). The assay procedure was as follows; 30 µl of G6P was pipetted into each well and then 70 µl of the assay solution was added to the wells. These mixtures were incubated for half an hour in the dark at room temperature. The absorbance was measured at 440 nm using a plate reader, and activities were followed for 30 min. 1 unit (U) of activity was the amount of enzyme, which is required to reduce one µmol of $NADP^+$ per min in the assay condition. Specific activity was defined as units/mg of protein. $NADP^+$ standards were treated in the same way as above to prepare the calibration curve.

Brain lipid profile

The profile of brain lipid was evaluated by measuring the level of brain cholesterol and phospholipids based on the method of Watson, 1960 and Connerty et al., 1961, respectively [23, 24].

ELISA assay for determination of Inducible nitric oxide synthase, Aβ-40, Aβ-42, and insulin-degrading enzyme (IDE)

Inducible nitric oxide synthase, Aβ-40, Aβ-42, and IDE in brain homogenate were determined using the iNOS ELISA Kits (RayBiotech, Canda), Amyloid-beta Peptide 1-40, 1-42 ELISA Kits (CUSABIO, China), and IDE ELISA Kit (CUSABIO, China), according to the manufacturer's instructions.

Total RNA isolation and PCR analysis

According to the Chomczynski and Sacchi method, 2006 [25], total RNA was isolated from the brain tissue using the phenol/guanidine-based Isol-RNA Lysis Reagent™ (5 PRIME GmbH, D-22767, Hamburg, Germany). One microgram of the isolated RNA was reverse transcribed into cDNA using reverse transcriptase (Maxime RT PreMix kit, iNtRON Biotechnology, Korea). cDNA was used as a template for subsequent PCR amplification using specific primers (GenBank) for G6PD of 99 bp (forward primer 5'-CGCCTGCGTTATCCTCA-3' and reverse primer 5'-TGGTTCTGCATCACGTC-3'), Tau of 65 bp (forward primer 5'-CGCCAGGAGTTTGACACAAT-3' and reverse primer 5'-CCTTCTTGGTCTTGGAGCATAGTG-3'), ADAM-17 of 228 bp (forward primer 5'-TAGCAGATGCTGGTCATGTG-3' and reverse primer 5'-TTGCACCACAGGTCAAAAG-3'), iNOS of 171 bp (forward primer 5'-AAGGATTCCTCTCGATCAATCTTG-3' and reverse primer 5'-GACTGCACAGAATGTTCCAG-3'), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of 224 bp (forward primer 5'-TACCCCACGGCAAGTTCAAT-3' and reverse primer 5'- AGGGGCGGAGATGATGATGA- 3') as internal control using PCR thermal cycler (ESCO, Swift MiniPro Thermal Cycler, USA). PCR analysis was performed using OneTaq® Quick –Load® 2X Master Mix with Standard Buffer (New England Biolabs, Inc, Japan). PCR products were analyzed by using 1.5% agarose gel electrophoresis, stained with ethidium bromide. DNA bands were revealed by Ultraviolet exposure on gel documentation system (Alpha – chem. Imager, USA) and the band intensity was analyzed by UVitec software. cDNA expression levels were determined by calculating the relative band intensity of each G6PD, Tau, ADAM-17, and iNOS compared to GAPDH.

Histopathological study of hippocampus tissues

According to Mirra SS et al., 1993 [26], the hippocampus of control and experimental groups were fixed in 10% neutral buffered formalin for 24 hours, washed with running water, dehydrated in ascending grades of alcohol (70%, 80%, 90%, and absolute alcohol) and cleaned by immersion in xylene and then impregnated in melted paraffin wax in an oven at 60° C for one hour. The specimens were embedded in paraffin and were left to solidify at room temperature. Using a rotary microtome, sections of 5 µm thick were cut and mounted on clean glass slides. Finally, the slides were stained using Bielschowsky technique. The examination of the slides for any histopathological changes (e.g. neuronal architecture, pyramidal neurons, glial cells, and neuro-inflammation) was carried out under a light microscope in Histopathology Department, Faculty of Medicine, Alexandria University, Egypt.

Statistical analysis

Data were given as individual values and as means and standard deviation (SD) for the control group and other groups. Comparison between the means of various treatment groups was analyzed using one-way ANOVA with Fisher's least significant difference test. $P<0.05$ was considered significant. All analyses were done using the statistical software SPSS, version 11.5.

RESULTS

Effect of BER/IPR mixture on the impairment of spatial memory of rats administrated scopolamine

All treated groups (BER, IPR, and mixture of BER/IPR) showed a significant decrease in the latency time during 5 days; that was more or less similar to control group in comparison to SCO-induced group. The mixture of BER/IPR showed a better result than other individual treatments. Generally, it was noticed that there was a gradual decrease in the latency time from the first day to the fifth day among all studied groups ($p<0.05$, figure 1A).

Concerning the probe trial for retention, the cognitive impairment of SCO-induced group recorded a significant increase in comparison to the control group ($p<0.05$). Berberine, ipriflavone, BER/IPR mixture, and donepezil treated groups showed a significant increase in cognitive improvement in comparison to the SCO-induced group ($p<0.05$); confirming that scopolamine induces memory impairment. That was expressed as the number of crossing over the platform position for 60 seconds as shown in figure 1B.

Moreover, it has been noticed that the body weight gain of SCO induced group recorded a significant decrease in comparison to the control group ($p<0.05$, data not shown). On the contrary, berberine, ipriflavone, berberine/ipriflavone mixture, and donepezil treated groups showed a significant increase in body weight gain when compared to the induced group ($p<0.05$, data not shown).

Effect of BER/IPR mixture on brain oxidative stress as well as antioxidants of rats administrated scopolamine

Brain oxidative stress was detectable by determining the levels of NO and TBARS. The measurement of NO and TBARS levels in the brain revealed that SCO-induced group had the highest elevation among all experimental groups ($p<0.05$). While all treatments decreased the brain TBARS and NO levels, than that of the induced group ($p<0.05$; Figures 2A and 2B).

Besides, the effect of BER/IPR mixture on brain antioxidant system of the scopolamine-induced group was evaluated. Results showed that administration of SCO decreased the brain activities of GST and GPx as well as GSH level in comparison to the control group ($p<0.05$; figures 3A, 3B and 3C). While BER/IPR mixture and donepezil treated groups increased significantly the brain activities of GST and GPx as well as GSH level in comparison to the SCO-induced group ($p<0.05$; figures 3A, 3B and 3C).

Effect of BER/IPR mixture on the brain activity of AChE of rats administrated scopolamine

SCO significantly increased the activity of brain AChE in comparison to the control group ($p<0.05$; figure 4). BER/IPR mixture significantly inhibited the activity of brain AChE effectively more than the reference drug (donepezil) in comparison to the SCO-induced group ($p<0.05$; figure 4).

Effect of BER/IPR mixture on the brain activity of G6PD of rats administrated scopolamine

After the scopolamine administration, the G6PD activity increased significantly in comparison to the control group**.** Upon BER/IPR mixed treatment, the G6PD activity decreased significantly and effectively more than donepezil treated group as compared to SCO induced group ($p<0.05$; figure 5).

