Comparative Study between Saffron (*Crocus sativus L.*) and / or Turmeric (*Curcuma longa L.*) Extracts on D-galactose Deleterious Brain Effects in Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author KEB designed the study and revised the manuscript. Author FHA managed the analyses of the study, performed the statistical analysis and managed the literature searches. Author AMSG designed the study, wrote the protocol, managed the literature searches, wrote the first draft of the manuscript and performed the statistical analysis. All authors read and approved the final manuscript.

Article Information

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ABSTRACT

Aims: This study was designed to investigate the active chemical constituents and antioxidant capacities of saffron stigmas and turmeric rhizomes ethanolic extracts (ESE and ETE) respectively. D- galactose deleterious brain effects as well as the role of ESE and ETE supplementation against D-galactose intoxication were evaluated on male rat's brain.
Place of study: Biochemistry and Nutrition Department, Faculty of Women for Arts, Science and Education, Ain Shams University.
Methodology: Fifty adult male Sprague-Dawley rats were divided into 5 groups; 10 rats each. Group (1): Healthy control; group (2): D-galactose control; rats were intoxicated with D-galactose (250mg/kg body weight /day/subcutaneously); group (3-5): D-galactose intoxicated rats and supplemented with (30mg /kg body weight /daily orally) of ESE, ETE and (15mg /kg body weight /daily orally*)* from each extract respectively for six weeks.
Results: Research results revealed that saffron and turmeric ethanolic extracts contain active

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chemical constituents including polyphenols and flavonoids that possess high antioxidant activity. Biochemical analysis of brain tissues documented that injection with D-galactose caused significant increase ($p \leq 0.05$) in oxidative stress parameters including [advanced glycation end products (AGEs), protein carbonyl group (PCG), malondialdehyde (MDA) and nitric oxide (NO) Levels], pro-inflammatory markers like [tumor necrosis factor alpha (TNF-α) and interleukin -6 (IL-6) levels] , epigenetic marker [p16^{INK4a} content] as well as neural cell markers [metallothoenins (MTs) and serotonin (5-HT) levels].On the other hand D-galactose intoxication caused significant decrease ($p \leq 0.05$) in brain antioxidants as [total antioxidant capacity (TAC), reduced glutathione (GSH) level and catalase (CAT) activity] as well as brain acetylcholinesterase (AChE) activity. All these results were proved by the microscopic examination and apoptotic markers immunohistochemical analysis of brain tissues that revealed degenerative changes in cerebral cortex and hippocampus. Oral administration of saffron and turmeric ethanolic extracts alone or in combination decreased brain oxidants, pro-inflammatory markers, epigenetic marker and neural cell markers levels while increased the levels and activities of antioxidants as well as AChE activity associated with an improvement of brain microscopic examination and immunohistochemical analysis. The most significant improvements ($p \leq 0.05$) were recorded in the group that supplemented with both extracts.

Conclusion: Study results proved that saffron and turmeric ethanolic extracts active components were able to correct deleterious brain effects induced by D-galactose and using their mixture was more efficient in ameliorating brain toxicity than using each extract alone evidenced by biochemical analysis, microscopic examination as well as immunohistochemical determination of apoptotic markers in bmrain tissues. It is advised to add saffron and turmeric to human foods and to prepare their ethanolic extracts to be available for human beings due to their ability to preserve brain functions and structure as well as their potential to inhibit and retard brain aging and neuro-degeneration.

1. INTRODUCTION

Brain disease has become a subject that can no longer be ignored. The causes of such disorders are heterogeneous leading to neuro-degeneration [1] and cause progressive deteriorations leading to disability and mortality [2].

The brain is the most important organ of the central nervous system (CNS). It is more susceptible to damage by free radicals because of its high use of oxygen and its high content of polyunsaturated fatty acids [3]. Also, the antioxidant defense system of the brain is weaker as compared to other body parts. So the oxidative stress rapidly leads to the hippocampus (major memory processing region) degeneration [4].

Galactose is a C-4 epimer of glucose. There are two enantiomers of galactose: D- and L-galactose. In nature, the main form of galactose is D-galactose. The major natural dietary source of galactose is milk and dairy products. Free galactose is also present in some vegetables and fruits, such as tomatoes, bananas, and apples. In addition, the lactose hydrolysate syrup, as a sweetener, has been intensively used in biscuits, confectionery, and some dairy desserts containing high monosaccharide galactose content [5].

Galactose is also formed endogenously in the human cells. A 70 kg adult male could synthesize up to 2 grams of galactose per day [6]. The level of galactose in the body can be elevated in two cases: (1) via increased consumption of foods rich in galactose, and (2) through metabolic disorders associated with genetic mutations in Leloir pathway enzymes that control galactose metabolism [7].

D-galactose can be metabolized by D-galactokinase and galactose-1-phosphate uridyltransferase at normal physiological concentrations. However, when excessive D-galactose accumulates, it is converted by D-galactose oxidase into aldoses and hydro peroxides. This results in the production of reactive oxygen species (ROS) causing increased oxidative stress [8].

Medicinal plants have a protective effect against acute and chronic diseases through increasing expression of antioxidants and scavenging of free radicals. Antioxidants present in medicinal plants have been reported to protect against

neurodegenerative diseases by increasing the activity of endogenous antioxidant enzymes and reducing lipid peroxidation [9].

Saffron *(Crocus sativus L.)* belongs to *Iridaceae* family, is commonly used as a food additive worldwide to enhance the flavor of food and a well-known medicinal herb. Saffron stigma contains more than 150 phytochemical ingredients including carotenoids, flavonoids, terpenoids, and anthocyanin [10]. Many pharmacological studies have shown that saffron and its phytochemicals have emerged as nutraceutical elements, endowed with beneficial effects on health showing antibacterial, antioxidant, antifungal, immunomodulatory, anti-mutagenic and antiplatelet effects [11,12].

Turmeric is an Indian rhizomatous herbal plant (*Curcuma longa L.)* of the ginger family *Zingiberaceae* of well-known medical benefits. The medicinal benefits of turmeric could be attributed to the presence of active principles called curcuminoids including curcumin, demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC).These yellow colored curcuminoids are isolated from rhizomes [13].Turmeric has been used in tradition as a medicinal herb due to its various advantages such as antioxidant, anti-inflammatory, anti-mutagenic, antimicrobial and several therapeutic properties[14].

This study was designed to investigate saffron stigmas and turmeric rhizomes ethanolic extracts active chemical constituents and antioxidant capacities, evaluation of deleterious brain effects induced by D- galactose intoxication in male rats as well as the role of saffron and turmeric extracts supplementation against D-galactose intoxicated rat's brain.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

Saffron stigmas and turmeric rhizomes were purchased fresh from Ministry of Agriculture, Cairo, Egypt.

2.1.2 Chemicals

D-galactose was obtained from Sigma Aldrich Chemical Co. (St. Louis, Missouri, USA). Ethanol (70%) and all other chemicals were of high analytical grade and purchased from El-Gomhouria Company for Chemicals, Cairo, Egypt. D-galactose was dissolved in saline and injected subcutaneously at dose of (250mg/kg body weight/day) to induce rat's brain toxicity according to Li et al. [15].

2.1.3 Animals

Fifty healthy adult male white albino rats (Sprague -Dawely- strain) weighing (200±10) g were used, the animals supplied from National Research Center, Dokki, Giza, Egypt.

2.1.4 Diet

Standard commercial pellet diet was used according to NRC [16] which obtained from Egyptian Organization for Biological Products and Vaccines Helwan, Egypt.

2.2 Methods

2.2.1 Preparation of plants ethanolic extracts

The stigmas of saffron or rhizomes of turmeric were washed, cleaned, dried at room temperature and powdered by electric blender. 30 g of dry powdered plant material were extracted on the basis of maceration method. The powdered plant was soaked in ethanol 70% for 3 days with occasional shaking and stirring. The clear solution obtained after filtration was collected in a glass container. The ethanol was removed at 50 ℃ by a rotary evaporator (WHEATONSP35) in the Faculty of Science, Central Lab, Ain Shams University. Dried extract was diluted with distilled water to give the final extract concentration of (30mg/ml) stored in a dark, cool place away from light and moisture [17]. The extracts were well shaken before giving to rats.

2.2.2 Determination of total poly phenols, flavonoids and anti-oxidants in plants ethanolic extracts

The total phenolic and flavonoids contents were determined according to the Folin-Ciocalteu procedure [18]. Total anti-oxidants in plants ethanolic extracts were determined using the stable 1, 1-diphenyl-2-picryl hydrazyl (DPPH) according to Hwang and Do Thi [19]. The experiments were repeated in triplicate.

2.2.3 Determination of plants ethanolic extracts active constituents using high performance liquid chromatography (HPLC)

HPLC analysis of plants ethanolic extracts were carried out according to Kim et al. [20] using Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and a diode-array detector.

2.2.4 Experimental design

All rats were individually housed with constant controlled environments in stainless steel cages with good ventilation, under conventional condition (22 ± 3°C and natural light/dark cycle) and fed on the balanced commercial diet with drinking water *ad libitum* for 7 days to be acclimatized. Animals were classified randomly into 5 groups of ten animals each as follow:

Group (1) HCG: Healthy control group, rats were given distilled water orally and injected with normal saline subcutaneously (s.c) daily.

Group (2) DGCG: D-galactose control group, rats were given distilled water daily orally and injected with D-galactose (250mg/kg /day/s.c) according to Li et al.[15]**.**

Group (3) DG+ESE: D-galactose intoxicated rats supplemented with ethanolic saffron extract, rats were injected with D-galactose dissolved in saline (250mg/kg/day/s.c) and supplemented with saffron extract (30mg /kg/daily orally) according to Ziaee et al. [21].

Group (4) DG+ETE: D-galactose intoxicated rats supplemented with ethanolic turmeric extract**,** rats were injected with D-galactose dissolved in saline (250mg/kg/day/s.c) and supplemented with turmeric extract (30mg /kg/daily orally) according to Kumar et al. [22].

Group (5) DG+ESE+ETE: D-galactose intoxicated rats supplemented with ethanolic saffron and turmeric extracts, rats were injected with D-galactose dissolved in saline (250mg/kg/day/s.c) and supplemented with a mixture of saffron extract (15mg /kg/daily orally**)** and turmeric extract (15mg/kg/daily orally).

