Original Article

The Potential Efficacy of Thymoquinone and Hesperidin as Prophylaxis for Endotoxin Induced Oxidative Stress in Rats

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ABSTRACT

Introduction

This experimental study evaluates the protective effect of Thymoquinone (TQ) and Hesperidin (HSP) as antioxidants on endotoxin Lipopolysaccharide (LPS) induced oxidative stress in rats.

Materials and methods

Total 56 rats were divided equally into seven groups; each contained eight rats. Group-1 rats served as control, given normal saline orally 14 days; Group-2 and Group-3 rats were given TQ and HSP respectively for 14 days; Group-4 rats were given a single dose LPS intraperitoneally on 14th day; Group-5 and Group-6 rats were given TQ and HSP respectively for 14 days followed by single dose LPS on 14th day; Group-7 rats were given TQ and HSP separately at 10-minutes interval for 14 days followed by single dose LPS on 14th day. After 14 days, rats were sacrificed and biochemical tests on (i) rats' serum; (ii) rats' brain tissue, with (iii) histopathology and (iv) immunohistochemistry in brain tissues were assessed among all seven groups.

Results

LPS induces significant increase in serum corticosteroid, tumor necrosis factor alpha (TNF-α) and decrease in total antioxidant capacity (T-AOC) compared to control group which were reversed with TQ and HSP treated group. Similarly, in brain tissue, LPS induces significantly increased brain Malondialdahyde (MDA), ATP, ADP, Cholesterol, Phospholipid, and significantly decreased Na+K+ ATPase and Superoxide dismutase (SOD) compared to control group which was reversed in TQ and HSP treated group. The histopathology and immunohistochemistry confirm the results.

Conclusion

Both TQ and HSP complement (synergistic) the antioxidant, anti-inflammatory, and anti-apoptotic properties of each other, and have neuroprotective effects against oxidative stress induced by LPS.

Keywords

Thymoquinone (TQ) Hesperidin (HSP), Antioxidants, oxidative stress, Lipopolysaccharide (LPS)

INTRODUCTION

Oxidative stress is the disturbance in the pro-oxidant and antioxidant balance in the body, with a greater amount of pro-oxidant activity occurring compared to antioxidant activity leading to potential cellular damage in the body[1,2]. Due to the oxidative process, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are generated in cells and tissues and excess ROS/RNS causes mitochondrial dysfunction, leading to deficits in energy production, damages cellular lipids, proteins or DNA and impair their normal function. The oxidative stress is associated with inflammation implicated in the etiology of many chronic and neuro degenerative diseases, and involved with the high mortality from several

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diseases such as endotoxic shock[3]. Reactive oxygen species (ROS) comprise both free radical and non-free radical oxygen containing molecules such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), singlet oxygen ($1/2O_2$), and the hydroxyl radical ($^{\cdot}OH$)[4,5].

Lipopolysaccharide (LPS) induces and accelerates oxidative stress together with the inflammation cascade[6]. The LPS, a principal component of the outer membrane of all Gram-negative bacteria, is the endotoxin considered to be responsible for initiation of inflammation and oxidative stress and contributes to the development and pathogenesis of bacterial infections[6,7]. LPS induces the production and release of several inflammatory cytokines and creates a status of oxidative stress[8]. Thus, the host response due to these microorganisms causes sepsis including neurodegenerative disorders, diabetes, liver injury, renal dysfunctions[6,7].

It is reported that naturally occurring plant-based antioxidants such as Thymoquinone (TQ)[9] and Hesperidin (HSP)[10] have the potential in reducing endotoxin-induced oxidative stress. Thus, reduction of LPS-induced oxidative stress may be a good target for prevention and treatment of sepsis and inflammatory response.

Thymoquinone, is a compound derived from *Nigella sativa* L seeds (black cumin), a plant in the Ranunculaceae family. This monoterpene molecule is characterized as 2-methyl-5-isopropyl-1,4-benzoquinone. This compound exhibits pharmacological effects of anti-inflammatory, antimicrobial, antioxidant, antiasthmatic, antihypertensive, and anticancer[11].

Hesperidin, is a flavonoid found in citrus fruits has a number of health benefits and pharmacological properties. This compound is identified as 3,5,7-trihydroxyflavanone 7-rhamnoglucoside, and demonstrates pharmacological actions such as anti-inflammatory effect, neuro-protective action, oxidative stress reduction etc[11].

The objective of this study was to examine the ability of TQ and HSP both individually and combined as antioxidant in reduction of LPS-induced oxidative stress and inflammatory responses in rats.

MATERIALS AND METHODS

Design of the study:

This was an experimental design of study on animal, conducted in the Department of Pharmacology at Faculty of Medicine, Al-Azhar University, Egypt. It was aimed to investigate the potential efficacy of TQ and HSP as prophylaxis for endotoxin induced oxidative stress in rats.

Study sample

A total of 56 adult male albino rats, weighing 150-200g each were used as sample for this animal experimental study.

Ingredients used

The following drugs, chemicals, buffer and ready-made kits were used in this experiment.

Drugs: The drugs used were TQ, HSP, and endotoxin LPS (Sigma-Aldrich Co.Ltd, USA).

Chemicals: The following chemicals were used in this study: n-Butanol (Merck), Catalase (Sigma-Aldrich), Concentrated hydrochloric acid (Merk), Dipotassium hydrogen phosphate anhydrous (El-Nasr), Formaldehyde solution 37% (El-Nasr) Malondialdehyde (MDA) (Sigma-Aldrich), Orthophosphoric acid (1%) (El-Nasr), Potassium dihydrogen phosphate (El-Nasr), Pyrogallol (Merck), Sodium chloride (El-Nasr), Thiobarbituric acid (TBA) (Sigma-Aldrich), Tris - HCl (Merck), Tween 80 (Sigma-Aldrich).

Buffer: The buffer solution used was Phosphate buffer solution (0.1M).