Effect of BER/IPR mixture on the brain cholesterol and phospholipids levels as well as cholesterol/total phospholipids ratio of rats administrated scopolamine

Figures 6A and 6B show significant changes in brain cholesterol and phospholipids. SCO-induced group showed the highest brain cholesterol level ($p<0.05$). BER/IPR mixed treatment decreased brain cholesterol level when compared to the induced group as shown in figure 6A. This effect is comparable to the effect of donepezil.

The induced group showed a significant decrease in phospholipids level in comparison to the control group ($p<0.05$). BER/IPR mixture and donepezil treated groups showed a significant elevation in brain phospholipids ($p<0.05$) in comparison to the induced group (Figure 6B). Based on the previous results of brain levels of cholesterol and phospholipids, SCO-induced group showed the highest brain cholesterol/phospholipids ratio ($p<0.05$). On the contrary, treatment with BER/IPR mixture or donepezil, significantly normalized brain cholesterol/phospholipids ratio, as shown in figure 6C ($p<0.05$).

Effect of BER/IPR mixture on the iNOS, Aβ-40, Aβ-42, and IDE in the brain of rats administrated scopolamine

ELISA test demonstrated that SCO administration for 4 weeks significantly increased iNOS and Aβ-42 in rat brain ($p<0.05$); while decreased the brain Aβ-40 and IDE in comparison to the control group ($p<0.05$). On the contrary, BER/IPR mixed treatment significantly decreased the brain iNOS and Aβ-42 ($p<0.05$), in comparison to the SCO-induced group (Figures 7A and 7B). Also, brain Aβ-40 and IDE were significantly increased in BER/IPR mixture treated group ($p<0.05$) in comparison to the SCO-induced group (Figure 7C and 7D).

Effect of BER/IPR mixture on the G6PD, iNOS, Tau, and ADAM-17 mRNA expression in the brain of rats administrated scopolamine

To investigate the effects of SCO and mixed treatment with BER/IPR on the expression levels of some genes that can be up-regulated during neurotoxicity induction, such as G6PD, iNOS, Tau, and ADAM-17, reverse transcription-polymerase chain reaction (RT-PCR) was performed. Results demonstrated that SCO induction increased the gene expression of G6PD, iNOS, and Tau while decreased ADAM-17 mRNA level. In contrast, BER/IPR mixture and donepezil treatment showed a significant decrease in the expression of G6PD, iNOS, and Tau in addition to significant up-regulation in ADAM-17 expression (Figure 8).

Histopathological study of hippocampus tissues

The histological sections of rat brain tissues in control and PEG groups revealed normal neuronal architecture with normal pyramidal neurons and glial cells (figure 9A and 9B, respectively). On the contrary, histopathological studies of the hippocampus of SCO group showed Alzheimer-like pathology with severe congestion in the blood capillaries; neuroinflammation; as well as the hippocampus with non-homogenous cytoplasm, reactive gliosis, and apparent flame-shaped cells. Also, together neurofibrillary tangles and senile plaques, as well as extensive vacuolation, are well seen in figure 9C and 9D. Interestingly, berberine and ipriflavone mixture showed a strong synergistic and inhibitory effect on Alzheimer-like pathology and neuroinflammation (figure 9E), which was similar to donepezil effect on SCO induced group (figure 9F).

DISCUSSION

This study evaluated the effect of SCO on cognitive abilities and rat performance on the MWM test**,** the cholinergic system, oxidative stress, brain lipid profile, and the relation between brain performance and glucose

metabolism. The study also evaluated the therapeutic ability of BER, IPR, and BER/IPR mixture in improving memory and learning deficits in rats injected with SCO.

Results showed that treatment with the mixture of BER/IPR improved scopolamine-induced cognitive dysfunctions in the MWM test and was significantly correlated to the increase in antioxidant levels, cholinergic function, and brain energy profile as well as the reduction in pro-oxidant levels, Alzheimer disease markers, and inflammatory markers. Also, BER/IPR mixture gave a better result than donepezil treatment through *in vivo* inhibition of G6PD gene expression, which led to decrease in the accumulation of amyloid plaques and tau tangles that were confirmed by histopathology analysis of brain tissues.

AD is correlated with the hyperactivation of glucose 6-phosphate dehydrogenase (G6PD), which leads to energy disruption. The defective metabolism of glucose produces defective metabolism of amyloid precursor protein and the faulty clearance of Aβ in addition to cascade events including hyperphosphorylated tau, intermediated breakdown of microtubular aggregation, and synaptic defect, leading to AD [4].

Ismail et al., 2013, and Malin et al., 2015, reported that SCO intake altered rats performances on Morris Water Maze (MWM) tests and gene expression of AD markers causing memory and learning defects, therefore SCO was accounted as dementia inducer [27, 28]. In agreement with these findings, our data demonstrated that MWM performance significantly declined in male rats administrated SCO in comparison to the control one. Gu et al., 2014, reported weight loss in patients with AD [29]. In this study, results revealed that administration of SCO to male rats decreased their body weight. Spatial memory and learning impairment in SCO-induced group were significantly improved by donepezil and the mixture of BER/IPR. Also, BER/IPR mixture showed better effect than what was shown by Lee, et al., 2012; and Hafez, et al., 2017; who reported that individual administration of berberine or ipriflavone improved MWM performance by directly improving spatial memory [8, 11].

Moreover, the data revealed that the group administered SCO had hypercholesterolemia and high level of lipid peroxidation [TBARS and NO] with consequent features of oxidative stress due to decrease in the scavenger endogenous antioxidant capacity [glutathione (GSH), glutathione peroxidase (GPx), and glutathione S-transferase (GST)] and hyperactivated acetylcholinesterase (AChE). Treatment with BER/IPR mixture increased GSH, GPx, and GST levels in addition to decreasing levels of NO and TBARS. Furthermore, the current study showed a substantial effect of BER and IPRI in the inhibition of *in vivo* activity of AChE.

The result also revealed that SCO administration significantly up-regulated the expression of Tau and increased the level of Aβ-42, which was associated with a progressive decrease in non-toxic Aβ-40 level in comparison to the control group.

Oxidative stress leads to neuroinflammation through the microglia activation, that stimulate Aβ generation [30]. Aβ causes the production of reactive oxygen species (ROS) and reactive nitrogen intermediates (RNIs) from microglia, astrocytes, neurons, and monocytes, in part by induction of the inducible isoform of NO synthase [31]. The data revealed that the induction of Alzheimer-like disease using SCO significantly increased gene expression and protein activity of iNOS. NO can upregulate the rate of lactate production and glucose consumption suggesting glycolysis activation, which is a phenomenon that is probably the result of the NO-mediated inhibition of mitochondrial respiratory chain [32]. Mitochondrial respiratory chain inhibition leads to the activation of G6PD (the rate-limiting enzyme in pentose phosphate dehydrogenase) [33]. Moreover, it is reported that the AChE hyperactivity decreased ATPase in the cerebral cortex and hippocampus as well as the induction of iNOS and hyperactivation of G6PD leading to energy disruption and neuronal dysfunction [34].

Another event correlated with AChE hyperactivity is hyperinsulinemia that alters the activity of the insulin-degrading enzyme (IDE) [35]. IDE is important in catalyzing the degradation of insulin and insulin-like growth factor as well as in the clearing Aβ in the brain [35, 36]. Therefore, whenever the activity of IDE decreases, β-amyloid plaques increase. The present data showed that administration of SCO significantly decreased IDE activity in comparison to the control group. Moreover, it is known that AChE hyperactivity is correlated with the reduction of glucose level in brain tissues [37] that is consistent with the present data, showing a significant decrease in glucose level of brain tissues.