2.2.5 Handling of brain samples

At the end of the experiment (6 weeks), the experimental animals were sacrificed under deep anesthesia. The brain was removed and washed with saline. Random brain samples were fixed in 10% formal saline for microscopic examination and the remaining brain samples were prepared to form tissue homogenate for other analysis. Brain tissues were rinsed in ice-cold phosphate buffered saline PBS (0.02mol/L, pH 7.0-7.2) to remove excess blood thoroughly. Then brain tissues were minced to small pieces and homogenized in 500µl of PBS with a glass homogenizer on ice. The resulting suspension was subjected to ultra-sonication to further break the cell membranes. After that, the homogenates were centrifuged for 15 minutes at 1500×g. Supernatant was removed and assayed immediately.

2.2.6 Biochemical analysis

The brain content of advanced glycation end products (AGEs) and protein carbonyl group (PCG) were determined according to Koschinsky et al. and Hopps et al.[23,24] using Enzyme linked immunosorbent assay (ELISA) kits of Mybiosource, USA. Malondialdehyde (MDA), nitric oxide (NO) levels, total antioxidant capacity (TAC), reduced glutathione (GSH) content and catalase (CAT) enzyme activity in brain tissue homogenate were determined according to Ohkawa et al., Montgomery and Dymock, koracevic et al., Beutler et al. and Aebi [25-29] respectively using Biodiagnostic kits, Giza, Egypt. Brain acetylcholinesterase (AChE) enzyme activity, metallothionein (MTs) and serotonin (5-HT) levels were determined according to kovarik et al., Özcelik et al., and Kittler et al. [30-32] using ELISA kits of BioAssay and Mybiosource, USA**.** Tumor necrosis factor-α (TNF-α) and interlukine-6 (IL-6) were determined in brain tissue homogenate according to Dowlati et al. and Hirano respectively [33,34] using ELISA koma biotech kits, Korea.

2.2.7 Determination of brain epigenetic marker P16^{INK4a} in brain tissuesusing quantitative real time PCR

2.2.7.1 RNA extraction

Total RNA was isolated using *Qiagen* instructions of manufacture tissue extraction kit *Qiagen, USA*.

2.2.7.2 cDNA synthesis

The total RNA (0.5–2 µg) was used for cDNA conversion using high capacity cDNA reverse transcription kit *Fermentas, USA*.

2.2.7.3 Real-time qPCR using SYBR Green I

Real-time qPCR amplification and analysis were performed using an Applied Bio-system with software version 3.1 (StepOne™, USA). The qPCR assay with the primer sets were optimized at the annealing temperature. The primer sequence was shown in Table 1.

2.2.8 Microscopic examination of brain tissues

Brain specimens were collected from all rats/ groups and then fixed in 10% neutral buffered formalin. Paraffin sections of 25 μm thickness were prepared and stained with hematoxylin and eosin (H&E) and then examined by a light microscope (Olympus BX50, Japan) [35]. Histopathological damage in the cerebral cortex and hippocampus were graded from (0-4) as follow: (0) indicated no changes; (1) indicated percentage area affected (<10%); (2) indicated percentage area affected (20-30%); (3) indicated percentage area affected (40-60%) and (4) indicated percentage area affected (>60%) [36].

2.2.9 Immunohistochemical analysis of apoptotic markers in brain tissues

2.2.9.1 Caspase-3 activity and Bax expression

Caspase-3 activity and Bax expression level in cerebral cortices and hippocampus sections were examined according to Matsushita et al. and Martín-Burriel et al. [37,38],using(Santa Cruz Biotechnology Inc., Dallas, TX, USA). The immune reaction was visualized using di-amino-benzidine tetrachloride (DAB, Sigma Chemical Co., St. Louis, MO, USA).

2.2.9.2 Bcl-2 expression

The secondary abtibody was detected using the ABC technique (Vector Labs, Burlingame, CA), using the peroxidase-DAB system.. No immunoreactivity was observed when the primary antibody was omitted from the protocol [39].

Quantification of caspase-3 activity, Bax and Bcl-2 proteins levels were determined by calculating the area percent expression in each section from 5 randomly chosen fields using image analysis software (Image J, version 1.46a, NIH, Bethesda, MD, USA) to determine the average.

2.3 Statistical Analysis

Data were statistically analyzed by Statistical Package for Social Science (SPSS) version 20. Values were presented as mean ± standard deviation (S.D.). Statistical Differences between groups were performed using one way ANOVA, the mean difference was significant at ($P \leq 0.05$) level according to Lévesque [40].

3. RESULTS AND DISCUSSION

3.1 Total Poly Phenols, Total Flavonoids Contents and Antioxidant Activity of Saffron and Turmeric Ethanolic Extracts

The result in Table (2) illustrated that each 1 g of the tested saffron extract contains 39.195 mg as gallic acid equivalent of total poly phenols ,2.787 mg as catechin equivalent of total flavonoids and 8.883 mg as trolox equivalent of total anti-oxidant capacity while each 1 g of the tested turmeric extract contains 1.955 mg as gallic acid equivalent of total poly phenols , 1.023 mg as catechin equivalent of total flavonoids and 2.012 mg as trolox equivalent of total anti-oxidant capacity, from these results it is found that saffron ethanolic extract contains higher phenolic ,flavonoids and antioxidant activities than turmeric ethanolic extract.

Spices and herbs are rich sources of phytochemicals. Phytochemicals are a large group that consists of flavonoids, phenolic compounds, carotenoids, and other sulphur-containing compounds. Saffron and turmeric contain number of antioxidants such as beta-carotene, ascorbic acid, terpenoids, alkaloids, polyphenols and flavonoids [41].

Table 1. The primer sequence of the studied gene

Primer sequence	
TG5	Forward primer :5'- ACGATGACCTGTGTCGAACT -3
	Reverse primer: 5'- AAACCAAATCTCACTAACATCTTCT -3
TG7	Forward primer :5'- GAGAGTACATCCCCACCGTC-3
	Reverse primer:5'- AGGGATCGTACACACCGACT -3
Beta Actin	Forward primer 5′-TGTTTGAGACCTTCAACACC-3′
	Reverse primer 5′-CGCTCATTGCCGATAGTGAT-3′

Table 2. Total poly phenols, total flavonoids contents and antioxidant activity of saffron and turmeric ethanolic extracts

Ethanolic Extract	Total Polyphenols Content(mg GAE/g)	Total Flavonoids Content(mg CE/g)	Antioxidant Activity (mg TE/g)
Saffron	39.195	2.787	8.883
Turmeric	1.955	1.023	2.012

Phenolics have a wide range of biological actions, including the ability to act as antioxidants, ameliorate inflammation, modulate enzyme activity and regulate gene expression [42].Saffron and turmeric ethanolic extracts have high content of polyphenols and this was confirmed previously [43-46].

Flavonoids represent the largest part of dietary polyphenols (up to 60%).Flavonoids exert their anti-oxidative activity by effectively scavenging various free radicals, regulating oxidative stress-mediated enzyme activity and chelation of transition metals involved in radical forming processes [47]. Results of flavonoids content of both saffron and turmeric extracts go hand in hand with other researcher results Baba et al. and Mushtaq et al. [48,49].

The antioxidant activities of plant extracts were usually linked to their phenolic content which attributed to hydrogen donating characteristics of the phenolic compounds that responsible for the inhibition of free radical and prevent lipid peroxidation [50]. Total antioxidant activities obtained results of saffron and turmeric extracts were linked with Ferrara et al. and Vallverdú-Queralt et al. [51,52] results.

3.2 Main Phenolic Compounds of Saffron and Turmeric Extracts by HPLC

The results presented in Figs. (1 and 2) and Fig.3 (A and B) showed the concentration of main active phenolic compounds in saffron and turmeric extracts by HPLC. Moreover, these result concluded that phenolic compounds of saffron extract by using HPLC technique were apigenin, chrysin, cateachin, qurecetin, cinnamic acid, sinapic acid, rutin, kaempferol, caffeic, vanillic, rosmarinic acids , apigenin-7-glucoside, p-coumaric , p-hydroxybenzoic and ferulic acids. Apigenin represents the highest concentration (8.26 mg/g extract) of saffron phenolic components when compared to all other components while ferulic acid represent the lowest concentration (0.02 mg /g extract). Also this result concluded that phenolic compounds of turmeric extract by using HPLC technique were apigenin-7-glucoside, ferulic acid, cateachin, vanillic, p-coumaric acids, qurecetin and p-hydroxybenzoic acid. Apigenin-7-glucoside represent the highest concentration (2.166 mg/g extract) of turmeric phenolic components when compared to all other components while p-hydroxybenzoic acid and qurecetin represent the lowest concentration (0.01 mg /g and 0.011 mg / g extract) respectively.

These results were confirmed by Esmaeili et al. [53] who documented that ethanolic saffron extract contain different phenolic compounds such as catechol, vanillin, salicylic, cinnamic, p-hydroxybenzoic, 2,5 dihydroxybenzoic (gentisic), syringic, p-coumaric , gallic, ferulic and caffeic acids. Also, Queralt et al. (2015) reported that ethanolic turmeric extract contain different phenolic compounds including p-hydroxybenzoic acid, cateachin ,vanillic, ferulic, p-coumaric acids, apigenin-7-glucoside and qurecetin [52].