Ready-made kits: ATP and ADP ELISA kit (Kamiya Biomedical Company USA), Na+/K+ ATPase assay kit (Avecon Healthcare Pvt. Ltd India), Cholesterol and Phospholipid assay kit (bioMérieux, France), Corticosterone, ELISA kit (DRG International, Inc, USA), Glucose assay kit, (bioMérieux France), TNF-α, ELISA kit (Ray Biotech, Inc., USA), total antioxidant capacity assay kit, (Cayman Chemical Company, USA).

METHODS

All 56 rats were housed in a conditioned atmosphere at 25±2°C, and fed with a standard laboratory diet and tap water. The rats were divided into 7 distinct groups, with each group consisting of 8 rats.

Group 1: Served as a control group, and rats here received normal saline orally for 14 days.

Group 2: Rats were given TQ 10 mg /kg orally by gavage tube for 14 days.

Group 3: Rats were given HSP 100 mg /kg orally for 14 days.

Group 4: Act as a stressed group, who were given a single dose of endotoxin LPS 2mg /kg intraperitoneally (IP).

Group 5: Rats were given TQ 10 mg/kg orally for 14 days followed by a single dose of LPS (IP).

Group 6: Rats were given HSP 100 mg/kg orally for 14 days followed by a single dose of LPS (IP).

Group 7: Rats here given TQ 10 mg/kg orally and HSP 100mg/kg orally 14 days followed by a single dose of LPS (IP).

Sample Collection and Storage for Analysis

After 14 days of treatment and followed by 4 hours after endotoxin administration, all 56 rats were sacrificed by cervical dislocation, and (I) serum analysis, (II) brain tissue homogenate examination and (III) histopathological and immunohistochemical examination of brain was done.

I. Serum analysis: Blood samples were collected from the retro-orbital plexus by heparinized capillary tube into centrifugation tubes and centrifuged at 3000 rpm for 10 min. Serum was taken by automatic pipette into Eppendorf tubes and kept at -80ºC till analysis.

As stressed markers, estimation of serum corticosterone, glucose, tumor necrosis factor alpha (TNF-α) and total antioxidant capacity (T-AOC) level were assessed. The serum corticosteroid level was determined using ELISA EIA-5186). The adopted method for serum glucose level followed the procedure described by Trinder (1969)[12]. The TNF-α was determined by using rat specific ELISA kit (Cat#: ELR-TNFalpha-001) and serum total antioxidant capacity was determined by following standard method by Rice-Evans and Miller (1994)[13].

II. Brain tissue homogenate analysis: Rats' brains were quickly excised and washed with saline and used for preparation of tissue homogenate (20%) in 0.9% NaCl saline solution using homogenizer (X620 CAT, Germany) and stored at -80°C until analysis.

Biochemical changes that occur due to the effect of TQ and HSP as antioxidants on endotoxin LPS induced oxidative stress in rats' brain tissue were studied through Malondialdehyde (MDA) content; Superoxide dismutase (SOD) enzyme activity; ATP; ADP; Na^+ - K^+ ATPase; Cholesterol and Phospholipid contents.

The MDA content (nmol/g tissue) was determined colorimetrically[14]. The SOD enzyme activity (U/g tissue) was determined by the difference between autoxidation of pyrogallol alone and in presence of homogenate fraction that contain SOD[15]. ATP was determined by ELISA Cat. No. KT-59182, and ADP was determined by ELISA Cat. No. KT-59190. Na+-K+ ATPase in brain homogenate was determined following standard procedure[16]. Determination of cholesterol was done according to Allain et al (1974)[17], and phospholipids were determined following a colorimetric method[18].

III. Histopathological examination and Immunohistochemical examination of brain: Brain of two animals from each group was kept in 5% formalin, which was used for histopathological and immunohistochemical examination and the examination was done following the method of Humason (1962)[19].

Immunohistochemical examination was done to investigate the expression of Caspase-3, Bax, and Bcl-2 and neuron apoptosis. Caspase-3 and Bax are pro-apoptotic proteins while Bcl-2 is an anti-apoptotic.

Rabbit anti-rat Caspase-3, Bax and Bcl-2 immunohistochemistry kits were employed to detect the expression of the three proteins. Assay procedures were performed according to the manufacturer's instructions. Following immunohistochemical staining, the brain tissue sections were placed under the microscope to observe Caspase-3-, Bax- and Bcl-2-positive neurons.

Statistical Analysis: The results were analysed using SPSS software, version 2020 (SPSS Inc., Chicago, IL, USA). Data was expressed as mean ± SD. Group comparisons were done by using One-way analysis of variance (ANOVA) followed by Tukey-Kramer post-hoc analysis. $P< 0.05$ was considered as statistically significant.

RESULTS

Biochemical tests results of serum among rats of different groups

Table 1 displayed the endotoxin LPS induced stressed rats of group-4 showed significant increase in serum corticosteroid, TNF-α and significant decrease in T-AOC in comparison with rats of group-1 (control), group-2 treated with TQ, and group-3 treated with HSP. Serum glucose concentration although statistically not significant but it was highly increased. Rats treated with TQ followed by LPS in group-5, group-6 rats treated with HSP followed by LPS and group-7 rats treated with both TQ and HPS followed by LPS showed significant

decrease in serum corticosteroid, TNF-α and significant increase in T-AOC in comparison with endotoxin LPS induced stressed rats of group-4; Glucose level also highly decreased, although it was statistically not significant.

Table 2 showed the biochemical tests results in brin tissue of rats among different groups. It revealed that endotoxin LPS induced stressed rats of group-4 have significantly increase in brain MDA, ATP, ADP, cholesterol, phospholipid, and significantly decrease in Na+K+ ATPase and SOD in comparison with rats of group-1, group-2 and group-3. The rats' brain in groups-5, 6 and 7 treated with TQ+LPS, HSP+LPS and TQ+HSP+LPS respectively have shown significant decrease of brain MDA, ATP, ADP, cholesterol and phospholipid, while it significantly increases brain Na+K+ ATPase and SOD, in comparison with endotoxin LPS induced stressed rats in group-4.