The present data showed a significant increase in brain G6PD activity and its level of gene expression by the scopolamine-induced group in comparison to the control group. Treatment with BER/IPR mixture or donepezil significantly decreased the brain G6PD activity and its expression level when compared to the induced group.

It is known that G6PD is regulated by the NADPH/$NADP^+$ ratio, accordingly, as the ratio decrease, the activity increases to provide more NADPH. G6PD is activated after cells exposure to several extracellular oxidants, leading to a decrease in NADPH level as a defense mechanism against oxidative stress, which is induced by SCO in rats [33, 34]. In post-mitotic cells like neurons, NADPH is mainly used as a cofactor to regenerate the pathway of the antioxidant glutathione

(GSH) that is reduced from GSSG (the oxidized form of GSH) through a reaction, which is catalyzed by glutathione reductase [33].

In addition, the data showed that SCO administration in male rats increased brain cholesterol level. It is known that cholesterol affects the enzyme activities included in the metabolism of the amyloid precursor protein and in the production of Aβ [38]. One probability, recently suggested that oxysterols and oxidized derivatives of cholesterol that pass through the blood-brain barrier may increase cholesterol levels throughout the body [38]. Moreover, Farooqui et al., 1997, showed that phospholipids are the main components of the membrane, determining various integrity and functions of the membrane [39]. Transport of essential substrates, signal transduction, and normal receptor function strongly depend on normal membrane phospholipid metabolism. The results showed that administration of SCO to male rats decreased the brain phospholipids, which significantly indicated elevation in cholesterol/phospholipid ratio. Also, the present data revealed that the genetic expression of ADAM-17 is significantly decreased in male rats administered SCO in comparison to the control group. ADAM-17 (α disintegrin and metalloproteinase) acts as α-secretases to cleave amyloid precursor protein and produce a soluble, non-amyloidogenic fragment, Aβ-40 [40]. All of the above-mentioned detrimental effects of scopolamine were normalized by the therapeutic effect of the berberine/ipriflavone mixture.

Histopathology of the present study revealed that scopolamine-induced a non-homogenous cytoplasm and apparent flame-shaped appearance of cells in the hippocampus, reactive gliosis in the cortex, as well as extensive vacuolation in the cortex associated with neuronal loss in addition to active microglial cells and apoptotic cells. Treatment with a mixture of berberine/ipriflavone resulted in normal neuronal tissues with a little number of dead neuronal cells and a lesser number of activated microglial cells.

A limitation of the study was that scopolamine induces biological effects and cognitive deficits but does not induce the neurological hallmarks of Alzheimer's disease (plaques tangles). That is why scopolamine presented as a model of "Alzheimer-like disease" inducer, but not Alzheimer disease inducer.

CONCLUSION

This study supports the protective effect of BER/IPR mixture against AD-like cognitive impairment, biochemical and molecular alteration of SCO-induced AD-like disease. Furthermore, the BER/IPR mixture exhibited a significant antioxidant and anti-inflammatory activity as well as the protective effect against the cognitive impairment induced by scopolamine through a mechanism involving G6PD.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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Abbreviations:

Aβ: Amyloid-β
ACh: acetylcholine
AChE: Acetylcholine esterase
AD: Alzheimer disease
ADAM-17: A disintegrin and metallopeptidase-17
BChE: Butyrylcholine esterase
BER: Berberine
G6PD: Glucose-6-phosphate dehydrogenase
GPx: Glutathione peroxidase
GSH: Glutathione (reduced form)
GST: Glutathione S-transferase
IDE: Insulin degrading enzyme
iNOS: Induced nitric oxide synthase
IPR: Ipriflavone
MAO: Monoamine oxidase
MWM: Morris water maze
NO: Nitric oxide
PA: *Phellodendron amurense*
Tau: Microtubule-associated protein that is mainly expressed in neurons
TBARS: Thiobarbituric acid-reactive substances
SCO: Scopolamine

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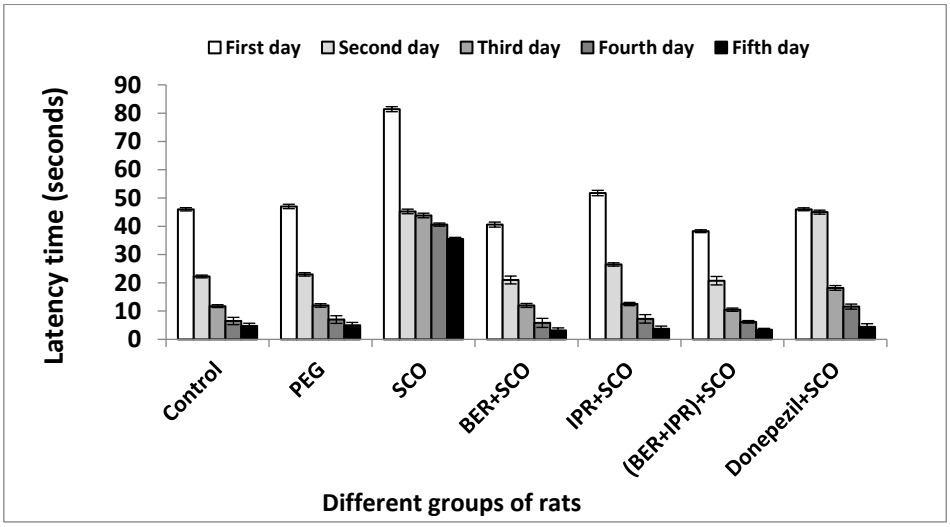