3.3 Effect of Saffron and / or Turmeric Ethanolic Extracts Supplementation on Brain Advanced Glycation End Products (AGEs), Protein Carbonyl Group (PCG), Malondialdehyde (MDA) and Nitric Oxide (NO) Levels in D-galactose Intoxicated Rats

The results tabulated in Table (3) revealed that D-galactose acted as potent oxidant, reached brain tissue and caused a state of oxidative stress, leading to a massive increase ($P \leq 0.05$) in the levels of oxidative biomarkers such as AGEs, PCG, MDA and NO in D-galactose control group when compared to healthy control group. On the other hand, it was observed from the results that saffron and turmeric ethanolic extracts contain potent antioxidants that caused noticeable decrement ($P \leq 0.05$) in the levels of oxidative biomarkers in D-galactose supplemented groups when compared with D-galactose control group. It was noticed that the supplementation with both extracts caused the most significant Improvements ($P \leq 0.05$) in brain oxidative biomarkers levels.
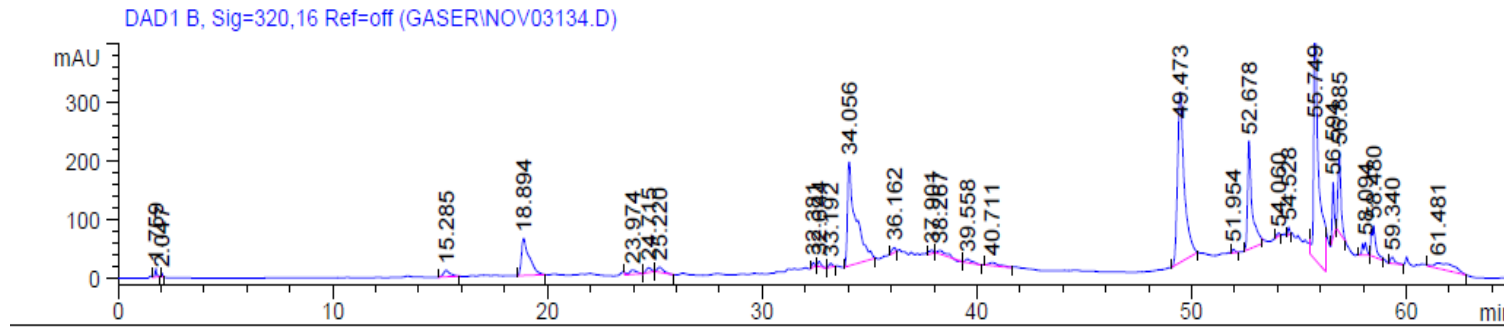
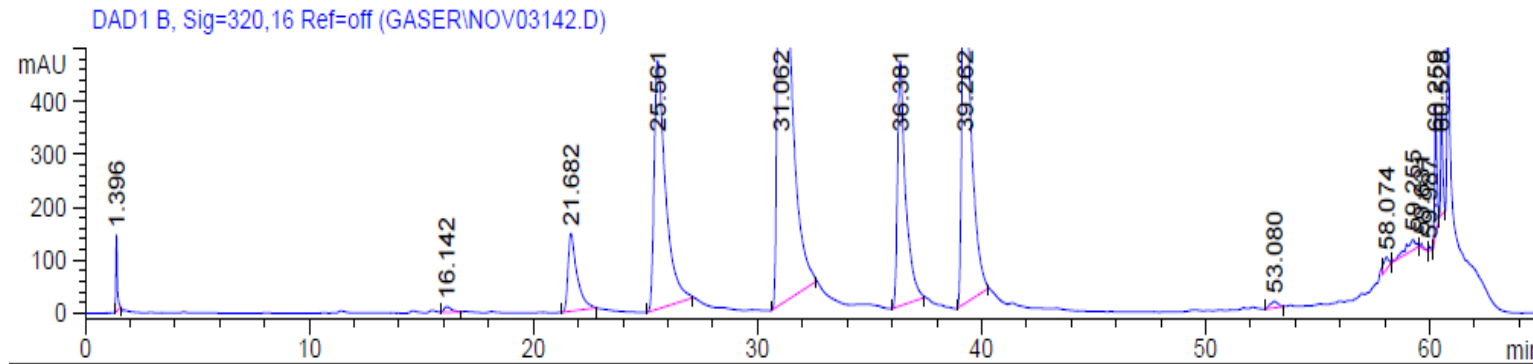**Fig. 1. HPLC chromatograms of nonvolatile constituents of saffron ethanolic extract**

Fig. 2. HPLC chromatograms of nonvolatile constituents of turmeric ethanolic extract.

The brain, in particular, is more susceptible to oxidative stress than other organs. Although the brain only accounts for ~2% of body mass it consumes 15–20% of the energy generated in the entire body. The high mass specific metabolic rate is attributed to the high proportion of omega-three polyunsaturated fatty acids (PUFAs) in brain tissue. These phospholipids are highly susceptible to peroxidation. Moreover, brain tissue contains high levels of redox active iron and copper further enhancing its vulnerability to oxidative stress. Brain also has little potential to replenish damaged cells since it is composed mostly of terminally differentiated neurons and glia [54].

It was reported that abnormal accumulation of galactitol from excess D-galactose by aldose reductase in cell, lead to osmotic stress and generation of ROS [55]. These results revealed that injection of D-galactose caused a state of oxidative stress and so caused significant increase in the levels of oxidative biomarkers as AGEs, PCG, MDA and NO as D-galactose is a reducing sugar that reacts readily with free amines of amino acids in proteins and peptides both *in vivo* and *in vitro* to form advanced glycation end products (AGEs) through non enzymatic glycation. The AGEs activate their receptors, coupled to biochemical pathways that stimulate free radical production. So lead to impairment of cholinergic neurons in the basal forebrain and neurogenesis in the hippocampus [56].

In agreement with these results, Prakash and Kumar, Qu et al. and Al-babily and Tawfeeq suggested that administration of D-galactose significantly raised brain MDA, nitrite and AGEs concentration when compared to the normal control group [57-59].
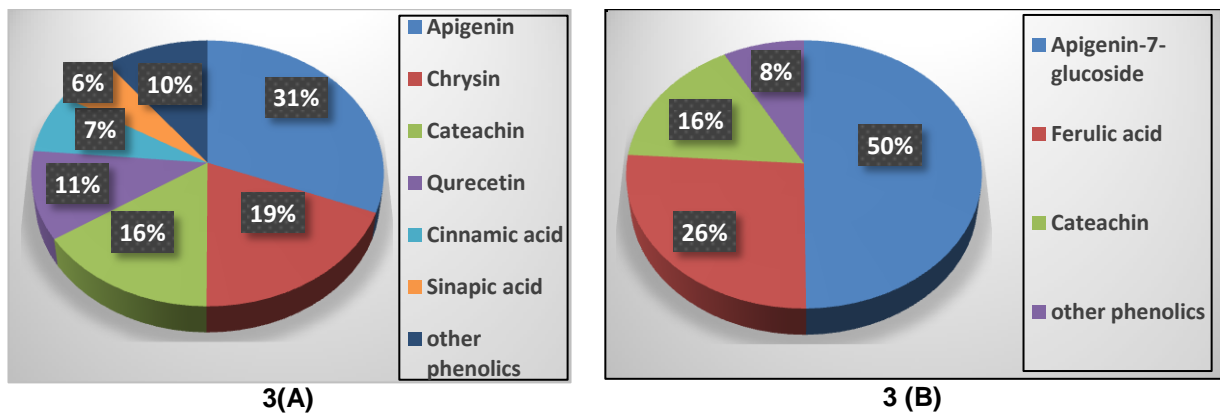

Fig. 3. Main phenolic compounds percent of (A): saffron ethanolic extract and (B): turmeric ethanolic extract

Table 3. Effect of saffron and / or turmeric ethanolic extracts supplementation on brain AGEs, PCG, MDA and NO levels in D- galactose intoxicated rats

Parameters / Groups	AGEs (ng/mg)	PCG (ng/mg)	MDA (nmol/g)	NO (mmol/g)
G1: (HCG)	39.06 ± 0.88[e]	26.47 ± 0.66[e]	35.13 ± 0.419[e]	20.93± 0.75[e]
G2: (DGCG)	153.34 ± 1.01[a]	109.35 ±0.70[a]	121.50 ± 0.63[a]	65.03 ± 0.50[a]
G3: (DG+ESE)	78.01 ± 1.18[c]	39.88 ± 0.54[c]	51.19 ± 0.71[c]	28.39 ± 0.19[c]
G4 : (DG+ETE)	86.02 ± 0.83[b]	67.65 ± 0.81[b]	56.63 ± 0.81[b]	35.73 ± 0.73[b]
G5:(DG+ESE+ETE)	64.84 ± 0.86[d]	34.75 ± 0.61[d]	39.26 ± 0.07[d]	21.50 ± 0.59[d]
LSD (p≤ 0.05)	0.869	0.610	0.567	0.533

Values are expressed as (mean ± S.D), n=10 rats. There was no significant difference between means that have the same alphabetical superscript letter in the same column (p≤ 0.05).HCG: Healthy control group, DGCG: D-galactose control group, DG+ESE: D-galactose intoxicated rats supplemented with ethanolic saffron extract; DG+ETE: D-galactose intoxicated rats supplemented with ethanolic turmeric extract; DG+ESE+ETE: D-galactose intoxicated rats supplemented with ethanolic saffron and turmeric extracts.

The antioxidants act as a major defense against radical mediated toxicity by protecting cells from the damages caused by free radicals [60].Oral administration of saffron extract led to a decrease in brain oxidative markers due to antioxidants that found in saffron such as volatile agents (e.g., safranal), bitter principles (e.g., picrocrocin), and dye materials (e.g., crocetin and its glycoside, crocin)**.** Saffron and its active constituents have free radical scavenging and antioxidant activities [61] causing decrease of MDA and PCG levels in the liver and brain of intoxicated rats [62,63].

Turmeric is traditionally used as a medicinal herb. The potential use of turmeric as an alternative medicine is attributed to its antioxidant properties. *In vitro* and *in vivo* studies showed that the active compound of turmeric extract, curcumin, prevented oxidative stress-induced neuro-degeneration in the nervous system [64] .Oral administration of ethanolic turmeric extract led to decrease MDA and PCG levels in the brain of intoxicated rats [13].

3.4 Effect of Saffron and / or Turmeric Ethanolic Extracts Supplementation on Brain Total Antioxidant Capacity (TAC), Reduced Glutathione (GSH) Level and Catalase (CAT) Activity in D- galactose Intoxicated Rats

Table (4) clarifies the brain total antioxidant capacity (TAC), reduced glutathione (GSH) level and catalase (CAT) activity in all experimental groups. The research results showed that D-galactose serves as a potent oxidant and caused a state of oxidative stress that significantly (P ≤ 0.05) decreased the levels of antioxidant defense by decreasing brain TAC and GSH levels as well as depletion of brain CAT activity in D-galactose control group when compared with healthy control group. It was understandable from the results that ethanolic extracts of saffron and turmeric contain antioxidants caused a detectable enhancement (P ≤ 0.05) in all brain antioxidant parameters of D-galactose supplemented groups when compared with D-galactose control group. Treatment with both of ESE and ETE have the most improving action on brain antioxidant status.