Table-1 Biochemical tests results of serum among different groups

Serum Parameters	Control Group	Experimental Groups						*p value*
	Group-1	Group-2	Group-3	Group-4	Group-5	Group-6	Group-7	
	Saline	TQ	HSP	LPS	TQ+LPS	HSP+LPS	TQ+HSP+LPS	
Corticosteroid	25.5167 ± 1.336	14.716 ±0.532	18.716 ± 0.792	87.566 ± 2.208	48.566 ± 1.506	58.983 ± 1.364	25.633 ± 1.238	0.00*
Glucose	96.666 ± 2.741	64.500 ± 1.727	72.666 ± 1.626	277.500 ± 11.459	159.500 ± 4.217	161.167 ± 3.070	78.000 ± 2.620	0.06
TNF-α	35.75 ± 1.816	19.716 ± 0.585	18.833 ± 0.623	131.417 ± 3.150	81.766 ±2.994	73.666 ± 2.689	28.283 ± 1.441	0.01*
T-AOC	116.050 ± 2.024	162.033 ± 3.117	125.350 ± 2.430	35.400 ± 1.864	70.166 ± 2.734	56.566 ± 5.587	95.666 ± 2.487	0.17

Items marked * are significant (p is < 0.05).

Table-2: Biochemical tests results of brain tissue among different groups

Brain tissue Parameters	Control Group	Experimental Groups						*p value*
	Group-1	Group-2	Group-3	Group-4	Group-5	Group-6	Group-7	
	Saline	TQ	HSP	LPS	TQ+LPS	HSP+LPS	TQ+HSP+LPS	
MDA nmol /g	8.000 ± 0.337	3.600 ± 0.171	4.475 ± 0.175	27.050 ± 1.147	15.233 ± 0.606	21.683 ± 0.733	8.583 ± 0.260	0.00*
SOD U /g	7.183 ± 0.426	11.966 ± 0.462	11.133 ± 0.390	1.883 ± 0.094	3.816 ± 0.130	4.183 ± 0.282	9.316 ± 0.256	0.01*
ATP µmol/g tissue	6.566 ± 0.301	3.700 ± 0.204	3.950 ± 0.152	19.983 ± 0.758	12.016 ± 0.340	12.033 ± 0.398	6.483 ± 0.170	0.00*
ADP µmol/g tissue	0.998 ± 0.0566	0.508 ± 0.031	0.661 ± 0.025	2.456 ± 0.241	1.515 ± 0.044	1.813 ± 0.037	1.146 ± 0.022	0.00*
Na+-K+ ATP ase umol /g tissue	2.658 ± 0.143	3.873 ± 0.069	4.063 ± 0.160	0.786 ± 0.038	1.595 ± 0.0343	1.271 ± 0.040	2.775 ± 0.106	0.00*
Cholesterol mg/g tissue	7.766 ± 0.359	3.933 ± 0.130	4.083 ± 0.142	24.283 ± 1.093	12.466 ± 0.555	12.383 ± 0.399	6.683 ± 0.219	0.00*
Phospholipid mg/g tissue	16.083 ± 0.778	11.283 ± 0.200	8.483 ± 0.454	39.350 ± 2.005	26.100 ± 0.778	19.600 ± 0.546	11.166 ± 0.275	0.01*

Items marked * are significant (p is < 0.05).

Histopathological alterations in brain tissue of rats among different groups

The histopathological examination of brain tissues in different groups are shown a range of effects from no histopathological changes to severe damages.

Figure-1a, Figure-1b and Figure-1c revealed no histopathological changes noted in brain tissues of rats in group-1, group-2 and group-3 respectively. Figure-2a, 2b, 2c, and 2d showed various histopathological changes in rats injected with a single dose of endotoxin LPS in group-4 rats. Figure-3a, 3b and 3c showed necrosis of neurons and neuronophagia in group-5 rats' brain; neuronal degeneration and neuronophagia in group-6 rats' brain; and little neuronal degeneration in group-7 rats' brain respectively.

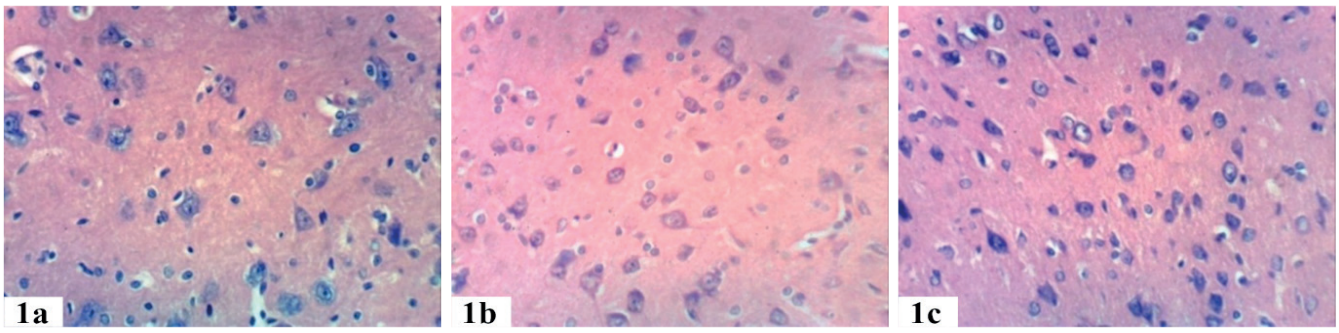

Figure-1a, 1b and 1c showed transverse sections of rats' brain tissue where no histopathological changes were observed in group-1 received normal saline 14 days, group-2 treated with TQ for 14 days and group-3 treated with HPS for 14 days respectively.