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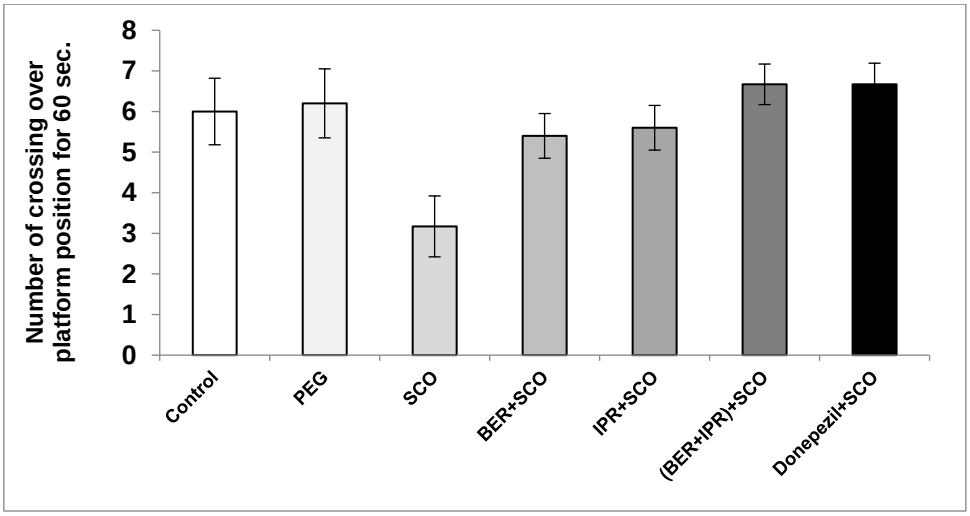
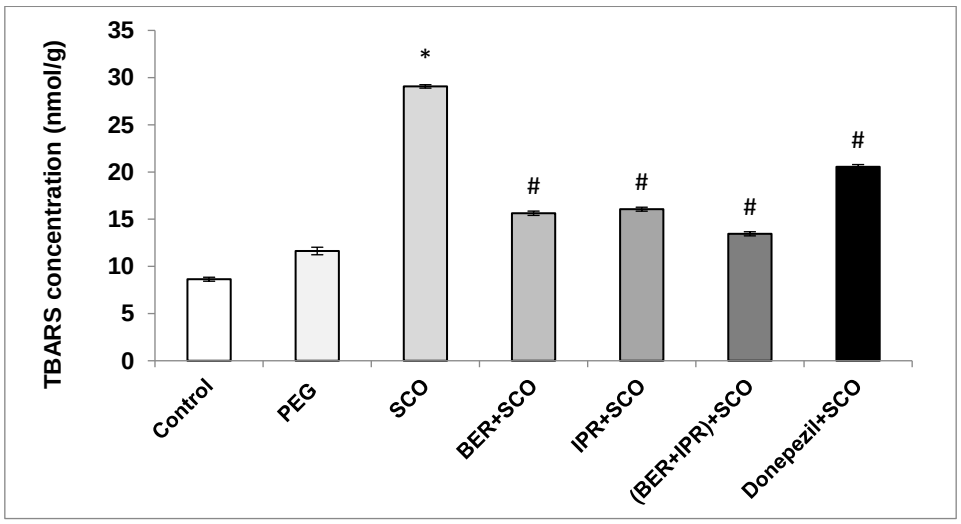


(B)

Figure 1: **(A)** Effect of berberine and ipriflavone mixture on the impairment of spatial memory of rats administrated scopolamine. Values represent the means ± SD of 8 male rats; ($p < 0.05$). **(B)** Effect of berberine and ipriflavone mixture on scopolamine-induced impaired performance in the MWM task. Values represent the means ± SD of 8 male rats; ($p < 0.05$).



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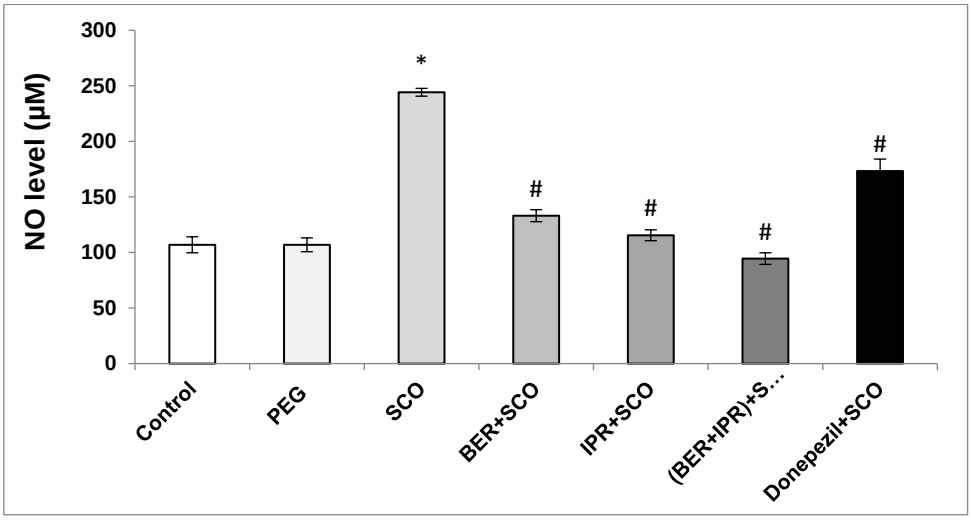
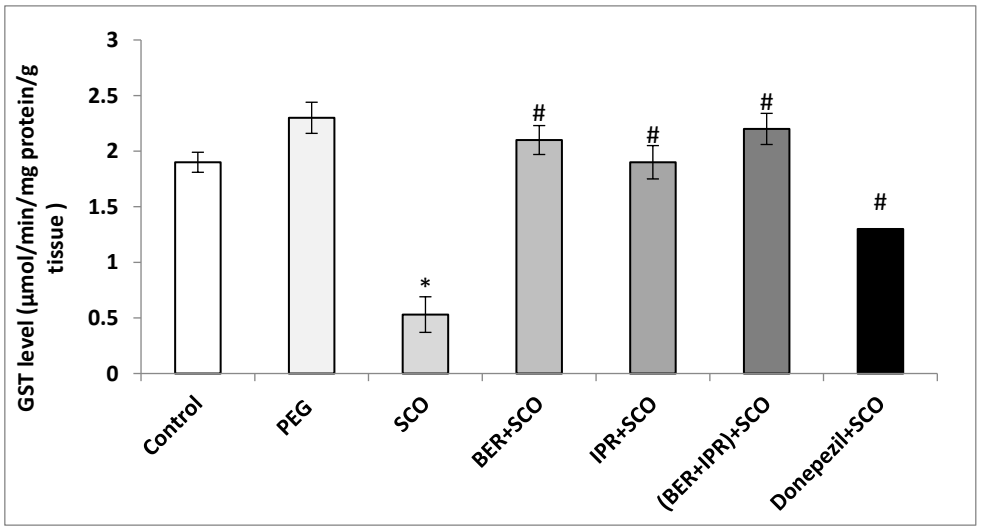


(B)

Figure 2: Effect of berberine and ipriflavone mixture on:
(A) brain TBARS level. The TBARS level was increased in the SCO group; **(B)** brain NO level. The NO level was increased in the SCO group.
Values represent the means ± SD of 8 male rats; *p < 0.05 in comparison to the control group. #p<.05 when compared to the SCO group.



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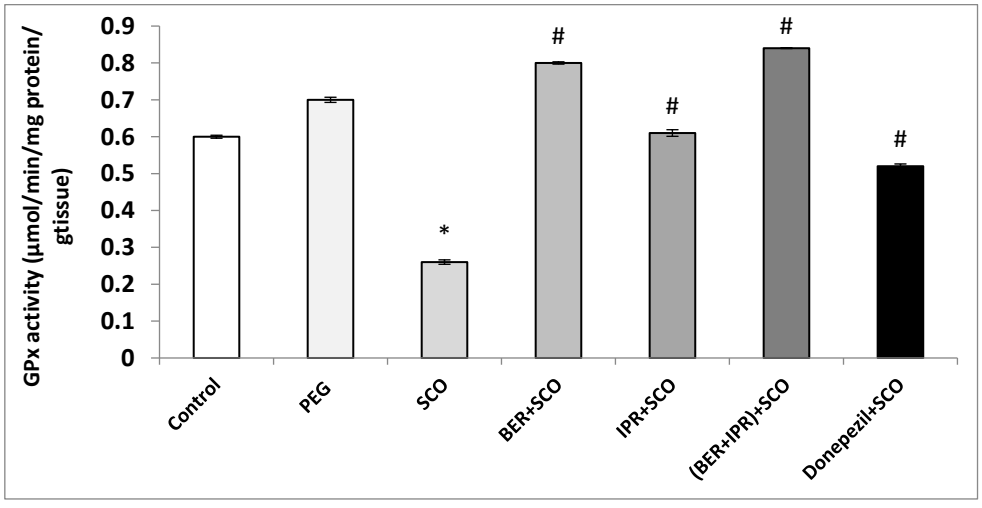