The present results were in agreement with the results of Prakash and Kumar [57]; Yu et al. [58];Qu et al. [65] and Chen et al. [66] who reported that injection with D-galactose resulted in significant decrease in TAC,GSH level and CAT activity, in the brain and this confirm that D-galactose induced mitochondrial dysfunction and biochemical changes by causing oxidative stress, apoptosis and decreasing antioxidant enzymes, oxidative phosphorylation, and cell viability in mice and rats.

The phenolic components of saffron and turmeric contribute to antioxidant properties as well as other biological activities. These phenolic components are able to scavenge the free radicals and maintaining the activities of antioxidant enzymes as result of their high antioxidant activities [67].These results were similar to previous studies that prove saffron extract led to increase TAC, GSH level and CAT activity [62,63]. Additionally, Yuliana et al. (2019) found that oral administration of ethanolic turmeric extract led to increase the GSH concentration and CAT activity in the brain as compared to normal control group and this prove that turmeric has antioxidant properties [13].

3.5 Effect of Saffron and / or Turmeric Ethanolic Extracts Supplementation on Brain Tumor Necrosis Factor Alpha (TNF-α), Interleukin -6 (IL-6) and p16[INK4a]Levels in D-galactose Intoxicated Rats

The results tabulated in Table (5) summarize the effects of D-galactose (250mg / kg body weight) along with different experimental groups on the levels of brain TNF-α, IL-6 and p16[INK4a]. The current results showed that D-galactose toxicity induces massive increase (P≤ 0.05) in the levels of brain TNF-α, IL-6 and p16[INK4a] in the D-galactose control group when compared with healthy control group. D-galactose was noticed to induce a state of oxidative stress that initiate and activate inflammatory pathways resulting in increased brain pro-inflammatory markers. On the other hand it was noticed from the results that administration of saffron and turmeric ethanolic extracts to intoxicated rats caused improvement (P≤0.05) in the levels of inflammatory and epigenetic markers when compared with   D-galactose control group due to presence of active components in the extracts that fight oxidants and inhibited inflammatory pathways. Results demonstrated that, treatment with mixture of extracts caused the most significant improvements (P≤0.05) in the brain compared to other treatments as a result of the synergistic effects between saffron and turmeric active constituents.

Table 4. Effect of saffron and / or turmeric ethanolic extracts supplementation on brain TAC, GSH level and CAT activity in D- galactose intoxicated rats

Parameters / Groups	TAC (mmol/g)	GSH (mmol/g)	CAT (U/g)
G1: (HCG)	40.45 ± 0.29^{a}	71.97 ± 0.45^{a}	118.11 ± 0.75^{a}
G2: (DGCG)	15.10 ± 0.67^{e}	30.44 ± 0.42^{e}	58.05 ± 0.88^{e}
G3: (DG+ESE)	30.60 ± 0.40^{c}	56.31 ± 0.17^{c}	101.73 ± 0.66^{c}
G4: (DG+ETE)	22.26 ± 0.46^{d}	51.27 ± 0.46^{d}	93.74 ± 0.45^{d}
G5:(DG+ESE+ETE)	36.39 ± 0.66^{b}	62.66 ± 0.64^{b}	117.02 ± 0.72^{b}
LSD (p≤ 0.05)	0.471	0.411	0.640

Values are expressed as (mean ± S.D), n=10 rats. There was no significant difference between means that have the same alphabetical superscript letter in the same column (p≤ 0.05).HCG: Healthy control group, DGCG: D-galactose control group, DG+ESE: D-galactose intoxicated rats supplemented with ethanolic saffron extract; DG+ETE: D-galactose intoxicated rats supplemented with ethanolic turmeric extract; DG+ESE+ETE: D-galactose intoxicated rats supplemented with ethanolic saffron and turmeric extracts.

Table 5. Effect of saffron and / or turmeric ethanolic extracts supplementation on brain TNF-α, IL-6 and p16[INK4a] levels in D- galactose intoxicated rats

Parameters / Groups	TNF-α (pg/mg)	IL-6 (pg/mg)	P16[INK4a]
G1: (HCG)	13.85 ± 0.64^{e}	31.92 ± 1.03^{e}	1.36 ± 0.17^{e}
G2: (DGCG)	83.74 ± 0.47^{a}	124.68 ± 0.634^{a}	7.28 ± 0.39^{a}
G3: (DG+ESE)	30.57 ± 0.24^{c}	54.01 ± 0.56^{c}	3.68 ± 0.135^{c}
G4: (DG+ETE)	36.29 ± 0.52^{b}	64.48 ± 0.73^{b}	4.45 ± 0.32^{b}
G5:(DG+ESE+ETE)	21.46 ± 0.61^{d}	38.78 ± 0.46^{d}	2.39 ± 0.12^{d}
LSD(p≤ 0.05)	0.468	0.643	0.229

Values are expressed as (mean ± S.D), n=10 rats. There was no significant difference between means that have the same alphabetical superscript letter in the same column (p≤ 0.05).HCG: Healthy control group, DGCG: D-galactose control group, DG+ESE: D-galactose intoxicated rats supplemented with ethanolic saffron extract; DG+ETE: D-galactose intoxicated rats supplemented with ethanolic turmeric extract; DG+ESE+ETE: D-galactose intoxicated rats supplemented with ethanolic saffron and turmeric extracts

The pro-inflammatory cytokines such as (TNF-α) and (IL-6) are involved in the up-regulation of inflammatory reactions, these pro-inflammatory cytokines are involved in the process of pathological disorders. So that, their levels in tissues of the experimental rats were determined. p16 [also known as p16[INK4a], cyclin-dependent kinase inhibitor 2A (CDKN2A), multiple tumor suppressor 1]. p16[INK4a] is a protein that slows cell division by slowing the progression of the cell cycle (the ordered sequence of events that occur in a cell in preparation for cell division).

In accordance with this study Zhu et al.; Gao et al. and Rehman et al. [68-70] reported that administration of D-galactose caused a significant increase in brain TNF-α and IL- 6

levels which led to systemic inflammatory response syndrome resulting in tissues inflammatory injury and damage. Also, Chen et al. reported that administration of D-galactose (200 mg/kg) caused a significant increase in brain TNF-α, IL- 6 and p16[INK4a] this was due to reactive oxygen species, lipid peroxidation and DNA damage in the cells resulted in inflammation. Also macrophages release pro-inflammatory cytokines, such as TNF-α and IL-6, to enhance inflammatory responses, which then results in further brain injury and also increase the gene expression (p16[INK4a]) in hippocampus of D-galactose intoxicated rats. Concentrations of p16[INK4a] increase dramatically as tissue ages. p16[INK4a] is regarded to be a biomarker of cellular senescence [65].

Saffron and turmeric extracts contain several different polyphenol-phytochemicals with several biological activities. Also, saffron stigma extract was showed to have inhibitory activities against acute and chronic inflammation in animal models. The phytochemistry and pharmacological actions of saffron and turmeric components suggest a wide range of clinical applications for the treatment and prevention of diseases where chronic inflammation is believed to play an essential etiologic role [71,72].

3.6 Effect of Saffron and / or Turmeric Ethanolic Extracts Supplementation on Brain Acetylcholinesterase (AChE) Activity, Metallothoenins (MTs) and Serotonin (5-HT) levels in D- galactose Intoxicated Rats

The results tabulated in Table (6) summarize the effects of D-galactose along with different experimental groups on brain AChE activity, MTs and 5-HT levels. The current results showed that D-galactose toxicity induced massive increase (P ≤ 0.05) in the levels of brain MTs and 5-HT with decreased activity of AChE in the D-galactose control group when compared with healthy control group. On the other hand it was noticed from the results that administration of saffron and turmeric ethanolic extracts to intoxicated rats caused improvements (P ≤ 0.05) in the levels of neural cell markers when compared with D-galactose control group due to presence of active components in the extracts that restore the loss of brain neurotransmitters. Results demonstrated that, treatment with mixture of ethanolic extracts caused the most significant improvements (P ≤ 0.05) compared to other treatments.

Acetylcholinesterase (AChE) is one of the most important enzymes that located in the nervous system and muscles; responsible for regulation of acetylcholine concentration during nerve signal transmission [73]. The activity of AChE is shown to decrease with aging and is reflected in AChE brain content. Loss of enzyme activity may indicate functional decline in the neuron population [74].These results were similar to Delwing de-lima et al. [75].

Metallothioneins (MTs) are low molecular weight, cysteine rich, zinc-binding proteins, which are down-regulated in older age groups. There is experimental evidence that MTs are induced in the aging brain as a defensive mechanism to attenuate oxidative and nitrative stress. MTs may also act as free radical scavengers, thus contributing to protecting mitochondrial function as a mechanism of neuro-protection in the aging brain induced by D-galactose [76].These results were in agreements with Wunderlich et al. [77].

Serotonin or 5-hydroxytryptamine (5-HT) is a neurotransmitter and hormone that contributes to the regulation of various physiological functions by its actions in the central nervous system (CNS) and in the respective organ systems. Abnormalities of the serotonergic nervous system are documented in pathologic studies of Alzheimer's disease and other age-related disease [78].5-HT turnover is increased and numbers of 5-HT receptors are reduced, as a result of brain aging [79].