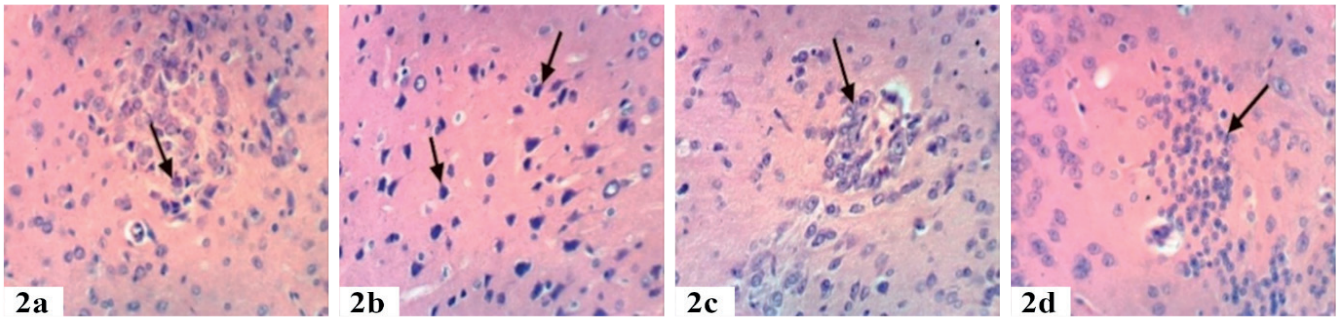

Figure-2a, 2b, 2c, and 2d showed transverse sections of rats' brain tissue where various histopathological changes were noticed in rats injected with a single dose of endotoxin LPS in group-4. The changes are focal necrosis (2a), multiple focal gliosis (2b), satellitosis (2c) and necrosis of neurons and neuronophagia (2d).

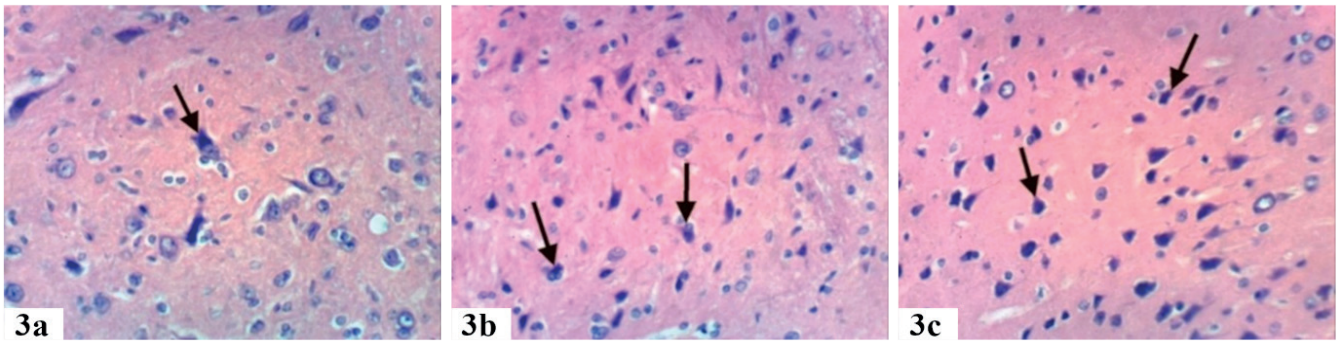

Figure-3a, 3b and 3c showed transverse sections of brain tissue of rats in group-5, group-6 and group-7 respectively. It showed necrosis of neurons and neuronophagia (3a) in group-5 rats' brain, treated 14 days with TQ followed by single dose of LPS; neuronal degeneration and neuronophagia (3b) in group-6 rats' brain, treated 14 days with HSP followed by single dose of LPS; little neuronal degeneration (3c) in group-7 rats' brain, treated 14 days with TQ + HSP followed by single dose of LPS.

Immunohistochemical alterations in brain tissue of rats among different groups

Figure 4 (4a-4i) showed the immunohistochemical expression of brain tissue of rats in group-1 (4a, 4b, 4c), group-2 (4d, 4e, 4f), and group-3 (4g, 4h, 4i) respectively, where no Ag-Ab reaction was observed. Figure 5 showed the immunohistochemical expression in brain tissue of group-4 rats with positive reactivity or Ag-Ab reaction of Caspase-3 (5a), Bax (5b), and Bcl-2 (5c) respectively. Figure 6 and 7 revealed immunohistochemical expression of brain tissue in rats of group-5 and group-6 respectively with mild positive reaction of Caspase-3 (6a, 7a), and no reactivity with Bax (6b, 7b), and Bcl-2 (6c, 7c). Figure 8 showed immunohistochemical expression in brain tissue of group 7 rats showed no reactivity or no Ag-Ab reaction of (8a) Caspase-3, (8b) Bax (8b), and (8c) Bcl-2.