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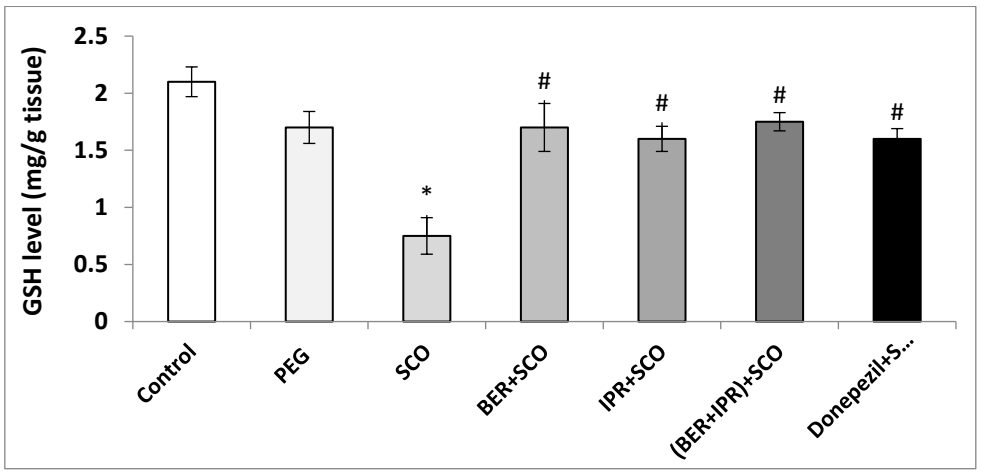


(C)

Figure 3: Effect of berberine and ipriflavone mixture on: **(A)** brain GST activity. The GST activity was decreased in the SCO group; **(B)** brain GPx activity. GPx activity was decreased in the SCO group; **(C)** brain GSH level. The GSH level was decreased in the SCO group.

Values represent the mean ± SD of 8 male rats; *p < 0.05 in comparison to control group. #p < 0.05 in comparison to the SCO group.

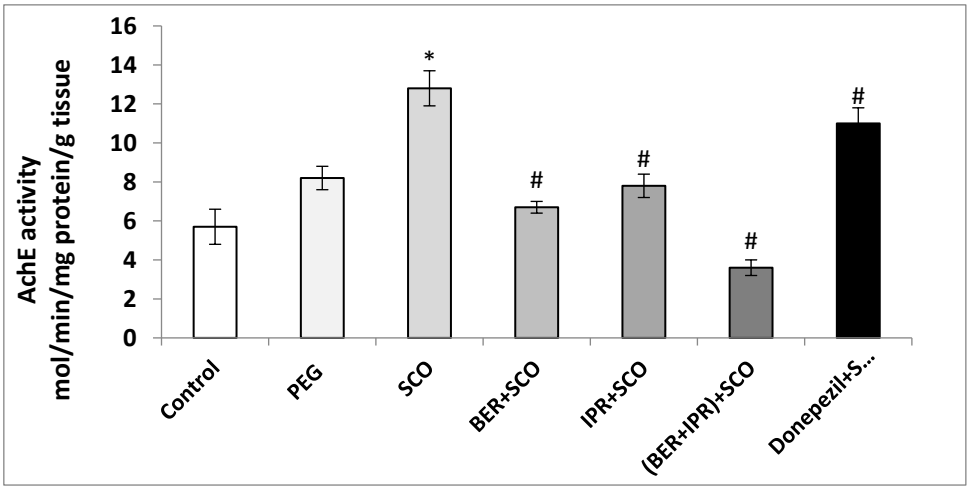


Figure 4: Effect of berberine and ipriflavone mixture on brain AChE activity. The AChE activity was increased in the SCO group. Values represent the means ± SD of 8 male rats; *p < 0.05 in comparison to the control group. #p<0.05 in comparison to the SCO group.

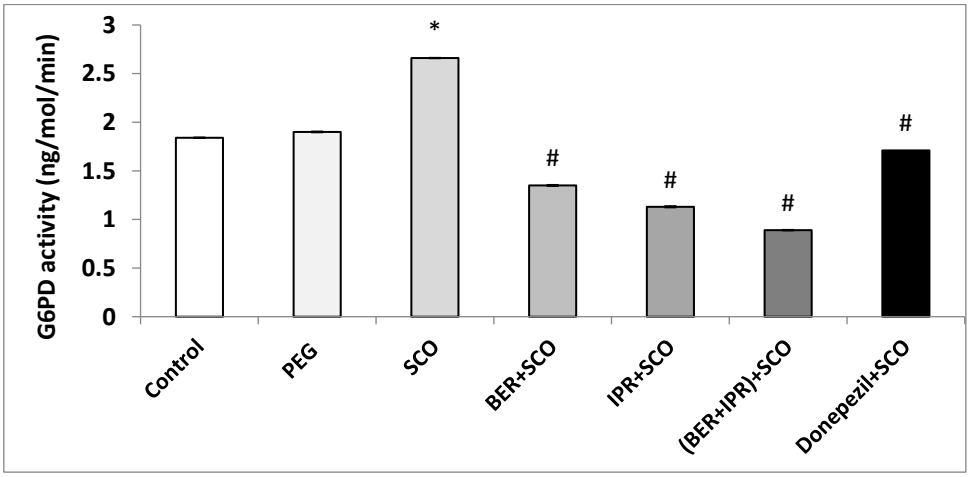
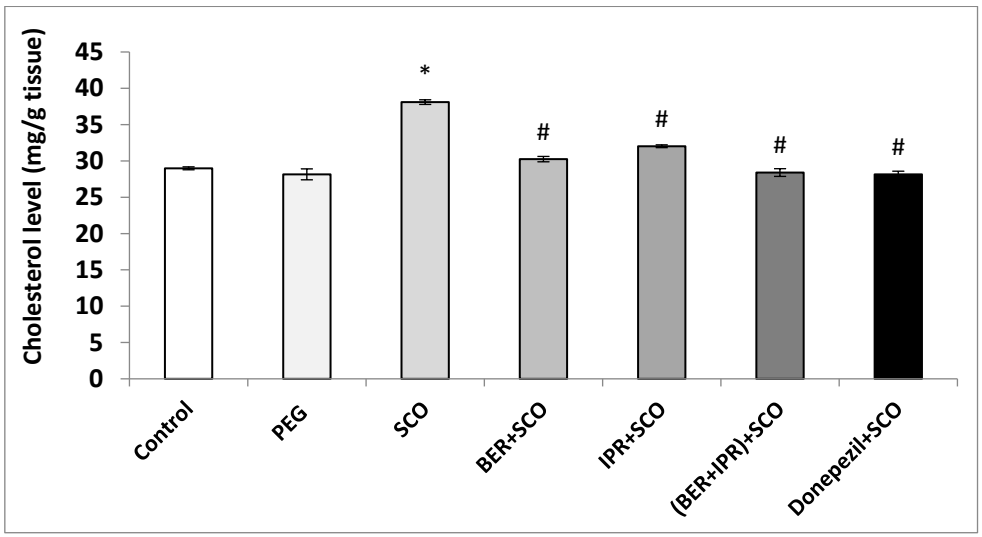


Figure 5: Effect of berberine and ipriflavone mixture on brain G6PD activity was determined. The G6PD activity was increased in the SCO group. Values represent the means ± SD of 8 male rats; *p<0.05 in comparison to the control group. #p<0.05 in comparison to the SCO group.