Table 6. Effect of saffron and / or turmeric ethanolic extracts supplementation on brain AChE activity, MTs and 5-HT levels in D- galactose intoxicated rats

Parameters / Groups	AChE (U/g)	MTs (ng/mg)	5-HT (ng/mg)
G1: (HCG)	41.94 ± 0.88^a	50.99 ± 0.80^e	44.92 ± 0.55^e
G2: (DGCG)	14.87 ± 0.30^e	131.22 ± 0.76^a	124.6 ± 0.668^a
G3: (DG+ESE)	31.99 ± 1.04^c	75.15 ± 0.53^c	53.42 ± 0.64^c
G4: (DG+ETE)	26.79 ± 0.72^d	80.53 ± 0.25^b	61.785 ± 0.800^b
G5:(DG+ESE+ETE)	34.83 ± 0.44^b	54.65 ± 0.54^d	50.38 ± 0.26^d
LSD	0.661	0.551	0.553

Values are expressed as (mean ± S.D), n=10 rats. There was no significant difference between means that have the same alphabetical superscript letter in the same column (p≤ 0.05).HCG: Healthy control group, DGCG: D-galactose control group, DG+ESE: D-galactose intoxicated rats supplemented with ethanolic saffron extract; DG+ETE: D-galactose intoxicated rats supplemented with ethanolic turmeric extract; DG+ESE+ETE: D-galactose intoxicated rats supplemented with ethanolic saffron and turmeric extracts.

Saffron has high amounts of anti-oxidative carotenoids and B vitamins. It is thought that saffron regulates the levels of neurotransmitters such as dopamine, norepinephrine, and serotonin in the brain. Also, the antioxidants in saffron are thought to help clean up free radicals in the body and to protect brain cells from oxidative stress [80]. These results were similar to Ghadrdoost et al. [81]. Naghibi et al. [82] and Ettehadi et al. [83] who reported that administration of ethanolic saffron extract improved learning and memory impairments and caused significant improvement in brain neurotransmitters and in moderating acetylcholinesterase (AChE) activity.

Turmeric contain several active components that have neuro-protective effects especially curcumin. It regulates the level of dopamine, norepinephrine, and serotonin in the brain and inhibits the enzyme monoamine oxidase (MAO) A and B, which decompose serotonin and dopamine. Turmeric modifies the dysfunction of these neurotransmitters and significantly reduces behavioral symptoms [84]. In line with these results, Nasir and Jaffat [85] found that administration of ethanolic turmeric extract regulated brain serotonin level in intoxicated groups. Curcumin oral administration improved the cognitive defects and neurotoxicity induced in rats by decreasing lipid peroxidation, protein oxidation, and cleaved caspase-3 expression, as well as increasing antioxidant content in the neuronal mitochondria. These results were in agreements with Abu-Taweel et al. [86] and Singh et al. [87].

3.7 Effect of Saffron and / or Turmeric Ethanolic Extracts Supplementation on Microscopic Examination of Brain Tissues in D- galactose Intoxicated Rats

The results of microscopic examination of brain tissue in all groups showed the degenerative changes caused by D-galactose and the corrective role of ESE, ETE and ESE+ETE on cerebral cortex and hippocampus provided supportive evidence for biochemical analysis.

I. Cerebral cortex: Microscopic examination of cerebral cortex of HCG reveled the normal histological structure (Fig. 4 A). On contrary, cerebral cortex of DGCG showed numerous neuro-pathological alterations which described as congestion of cerebral blood vessel,

meningeal infiltration with mononuclear cells (Fig. 4 B), marked necrosis, pyknosis and atrophy of neurons associated with neuronophagia (Fig. 4 C) and neurofibrillary tangles (Fig. 4 D). Meanwhile, cerebral cortex of rats from group three (co-treated with DG+ESE) showed improvement in the histopathological picture, examined sections showed necrosis of some neurons and neuronophagia (Fig. 4 E). Moreover, cerebral cortex of rats co-treated with DG+ETE revealed moderate regression in the neuropathic changes. Examined sections showed necrosis and atrophy of some neurons and neuronophagia(Fig. 4 F). On the other hand, marked regressed lesions was noticed in cerebral cortex of rats co-treated DG+ESE+ETE, most sections showed no histopathological alterations (Fig. 4 G).

II.Hippocampus: Microscopic examination of hippocampal current area (CA1) that is the first region in the hippocampal circuit from which a major output pathway goes to other layers of HCG revealed the normal histological structure of pyramidal neurons (Fig. 5 A). Meanwhile, sections from DGCG showed necrosis and atrophy of pyramidal neurons associated with neurofibrillary tangles (Fig. 5 B). However, hippocampal sections of rats co-treated with DG+ ESE revealed marked improvement with minimal changes, examined sections showed necrosis and atrophy of few pyramidal neurons (Fig. 5 C). Moreover, hippocampus of rats co treated with DG+ETE showed necrosis and atrophy of some pyramidal neurons associated with neurofibrillary tangles (Fig. 5 D). On the other hand, hippocampus of rats co- treated with DG+ ESE+ETE maintained their normal histology (Fig. 5 E).

These results were similar to Yellamma [88] Sun et al. [89] Mohamed et al. [90] and Rusu et al. [91] who reported that D-galactose caused severe structural damage, such as necrosis and apoptosis with a decline in the number of surviving neurons in the cortex and hippocampus of D- galactose rats compared to normal control rats .D-galactose decreased the neural cell density. On the other hand saffron and turmeric extracts antioxidants, polyphenols and active constituents preserved brain structure from D-galactose degeneration. This was confirmed by Sharma et al. [92] Sarona et al. [93] and Baghishani et al. [94] who proved that saffron and turmeric contain active components that preserve the brain structure.
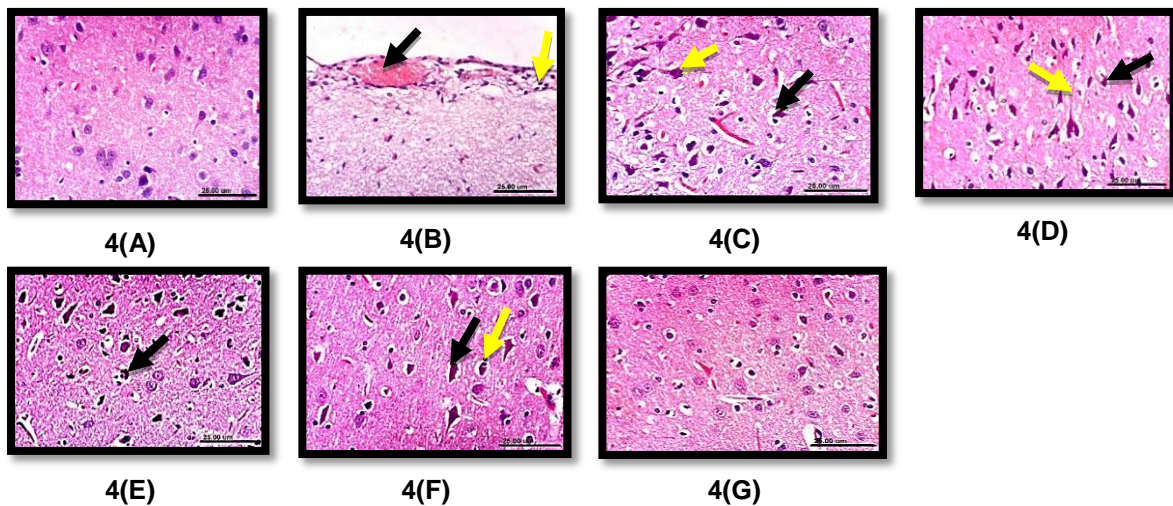
4(A) 4(B) 4(C) 4(D)

4(E) 4(F) 4(G)

Fig. 4. (A): Photomicrograph of cerebral cortex of HCG showing normal histological structure. (B): Photomicrograph of cerebral cortex of DGCG showing congestion of cerebral blood vessel (black arrow) and meningeal infiltration with mononuclear cells (yellow arrow).(C): Photomicrograph of cerebral cortex of DGCG showing marked necrosis, pyknosis and atrophy of neurons (black arrow) associated with neuronophagia (yellow arrow).(D): Photomicrograph of cerebral cortex of DGCG showing marked necrosis, pyknosis and atrophy of neurons (black arrow) associated with neurofibrillary tangles (yellow arrow).(E): Photomicrograph of cerebral cortex of rat co-treated with DG+ESE showing necrosis of some neurons and neuronophagia (black arrow) .(F): Photomicrograph of cerebral cortex of rat co-treated with DG+ETE showing necrosis of some neurons (black arrow) and neuronophagia (yellow arrow).(G): Photomicrograph of cerebral cortex of rat co-treated with DG+ESE+ETE showing no histopathological alterations. (H&E, scale bar 25um, X 400)

5(A) 5(B) 5(C)

5(D) 5(E)
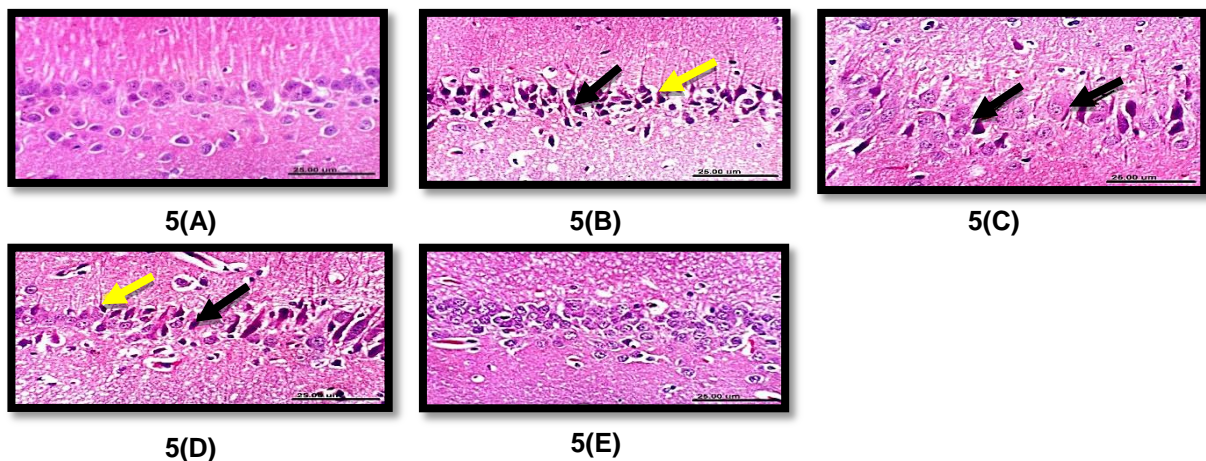
Fig. 5. (A): Photomicrograph of CA1 region of hippocampus of HCG showing the normal histological structure of pyramidal neurons. (B): Photomicrograph of CA1 region of hippocampus of DGCG showing necrosis and atrophy of pyramidal neurons (black arrow) associated with neurofibrillary tangles (yellow arrow).(C): Photomicrograph of CA1 region of hippocampus of rat co- treated with DG+ESE showing necrosis and atrophy of few pyramidal neurons (black arrow). (D): Photomicrograph of CA1 region of hippocampus of rat co-treated with DG+ETE showing necrosis and atrophy of some pyramidal neurons (black arrow) associated with neurofibrillary tangles (yellow arrow). (E): Photomicrograph of CA1 region of hippocampus of rat co-treated with DG+ESE+ETE showing normal neurons with no histopathological changes. (H&E, scale bar 25um, X 400)

45

3.8 Effect of Saffron and / or Turmeric Ethanolic Extracts Supplementation on Immunohistochemical Analysis of Brain Tissues in D- galactose Intoxicated Rats
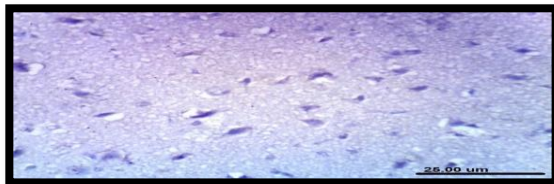
The results of immunohistochemical analysis of brain tissue in all groups showed the degenerative changes caused by D-galactose and the corrective role of ESE, ETE and ESE+ETE on hippocampus and cerebral cortex and provided supportive evidence for biochemical analysis.