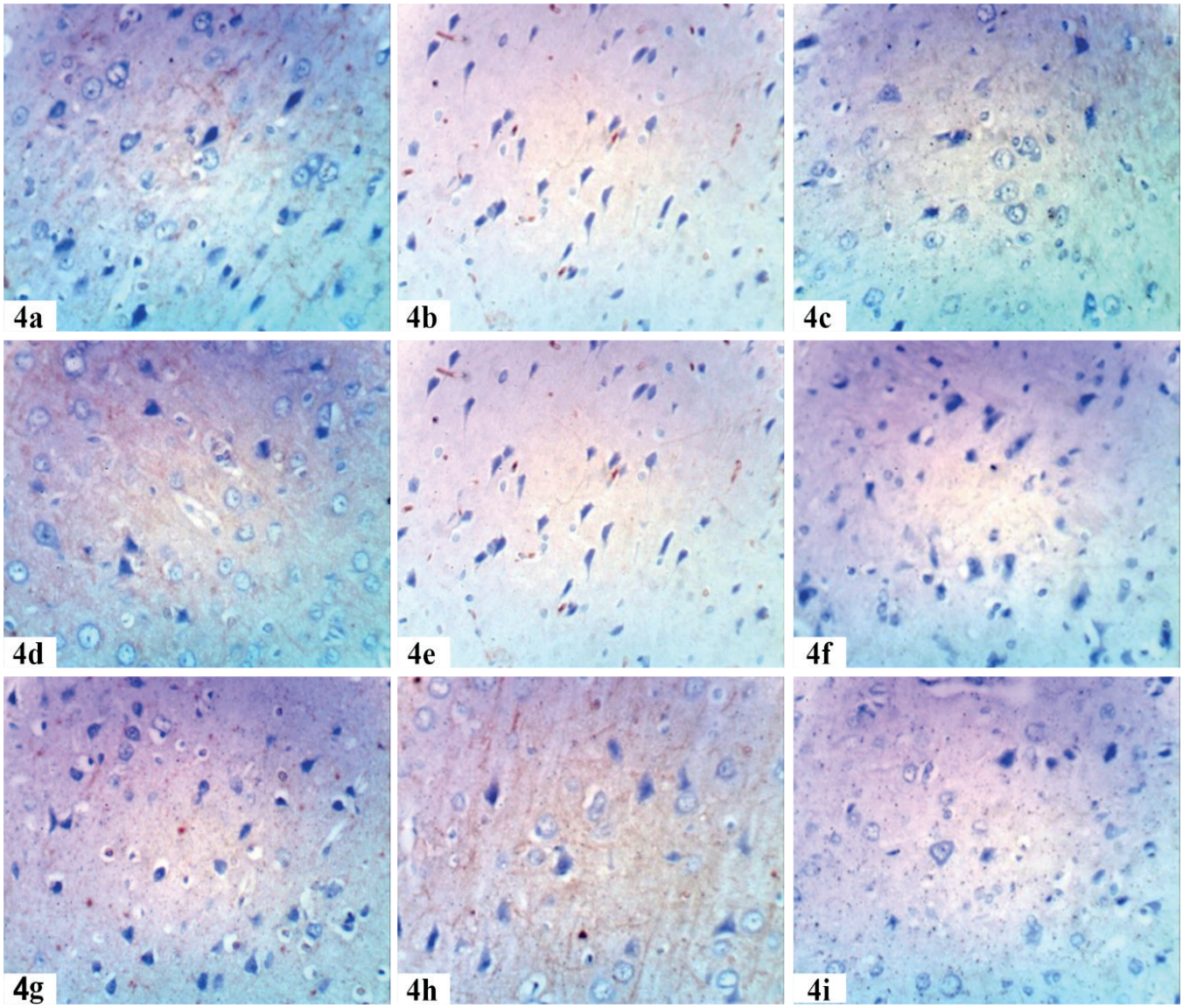

Figure-4 revealed transverse section of immunohistochemical expression in brain tissue of rats in Group-1 (4a, 4b, 4c); Group-2 (4d, 4e, 4f) and Group-3 (4g, 4h, 4i) respectively where no Ag-Ab reaction of Caspase-3, Bax nor Bcl-2 was observed.

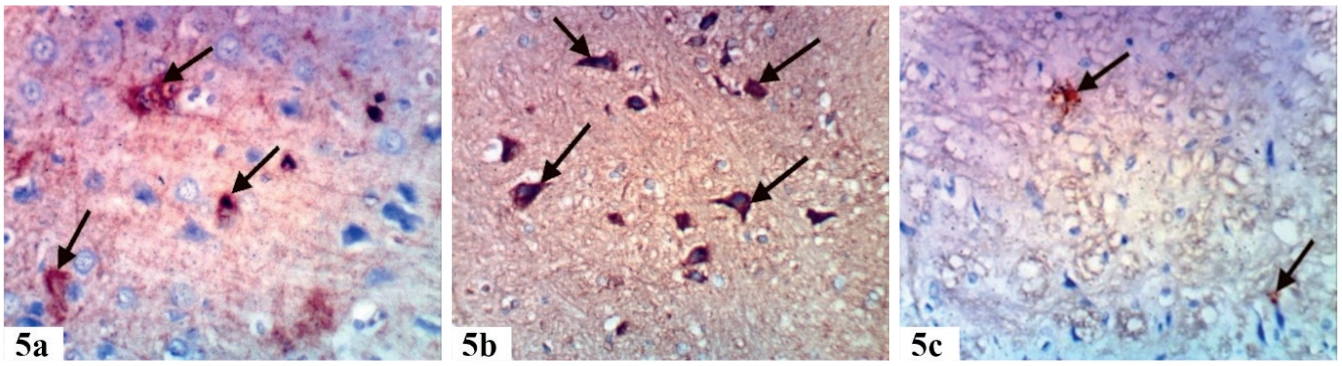

Figure-5a, 5b and 5c revealed transverse section of immunohistochemical expression in brain tissue of Group-4 rats treated with endotoxin LPS showed positive Ag-Ab reaction of Caspase-3 (5a), Bax (5b), and Bcl-2 (5c) respectively.

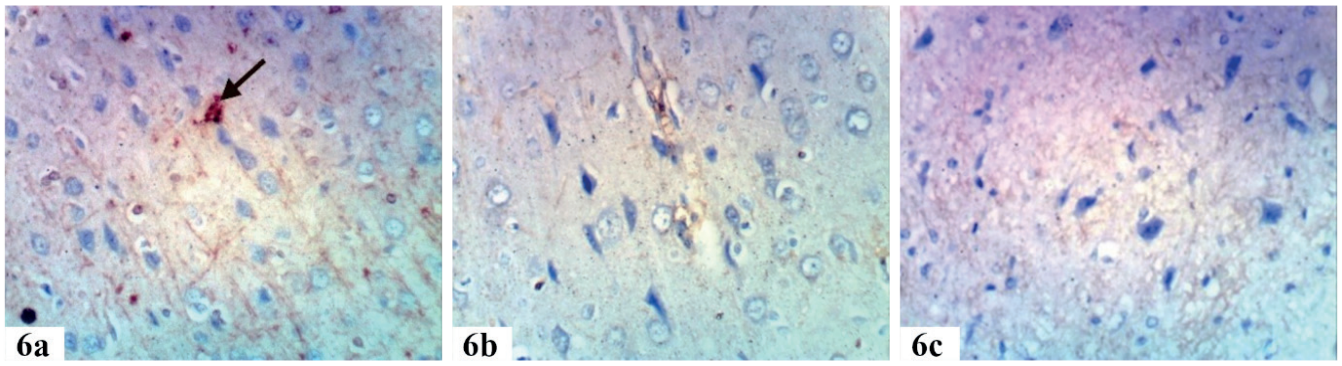

Figure-6a, 6b, 6c showed transverse section of immunohistochemical expression in brain tissue of Group-5 treated with TQ for 14 days followed by endotoxin LPS showed mild positive reactivity or mild Ag-Ab reaction of Caspase-3 (6a), while no reactivity of Bax (6b), and Bcl-2 (6c).