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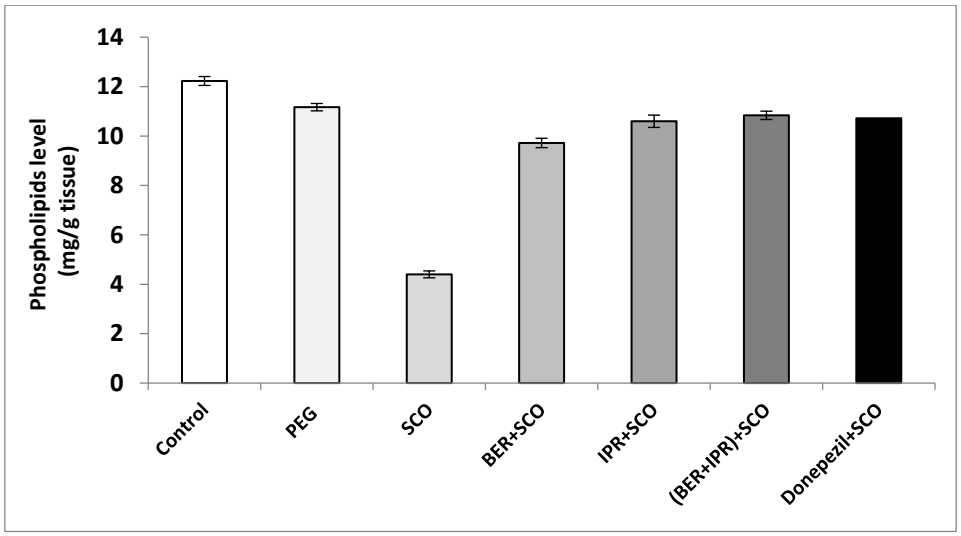

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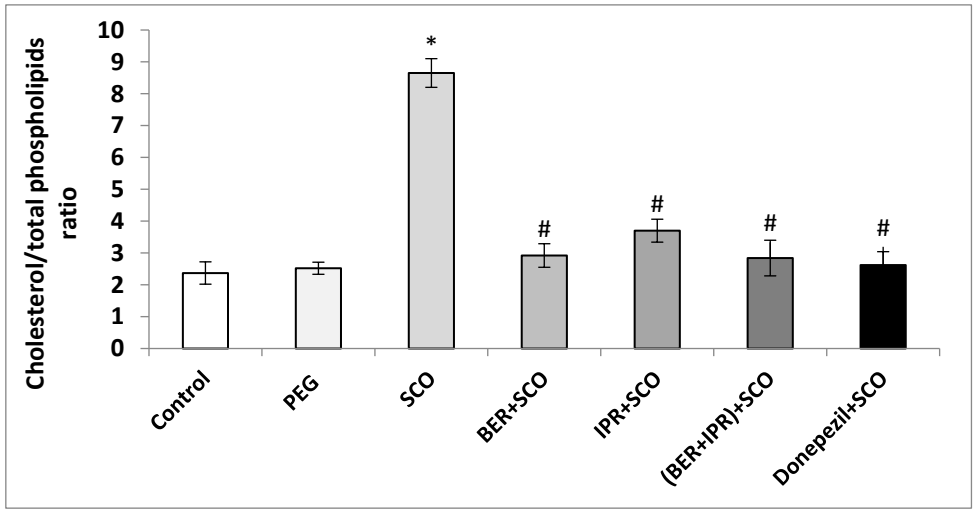
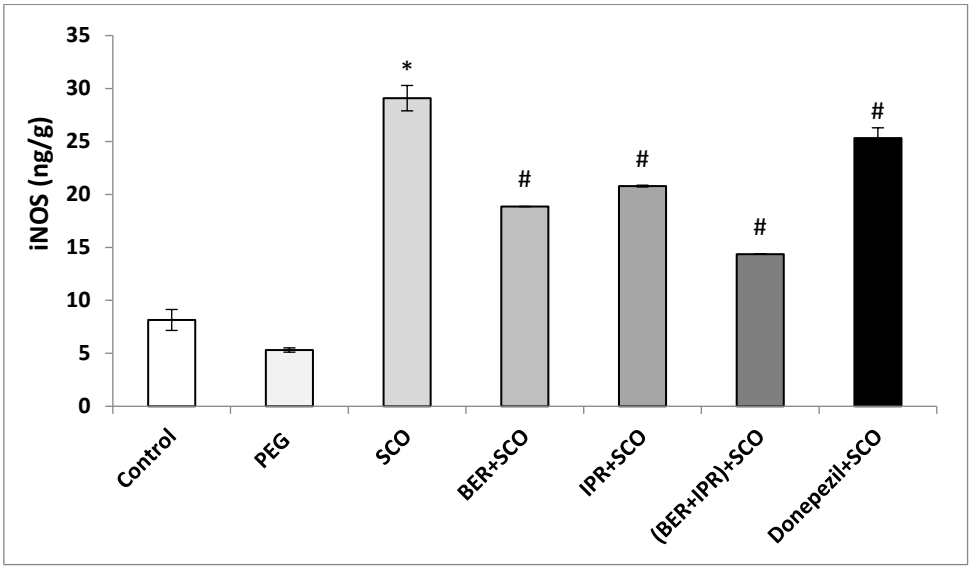


(C)

Figure 6: Effect of berberine and ipriflavone mixture on:
(A) brain total cholesterol. The total cholesterol was increased in the SCO group; **(B)** brain phospholipids level. The total phospholipids level was decreased in the SCO group; **(C)** brain total cholesterol/phospholipids ratio. The total cholesterol/phospholipids ratio was increased in the SCO group. Values represent the mean ± SD of 8 male rats; *p<0.05 in comparison to the control group. #p<0.05 in comparison to the SCO group.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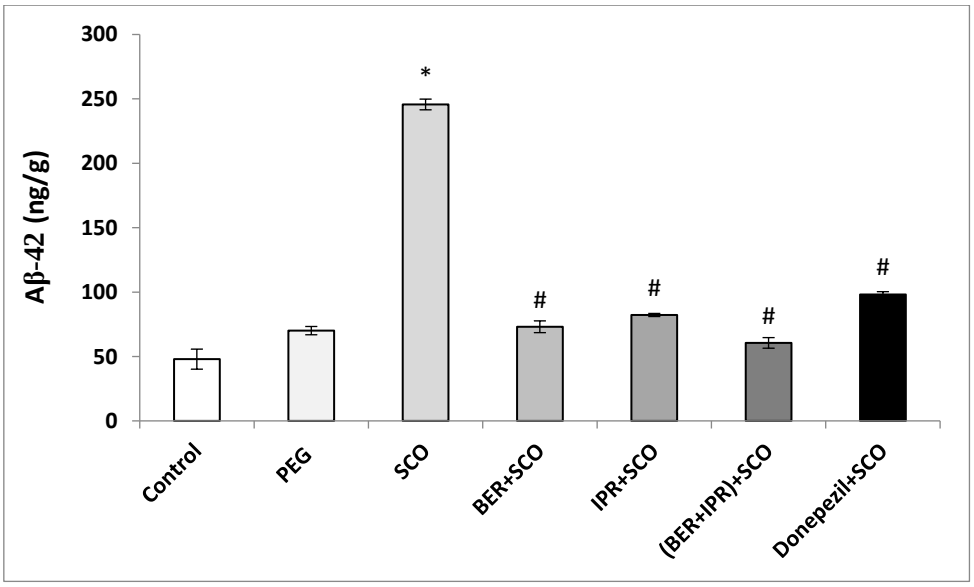
IPR+SCO
(BER+IPR)+SCO
Donepezil+SCO