I.Caspase-3 expression: Immunohistochemical analysis of caspase-3 in the cerebral cortex and hippocampus of HCG revealed no immune expression (Fig. 6 A and B). On contrary, intensely stained immune-reactive neurons were noticed in examined sections rats treated with D-galactose (Fig. 7 A and B). Meanwhile, cerebral cortex and hippocampus of rats co-treated with DG+ESE showed weak positive immune expression (Fig. 8 A and B). On the other hand, moderate positive caspase-3 expression was recorded in the cerebral cortex as well as hippocampus of rats co-treated with DG+ETE (Fig. 9 A and B). However, no or very weak immune reaction was noticed in the cerebral cortex and hippocampus of rats co-treated with the combination (Fig. 10 A and B) respectively.

Table 7. Histopathological lesions score in cerebral cortex and hippocampus of all experimental group

| Histopathological lesions	I. Cerebral cortex				
	HCG	DGCG	DG+ESE	DG+ETE	DG+ESE+ETE
-Congestion of meningeal blood vessel + mononuclear cells infiltration	0	2	0	0	0
-Necrosis of neurons	0	4	2 / 1	2	1
-Neurofibrillary tangles	0	3	0	1	0
-Neuronophagia	0	3	1	1	1
-Gliosis	0	2	0	0	0
II. Hippocampus					
-Necrosis of pyramidal neurons	0	4	2	2	1
-Neurofibrillary tangles	0	3	0	1	0

6(A)
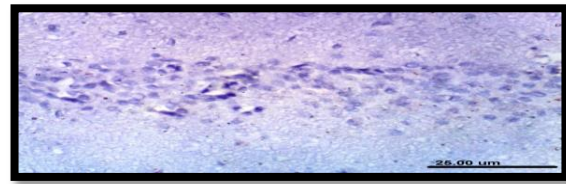
6(B)

Fig.6. (A): Immunohistochemical analysis of caspase-3 in the cerebral cortex of HCG showing negative expression. (B): Immunohistochemical analysis of caspase-3 in the hippocampus of HCG showing negative expression (scale bar 25 um)
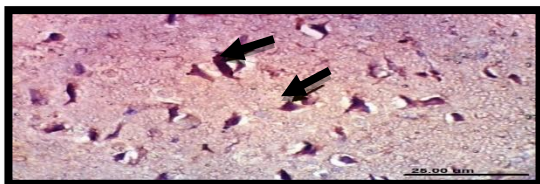
7(A)
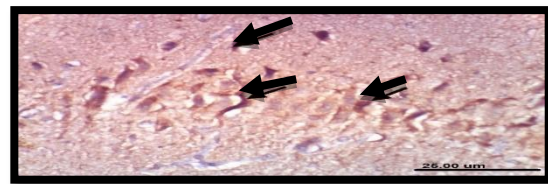
7(B)

Fig.7.(A):Immunohistochemical analysis of caspase-3 in the cerebral cortex of DGCG showing intense positive immune staining (immunopositivity indicated by brown colour).(B):Immunohistochemical analysis of caspase-3 in the hippocampus of DGCG showing intense positive immune staining (immunopositivity indicated by brown colour) (scale bar 25 um)
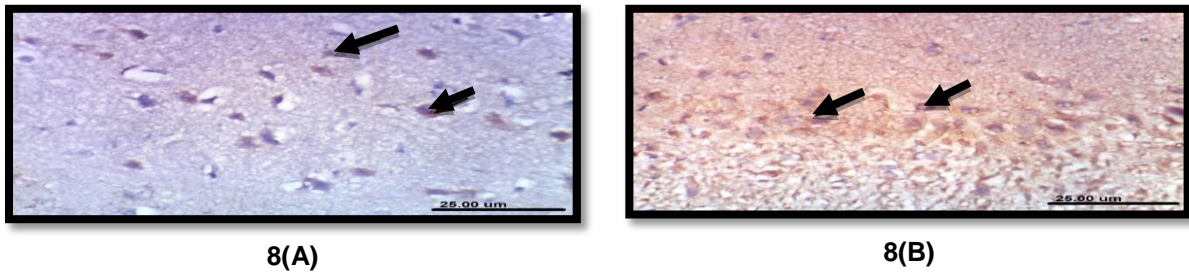
8(A)

8(B)

Fig. 8. (A):Immunohistochemical analysis of caspase-3 in the cerebral cortex of rat co-treated with DG+ESE showing weak positive immune reaction (immunopositivity indicated by brown color) .(B):Immunohistochemical analysis of caspase-3 in the hippocampus of rat co-treated with DG+ESE showing weak positive immune reaction (immunopositivity indicated by brown colour) (scale bar 25 um)
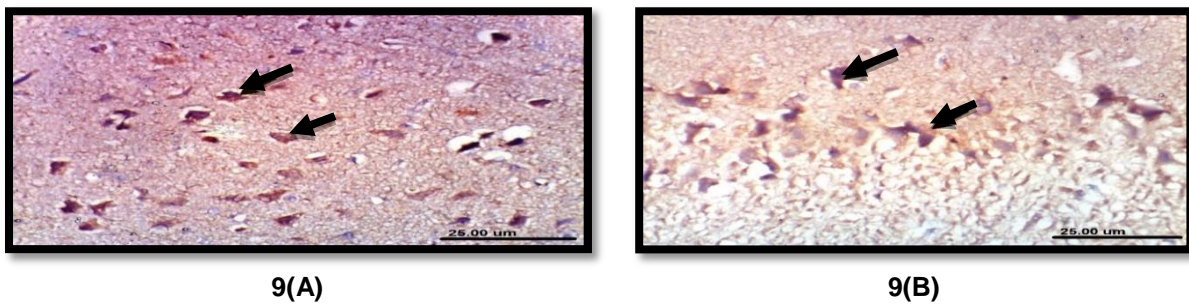

9(A)

9(B)

Fig. 9. (A):Immunohistochemical analysis of caspase-3 in the cerebral cortex of rat co-treated with DG+ETE showing moderate positive immune reaction(immunopositivity indicated by brown colour).(B):Immunohistochemical analysis of caspase-3 in the hippocampus of rat co-treated with DG+ETE showing moderate positive immune reaction (immunopositivity indicated by brown colour) (scale bar 25 um)
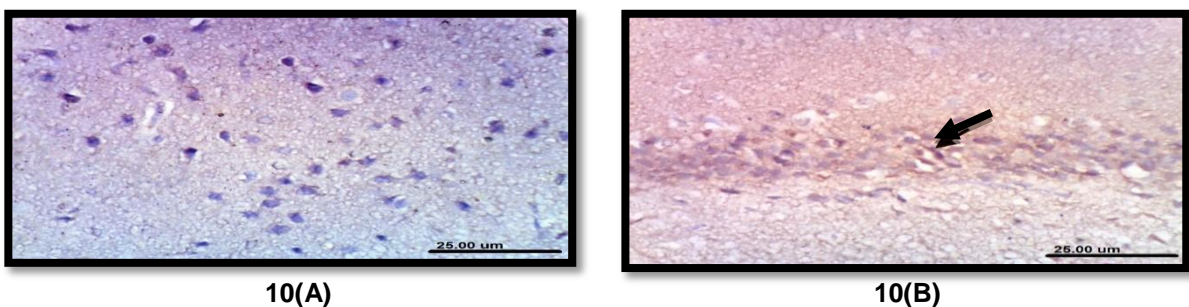

10(A)

10(B)

Fig.10.(A):Immunohistochemical analysis of caspase-3 in the cerebral cortex of rat co-treated with DG+ESE+ETE showing no immune expression .(B): Immunohistochemical analysis of caspase-3 in the hippocampus of rat co-treated with DG+ESE+ETE showing very weak positive immune reaction (immunopositivity indicated by brown colour) (scale bar 25 um)

II.Bax expression: Immunohistochemical analysis of Bax in the cerebral cortex and hippocampus of HCG revealed no immune expression (Fig. 11 A and B) respectively. On contrary, intensely stained immune-reactive neurons were noticed in examined sections rats treated with D- galactose (Fig. 12 A and B). Meanwhile, cerebral cortex and hippocampus of rats co-treated with DG+ESE showed weak positive immune expression (Fig. 13 A and B). On the other hand, moderate positive Bax expression was recorded in the cerebral cortex of rats co-treated with DG+ETE (Fig. 14 A and B). However, no immune reaction was noticed in the cerebral cortex and hippocampus of rats co-treated with the combination (Fig. 15 A and B).
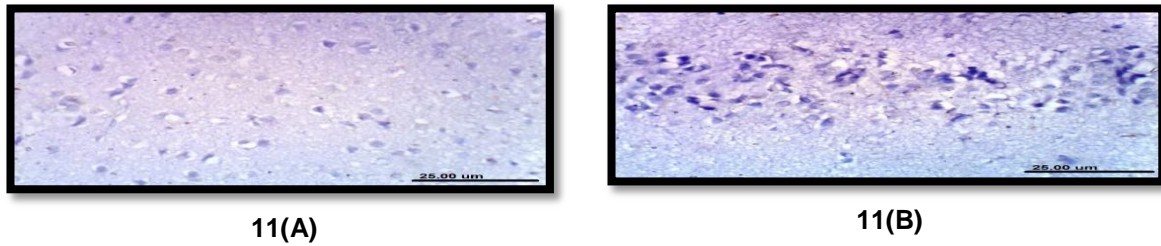
11(A)