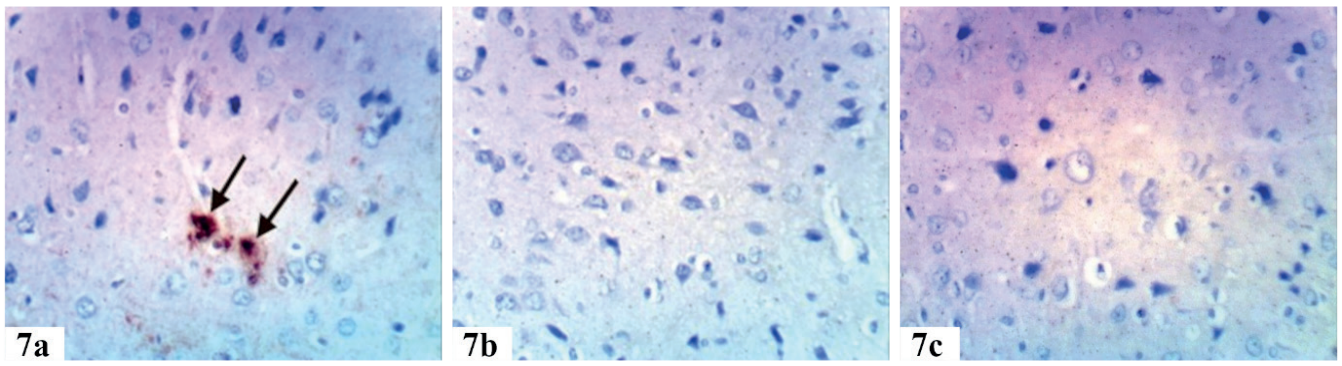

Figure-7a, 7b, 7c showed transverse section of immunohistochemical expression in brain tissue of Grope-6 rats treated with HSP for 14 days followed by endotoxin LPS showed mild positive reactivity or mild Ag-Ab reaction of Caspase-3 (7a), while no reactivity or no Ag-Ab reaction of Bax (7b), and Bcl-2 (7c).

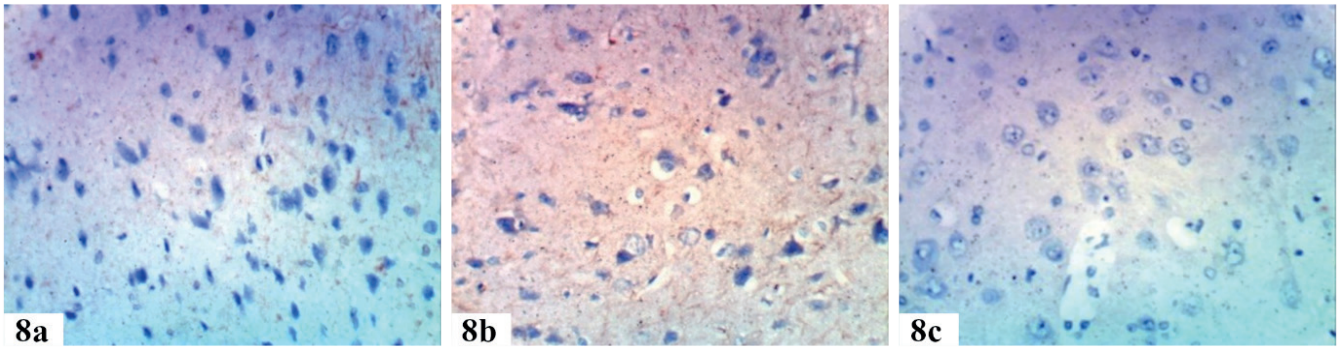

Figure-8a, 8b, 8c revealed the transverse section of immunohistochemical expression in brain tissue of Group-7 rats treated with TQ and HPS prior to induction with endotoxin LPS stress showed no reactivity or no Ag-Ab reaction of Caspase-3 (8a), Bax (8b) and Bcl-2 (8c).

DISCUSSION

Effect of endotoxin LPS on serum and brain tissue in rats

Our study results revealed that rats subjected to endotoxin LPS injection showed a significant increase in stress indices in serum marked by increase in corticosterone, TNF-α, and significant decrease in T-AOC compared to normal control. Glucose level although increased, does not showed significantly high. The effect of endotoxin LPS on the brain alters oxidative status of the brain and increases free radical generation, reflected by an increase in brain MDA, ATP, ADP, phospholipid and cholesterol and reduction in SOD, Na^+-K^+ ATPase activity.

Under physiological conditions, a homeostatic balance exists between the formation of reactive oxygen species (ROS) and their removal by endogenous antioxidants[20]. The exposure to stress deranged this balance and causes activation of the hypothalamic-pituitary-adrenal (HPA) axis causing increased secretion of pituitary endorphins, adrenocorticotrophic hormone (ACTH) and adrenal corticosteroids[21,22]. There is increased availability of metabolic substrates for energy generation, most notably glucose (Kheir-Eldin et al 2001)[8]. Moreover, stress induced surges in corticosterone, glucagon, and catecholamines promote hyperglycemia by enhancing liver glucose production via gluconeogenesis and glycogenolysis and reducing glucose uptake by peripheral tissues, a process also known as insulin resistance[23]. As endotoxins are potent activator of the innate immune system and triggers a massive release of pro-inflammatory cytokines, causing high TNF-α level that contribute to the neuronal damage and sustain neurodegenerative diseases and behavior impairment[10,24]. Endotoxin induced oxidative stress causes a significant decrease in serum T-AOC compared to normal control as reported in previous study[25].