(A)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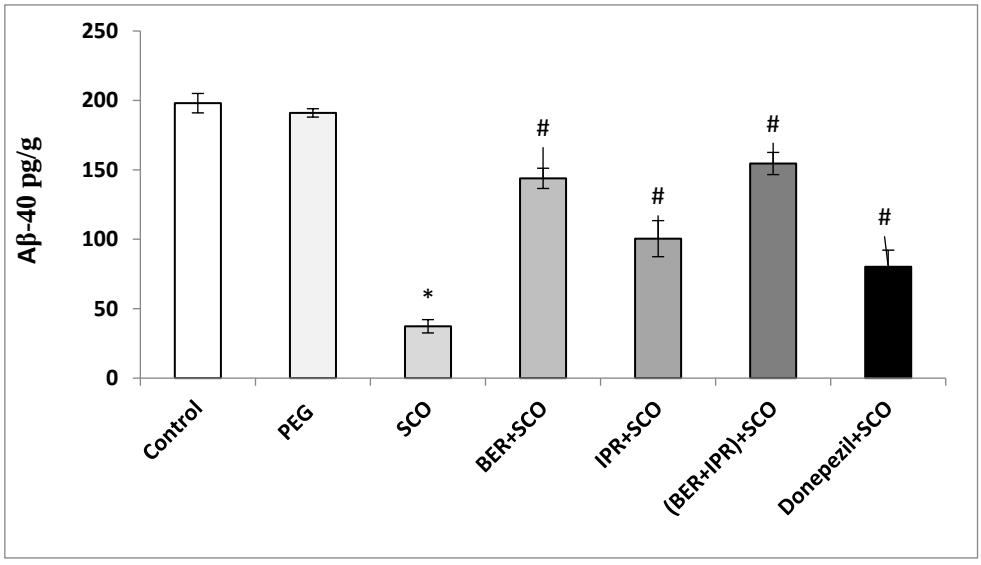
(BER+IPR)+SCO
Donepezil+SCO

(B)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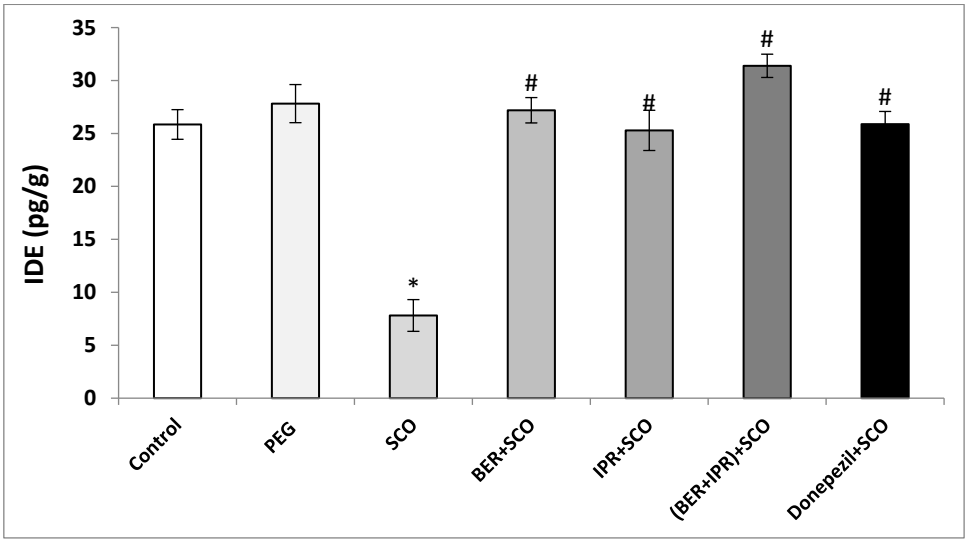
(C)



(D)

Figure 7: Effect of berberine and ipriflavone mixture on:
(A) brain iNOS level. The iNOS level was increased in the SCO group; **(B)** brain Aβ-42 level. The Aβ-42 level was increased in the SCO group; **(C)** brain Aβ-40 level. The Aβ-40 level was decreased in the SCO group; **(D)** brain IDE level. The IDE level was decreased in the SCO group; Values represent the mean ± SD of 8 male rats; *p<0.05 in comparison to the control group. #p<0.05 when compared to the SCO group.

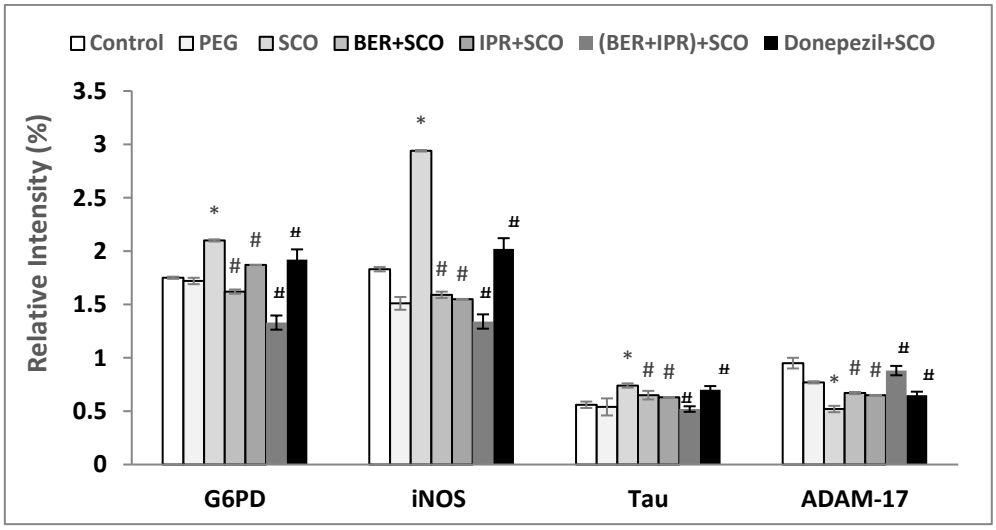
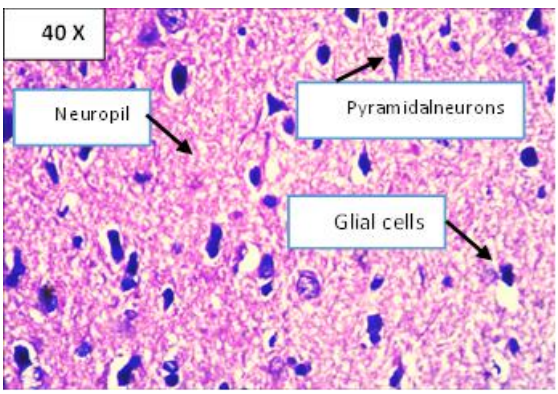


Figure 8: Effect of berberine and ipriflavone mixture on the mRNA expression level of G6PD, iNOS, Tau and ADAM-17 in brain tissue. Values represent the mean ± SD. *p<0.05 in comparison to the control group. #p<0.05 in comparison to the SCO group.