11(B)

Fig.11. (A): Immunohistochemical analysis of Bax in the cerebral cortex of HCG showing negative expression. (B): Immunohistochemical analysis of Bax in the hippocampus of HCG showing negative expression (scale bar 25 um)
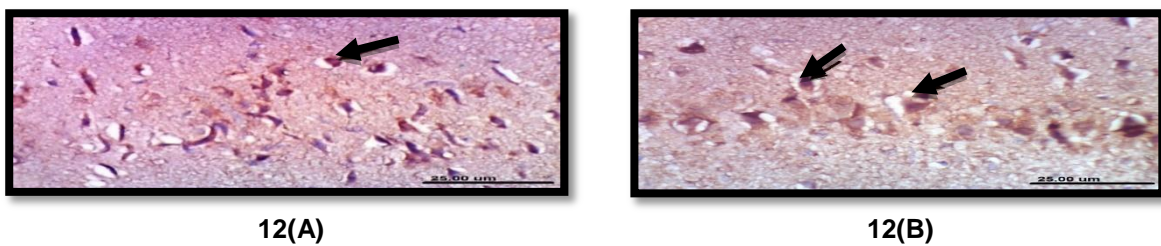

12(A)

12(B)

Fig. 12. (A): Immunohistochemical analysis of Bax in the cerebral cortex of DGCG showing intense positive immune staining (immunopositivity indicated by brown colour). (B): Immunohistochemical analysis of Bax in the hippocampus of DGCG showing intense positive immune staining (immunopositivity indicated by brown colour) (scale bar 25 um)
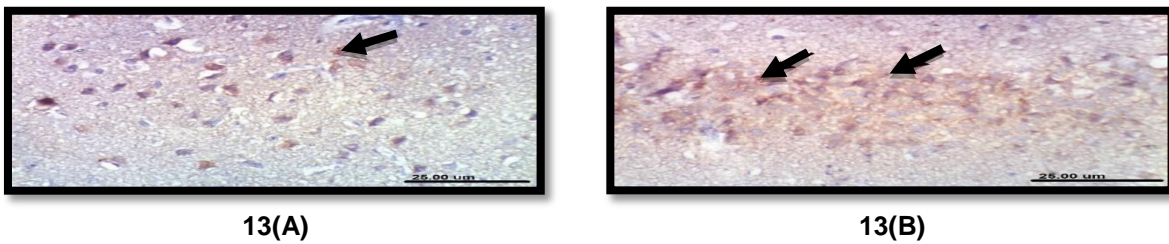

13(A)

13(B)

Fig. 13. (A): Immunohistochemical analysis of Bax in the cerebral cortex of rat co-treated with DG+ESE showing weak positive immune reaction(immunopositivity indicated by brown colour).(B): Immunohistochemical analysis of Bax in the hippocampus of rat co-treated with DG+ESE showing weak positive immune reaction (immunopositivity indicated by brown colour) (scale bar 25 um)
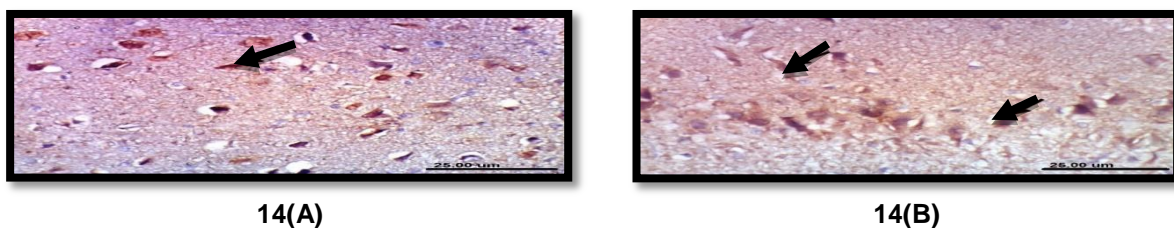

14(A)

14(B)

Fig. 14. (A): Immunohistochemical analysis of Bax in the cerebral cortex of rat co-treated with DG+ETE showing moderate positive immune reaction(immunopositivity indicated by brown colour).(B): Immunohistochemical analysis of Bax in the hippocampus of rat co-treated with DG+ETE showing moderate positive immune reaction (immunopositivity indicated by brown colour) (scale bar 25 um)
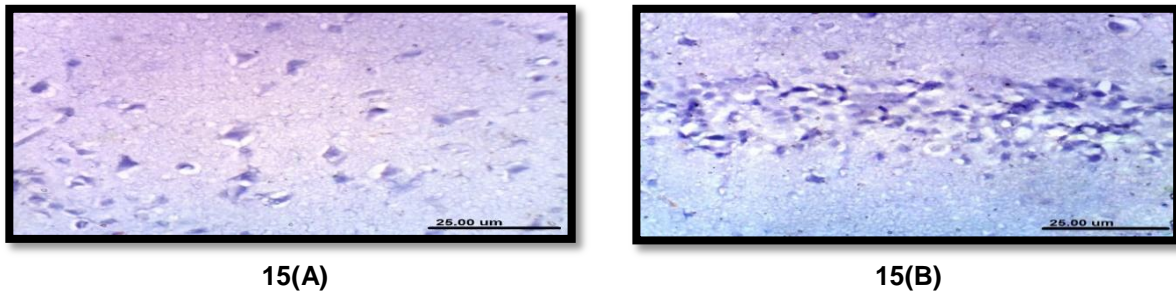
15(A)

15(B)

Fig. 15. (A): Immunohistochemical analysis of Bax in the cerebral cortex of rat co-treated with DG+ESE+ETE showing no immune expression. (B): Immunohistochemical analysis of Bax in the hippocampus of rat co-treated with DG+ESE+ETE showing no immune reaction (scale bar 25 um)

III.Bcl-2 expression: Immunohistochemical analysis of Bcl-2 in the cerebral cortex and hippocampus of HCG revealed strong Bcl-2 expression (Fig. 16 A and B). Meanwhile, sections from rats treated with D-galactose showed no expression of Bcl-2 (Fig. 17 A and B). Brain of rats co- treated with DG+ESE revealed strong positive expression (Fig. 18 A and B). However, sections from rats co-treated with DG+ETE showed moderate or weak positive reaction (Fig. 19 A and B). Moreover, strong positive expression of Bcl-2 was observed in cerebral cortex and hippocampus of rats treated with D-galactose and combination of saffron and turmeric ethanolic extracts (Fig. 20 A and B).

Apoptosis is the main process contributing to neuro-degenerative disorders. It seems that reactive oxygen production after mitochondrial dysfunction play an important role in the progression of cell death and neurotoxicity [95].Many apoptotic proteins and anti-apoptotic proteins are closely related to the neurotoxicity induced by injection of D-galactose. The Bcl-2 protein is a key factor in the inhibition of apoptosis; it is a known factor in cell ageing, and its over expression can effectively prevent the apoptosis induced by hydrogen peroxide. On the other hand, the main function of Bax is to accelerate apoptosis and, together with Bcl-2, regulate cell apoptosis. However, the core molecule in apoptosis is caspase-3 (cysteine protease), which is known to be a key factor of apoptosis in mammals. It is commonly believed that Bcl-2 acts downstream of caspase-3 activation, and thus, apoptosis is inhibited by inhibiting the activation of caspase-3, which plays an irreplaceable role in apoptosis. Previous studies indicated that neuronal apoptosis and or autophagy are main pathologic biomarkers of neuro-degeneration, increasing the apoptotic proteins caspase-3 as well as Bax, and decrease and anti-apoptotic Bcl-2protein [96].

In line with these results yang et al .(2016) and Rehman et al. (2017) found that administration of D-galactose caused significant increase in the levels of Bax and caspase-3 and caused decrease in the level of Bcl-2 in the D-galactose group when compared to healthy control group and lead to neuronal cell damage [97,98].
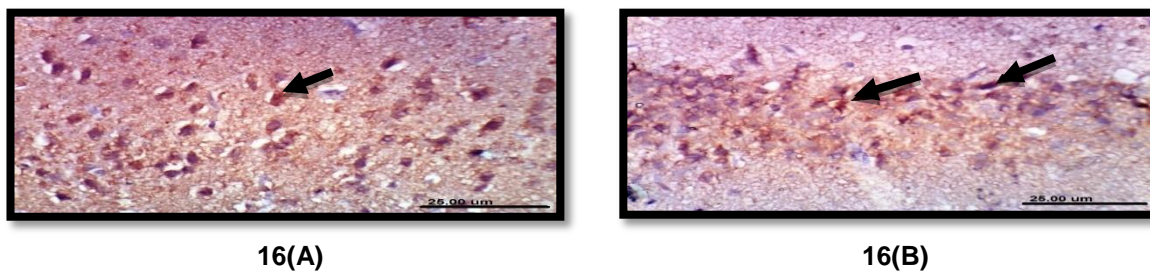

16(A)

16(B)

Fig.16. (A):Immunohistochemical analysis of Bcl-2 in the cerebral cortex of HCG showing intense positive immune staining (immunopositivity indicated by brown colour). (B): Immunohistochemical analysis of Bcl-2 in the hippocampus HCG showing intense positive immune staining (immunopositivity indicated by brown colour) (scale bar 25 um)