The effects of LPS on the brain showed a significant increase in brain oxidative status observed as elevation of MDA and significant decrease in SOD compared to normal control. This increase in MDA and decreased SOD indicates that LPS induces cellular damage by triggering oxidative stress, leading to an imbalance of antioxidants and damage to the cell membranes[10,26]. Endotoxins causing oxidative stress and neuroinflammation increases extracellular ATP, ADP release from damaged brain tissue[3]. The brain Na^+-K^+ ATPase activity of stressed rats decreases during endotoxemia is through NADPH oxidase activation[27]. The cholesterol and phospholipid content in stressed brain are increased as a result of LPS induced infection and neuro-inflammation through alterations in lipid metabolism[28].

Effect of TQ and HSP alone or in combination on serum and brain tissue in endotoxin-stressed rats

Concerning the effect of TQ and HSP alone or in combination on serum corticosterone, glucose and TNF-α level in endotoxin-stressed rats, our result showed that these antioxidants caused an apparent decrease in serum corticosterone level, glucose level and TNF-α level compared with stressed group. Study by Cai et al (2013)[29] showed agreement with our results who found decrease in the corticosterone level by administration of HSP for three weeks in stressed rats. Regarding the effect on glucose, Abdel-Wahab (2013)[30] showed TQ supplementation counteracts the

sodium fluoride-induced hepatotoxicity and reduces hyperglycemia without improving the insulin level. These results indicated that TQ induced hypoglycemia could be facilitated through enhanced peripheral glucose oxidation and/or reduction of gluconeogenesis[30]. Similar results were shown by Farah et al (2005)[31] exhibiting significant reduction in liver glucose release in diabetic hamsters treated with TQ, partially mediated through a decrease in hepatic gluconeogenesis. The decrease in TNF-α by the action of antioxidants TQ and HSP in endotoxin–stressed animal compared with stressed group are in harmony with a previous study where TNF-α levels were decreased more in the combination group of HSP and TQ[11]. Our study results of endotoxin induced significant decrease in serum T-AOC compared to normal control was ameliorated after administration of TQ and HSP which is in harmony with the results obtained by Abdel-Wahab (2014)[30]. He found that supplementation with TQ significantly normalized the suppressed antioxidants in the Bisphenol A-treated male rats and to a large extent restored their values toward that of the control group[30].

Treatment of TQ, HSP reversed the MDA level towards normal. One study noted the combination therapy showed the most significant improvement on MDA, with HSP demonstrating better efficacy compared to TQ when administered individually (Farooq et al 2014)[11]. The TQ and HSP treatment reduces lipid peroxidation in the rat brain induced by LPS and manifests as decreased MDA content. These results are attributed to the potential antioxidant effect of TQ and HSP. The decreased brain activity of SOD by endotoxin single dose ameliorated by anti-oxidant TQ and HSP treatment is compatible with previous studies where use of anti-oxidants TQ and HSP was found to significantly elevating the SOD level and improves tissue damage[11,32,33]. Superoxide dismutase has therapeutic potential for antioxidant therapy as this antioxidant enzymes degrade superoxide (O_2^-) into oxygen and hydrogen peroxide. Subsequently, H_2O_2 is reduced to water by the catalase enzyme, glutathione peroxidase, and/or thioredoxin-dependent peroxiredoxin enzymes[34,35].

The significant increase in brain ATP and ADP content compared to normal control in endotoxin-stressed rats was ameliorated by administration of TQ and HSP. The effect of TQ and HSP as potent antioxidant and anti-inflammatory alleviates oxidative stress and neuroinflammation, and help regulate the aberrant energy metabolism caused by endotoxin by protecting mitochondrial function and restoring cellular homeostasis[36,37]. The decreased activity of Na^+-K^+ ATPase activity was significantly increased with pre administration with TQ and/or HSP in endotoxin stressed rats. The strong protective effects of TQ in traumatic brain injury is the reduction in oxidative stress in the brain[38].

With respect to the pre administration effect of TQ and HSP alone or in combination on brain cholesterol and phospholipid contents in endotoxin–stressed rats, a significant decrease was observed in comparison to the corresponding stressed control. The reduction in cholesterol and phospholipids suggests that TQ and HSP protect brain cells from damage and dysfunction caused by the endotoxin-induced stress[39].

Histopathological and immunohistochemistry examination

Histopathological study, demonstrated that endotoxin administration produces focal necrosis, multiple focal gliosis, satellitosis, necrosis of neurons and neuronophagia whereas pre-treatment of TQ and HSP alone or in combination ameliorated oxidative stress injuries in brain tissue.

Apoptosis of endothelial cells is the characteristic of sepsis[40]. In our study, immunohistochemistry examination showed endotoxin administration produces positive reactivity of Caspase-3, a pro-apoptotic protein revealing LPS induces apoptosis mediated by the activation of caspase-3, a key apoptotic effector. In our study, both TQ and HSP prior to LPS showed no reactivity (no Ag-Ab reaction) of Caspase-3, Bax nor Bcl-2 showed improved cell viability. It is in harmony with Duarte et al (2014)[41]; who reported that apigenin, an anti-inflammatory flavonoid, protects endothelial cells from damage caused by the inflammatory agent LPS by reducing cell death (apoptosis) and improving mitochondrial function, specifically by lowering the activation of the cell-death enzyme caspase-3, thereby preserving cell viability.