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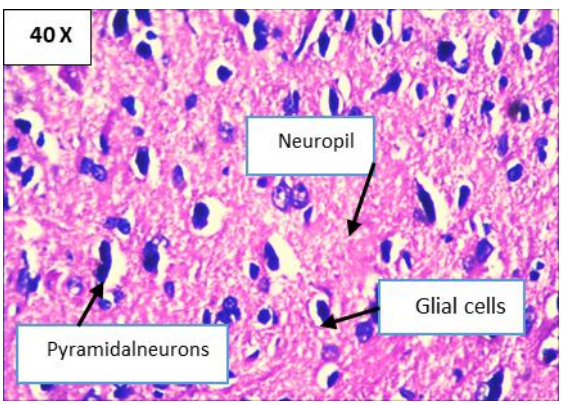

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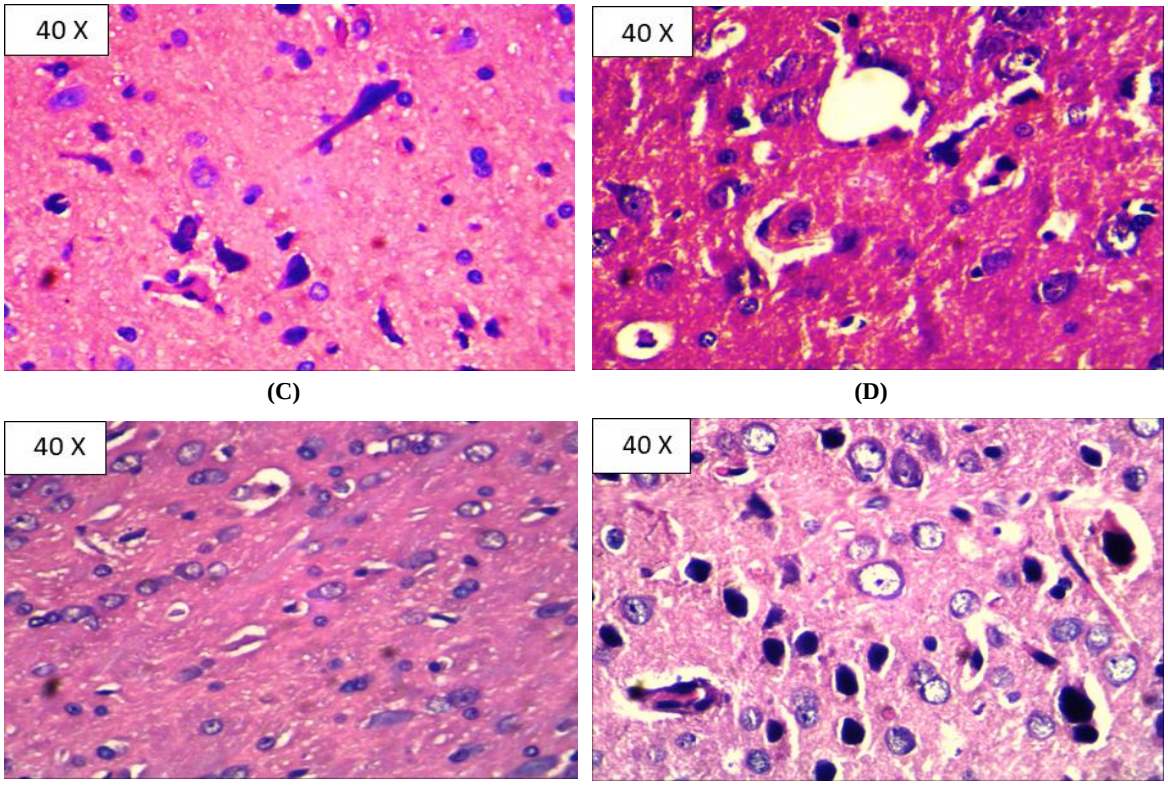
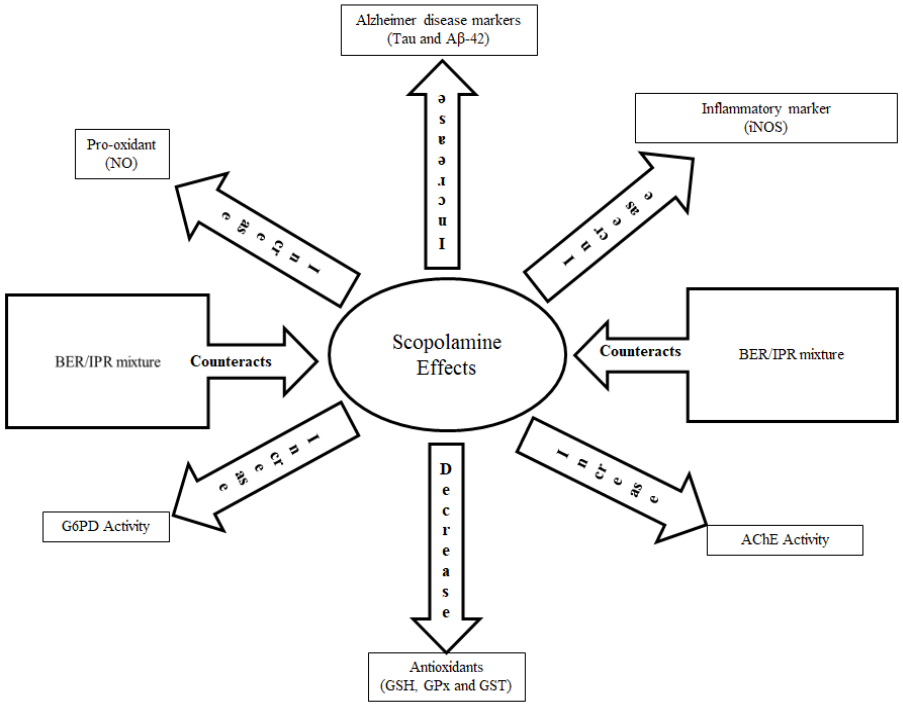


Figure 9: The histological sections of the brain tissue:
(A) control group and **(B)** PEG group, showed normal neuronal tissue formed of pyramidal neurons, glial cells as well as neuropil.
(C) and **(D)** a photomicrograph in brain tissue of SCO induced group revealed: **(C)** eosinophilic degenerating Purkinje neurons with condensed nuclei, pyknotic nuclei, and active microglia; in addition to; **(D)** neuron swelling, vacuolation, and apoptotic cells. **(E)** berberine and ipriflavone mixture treated group revealed normal neuronal tissue with a little number of dead neuronal cells and a lesser amount of activated microglial cell. **(F)** donepezil (reference drug) treated group in comparison to the heterogeneous sections with little neuronal swelling and vacuolation as well as pyknosis and some active microglial cells



Graphical Abstract