17(A)   **17(B)**
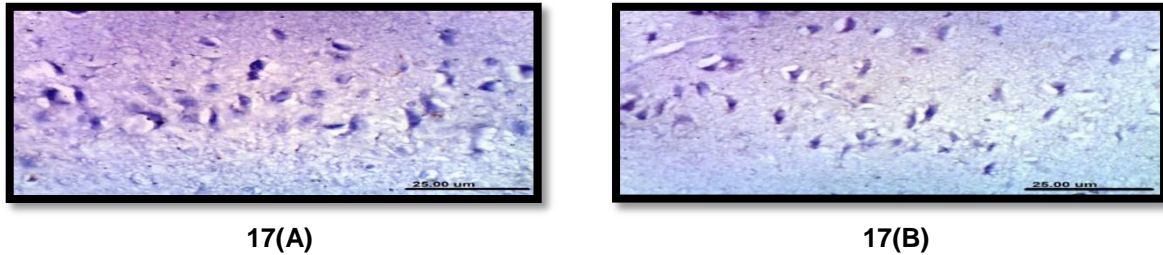
Fig. 17. (A): Immunohistochemical analysis of Bcl-2 in the cerebral cortex of DGCG showing no immune reaction (scale bar 25 um). (B): Immunohistochemical analysis of Bcl-2 in the hippocampus of DGCG showing no immune reaction (scale bar 25 um)

18(A)   **18(B)**
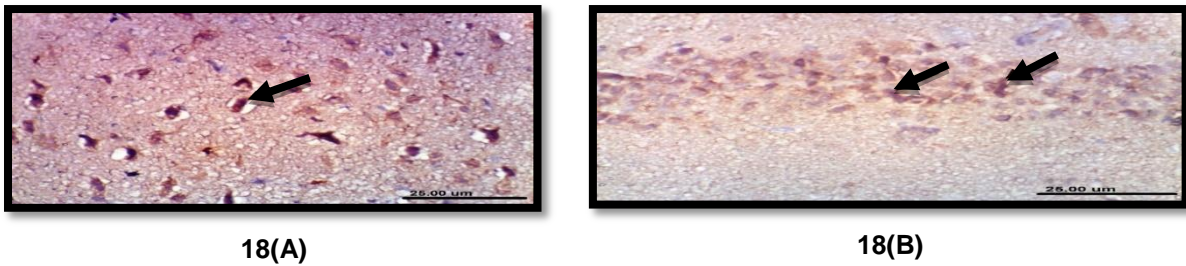
Fig. 18. (A): Immunohistochemical analysis of Bcl-2 in the cerebral cortex of rat co-treated with DG+ESE showing strong positive immune reaction (immunopositivity indicated by brown colour) .(B): Immunohistochemical analysis of Bcl-2 in the hippocampus of rat co-treated with DG+ESE showing strong positive immune reaction (immunopositivity indicated by brown colour) (scale bar 25 um)

19(A)   **19(B)**
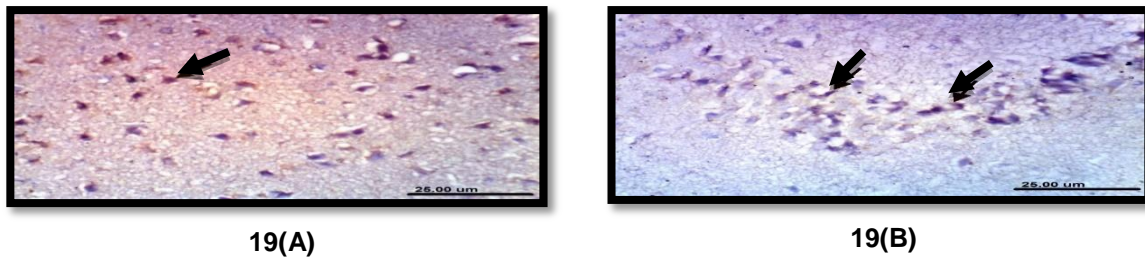
Fig. 19. (A): Immunohistochemical analysis of Bcl-2 in the cerebral cortex of rat co-treated with DG+ETE showing moderate positive immune reaction (immunopositivity indicated by brown colour).(B): Immunohistochemical analysis of Bcl-2 in the hippocampus of rat co-treated with DG+ETE showing weak positive immune reaction (immunopositivity indicated by brown colour) (scale bar 25 um)

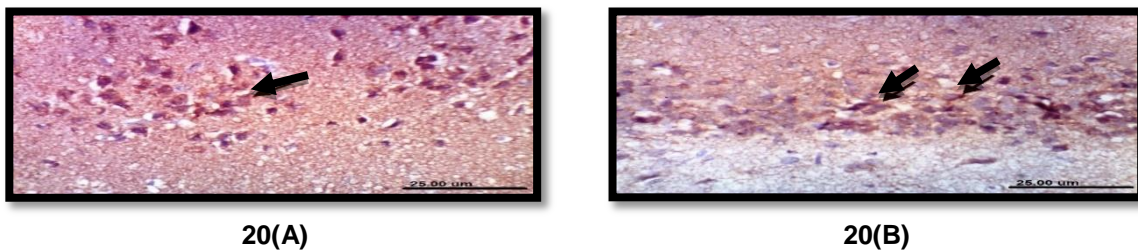**20(A)**   **20(B)**

Fig. 20. (A): Immunohistochemical analysis of Bcl-2 in the cerebral cortex of rat co-treated with DG+ESE+ETE showing intense positive immune expression (immunopositivity indicated by brown colour).(B): Immunohistochemical analysis of Bcl-2 in the cerebral cortex of rat co-treated with DG+ESE+ETE showing intense positive immune expression (immunopositivity indicated by brown colour) (scale bar 25 um)
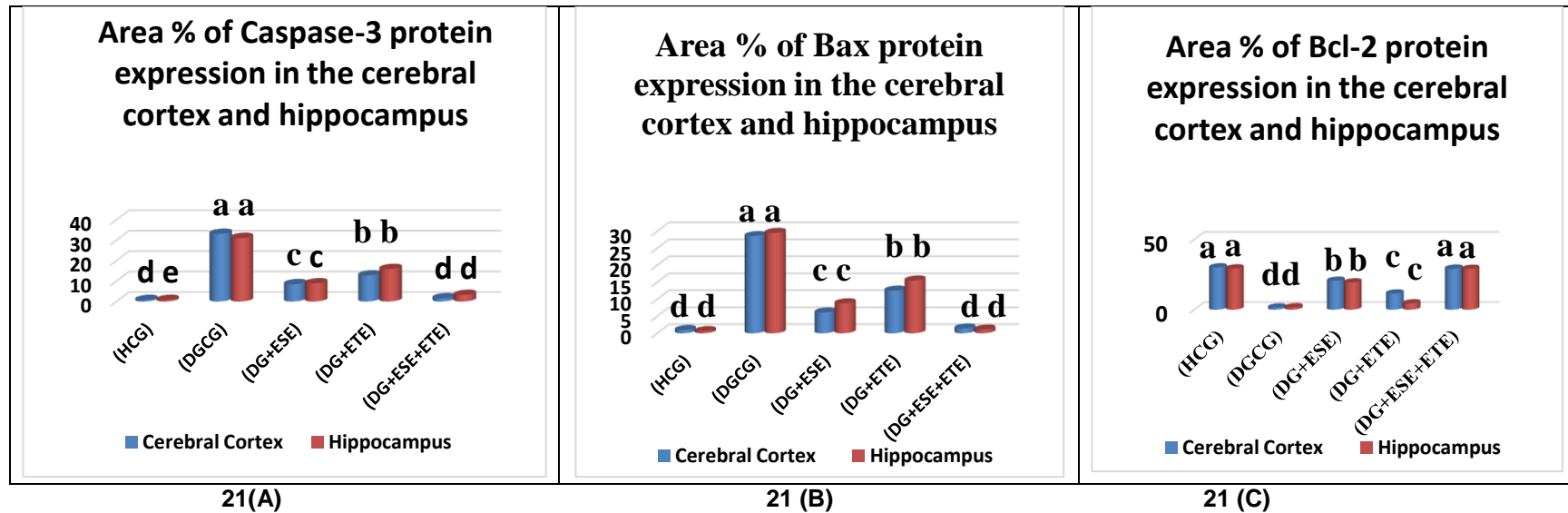
21(A)
21 (B)
21 (C)

Fig. 21. (A): Immunostaining area % of caspase-3 expression in the cerebral cortex and hippocampus. (B): Immunostaining area % of Bax expression in the cerebral cortex and hippocampus. (C)Immunostaining area % of Bcl-2 expression in the cerebral cortex and hippocampus. Data are shown as Mean ± SD; n= 5, 'a, b, c, d' statistically significant at (p≤ 0.05)

Also; Abdel-Rahman et al. [99] reported that saffron extract reduced apoptosis as revealed by a decrease in caspase-3 and Bax protein expression with a marked decrease in the apoptotic neuronal cells compared to control group [98]. Crocin is a protective agent against neurodegenerative disorders and other similar conditions by preventing apoptosis. Previous results demonstrated the anti-apoptotic effects of crocin as shown by the reduction of Bax and increase of Bcl-2 level in the brain. Crocin treatment can also attenuate caspase-3 induced cleavages, which induces Bax production and nuclear condensation resulting from some neurodegenerative processes .Thus, crocin can inhibit cell death in the apoptosis process during neuro-degeneration.

Study results go hand in hand with, Yuliana et al. [100] who documented that turmeric extract caused significant decrease in caspase-3 in neurotoxicated rats as compared to normal control group and this confirm that turmeric has anti-apoptotic properties on neurons Also, Hashish and Kamal. [101] found that administration of curcumin reduced caspase- 3, and improved Bcl- 2 expression as compared to normal control group and this due to the anti-apoptotic effect of curcumin that protect cells from apoptosis.

4. CONCLUSION

From this research results, it can be concluded that saffron and turmeric ethanolic extracts contain significant amounts of polyphenols, flavonoids and possess antioxidant activity that protected brain tissues from the degeneration caused by subcutaneous injection of D-galactose. Co-administration of saffron and turmeric ethanolic extracts mixture with D-galactose caused the most significant improvements compared to treatment with saffron or turmeric extracts alone as a result of the synergistic effects between their active constituents. It is advised to add saffron and turmeric to human foods and to prepare their ethanolic extracts to be available for human beings due to their ability to preserve brain functions and structure as well as their potential to inhibit and retard brain aging and neuro-degeneration.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It's not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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