CONCLUSION

The results obtained in our study in addition to histopathological and immunohistochemistry examination revealed that, TQ and HSP complements the antioxidant, anti-inflammatory and anti-apoptotic properties. The endotoxin induced biochemical

changes in brain (MDA, ATP, ADP, cholesterol, phospholipid and SOD, Na+ - K+ ATPase) and in serum stress markers (corticosterone, glucose, TNF-α, and anti AOC) were restored toward normal. The combined approach of TQ and HSP provides enhanced neuroprotective effects against LPS-induced oxidative stress and neuroinflammation in rat brains compared to either agent alone. Both TQ and HSP are natural compounds with antioxidant and anti-inflammatory properties, with their synergistic action offering a more significant protective outcome by mitigating oxidative damage and inflammatory responses in the brain. TQ and HSP are nearly equal in their activities with all the measured parameters except with serum corticosterone, and brain MDA, where TQ was more potent than HSP, while with brain phospholipid, HSP was more potent.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Authors' Contribution:

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Acquisition, analysis, and interpretation of data: Rabia, Abdelhalim, Gaber, Salam

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REFERENCES

1. Poljsak B, Šuput D, Milisav I. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. *Oxid Med Cell Longev*. 2013; **2013**:956792. doi:10.1155/2013/956792.
2. Neki NS. Oxidative Stress and Aging. *Bangladesh J Med Sci.* 2015;**14**(3):221-227. doi:10.3329/bjms.v14i3.23468.
3. Olufunmilayo EO, Gerke-Duncan MB, Holsinger RMD. Oxidative Stress and Antioxidants in Neurodegenerative Disorders. *Antioxidants.* 2023; **12**: 517. doi:10.3390/ antiox12020517
4. Faraguna S, Milinković Tur S, Sobočanec S, Pinterić M, Belić M. Assessment of Oxidative Stress and Associated Biomarkers in Wild Avian Species. *Animals (Basel)*. 2025; **15**(9):1203. doi: 10.3390/ani15091203.
5. Yusof N, Yasin NM, Yousuf R, Wahab AA, Aziz SA. Comparison of neutrophil respiratory oxidative burst activity between flow cytometry using dihydrorhodamine (DHR) 123 and conventional nitroblue tetrazolium test (NBT). *Bangladesh J Med Sci*. 2022; **21**(3):626-33. doi: 10.3329/bjms.v21i3.59577
6. Li X, Yao L, Cho YC, Lee DY, Cho N, Yoo G, et al,. Alleviation of LPS-induced oxidative stress and inflammation by lesbicoumestan (7) via the increase of Nrf2 expression in mouse Kupffer cells. *Toxicology and Applied Pharmacology* 2025; **501**: 117405. doi:10.1016/j.taap.2025.117405.
7. Swain P, Nayak SK, Nanda PK, Dash S. Biological effects of bacterial lipopolysaccharide (endotoxin) in fish: A review. *Fish Shellfish Immunol.* 2008; **25**(3): 191-201. doi: 10.1016/j.fsi.2008.04.009.
8. Kheir-Eldin AA, Motawi TK, Gad MZ, Abd-El Gawad HM. Protective effect of vitamin E, beta-carotene and N-acetylcysteine from the brain oxidative stress induced in rats by lipopolysaccharide. *Int J Biochem Cell Biol*. 2001; **33**(5):475-82. doi: 10.1016/s1357-2725(01)00032-2.
9. Jakaria M, Cho DY, Ezazul Haque M, Karthivashan G, Kim IS, Ganesan P, Choi DK. Neuropharmacological potential and delivery prospects of thymoquinone for neurological disorders. *Oxid Med Cell Longev*. 2018; **2018**(1):1209801. doi: 10.1155/2018/1209801.
10. Kaur G, Tirkey N, Chopra K. Beneficial effect of hesperidin on lipopolysaccharide-induced hepatotoxicity. *Toxicology*. 2006; **226**(2-3):152-60. doi: 10.1016/j.tox.2006.06.018.
11. Farooq J, Sultana R, James JP, Fathima C Z, Almutairy AF, Hussain ASM. Efficacy of Thymoquinone and Hesperidin in Attenuating Cardiotoxicity from 5-Fluorouracil: Insights from In Vivo and In Silico Studies. *Toxics*. 2024;**12**(9):688. doi: 10.3390/toxics12090688.
12. Trinder P. Determination of glucose in blood using glucose oxidase with an alternative oxygen receptor. *Ann. Clin. Biochem*. 1969: **6**:24–27.
13. Rice-Evans C, Miller NJ. Total antioxidant status in plasma and body fluids. *Methods Enzymol*. 1994; **234**:279-93. doi: 10.1016/0076-6879(94)34095-1.
14. Uchiyama M. and Mihara M. Determination of malonaldieldhyde precursor in tissues by thiobarbituric acid test. *Anal.Biochem*. 1978: **86**(1): 271-278.
15. Marklund SL. Pyrogallol autooxidation. In: Greenwald, RA(Ed), *Handbook of Methods for Oxygen Radical Research*, 1st ed; Boca Raton, FL, USA: CRC Press, 1985; pp. 243–247.
16. Sarkar PK. A quick assay for Na+-K+-AtPase specific activity. *Z Naturforsch C J Biosci*. 2002; **57**(5-6):562-564. doi: 10.1515/znc-2002-5-628.
17. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic

determination of total serum cholesterol. *Clin Chem*. 1974; **20**(4):470-475.

18. Raheja RK, Kaur C, Singh A, Bhatia IS. New colorimetric method for the quantitative estimation of phospholipids without acid digestion. *J Lipid Res*. 1973;**14**(6):695-7. Doi: https://doi.org/10.1016/S0022-2275(20)36853-X.
19. Humason GL. *Animal tissue techniques*, III edition, 1962, San Francisco, Freeman WH. doi:10.5962/bhl.title.5890
20. Victor VM, Rocha M, De la Fuente M. Immune cells: free radicals and antioxidants in sepsis. *Int Immunopharmacol*. 2004; **4**(3):327-347. doi: 10.1016/j.intimp.2004.01.020.
21. Gibb J, Hayley S, Gandhi R, Poulter MO, Anisman H. Synergistic and additive actions of a psychosocial stressor and endotoxin challenge: Circulating and brain cytokines, plasma corticosterone and behavioral changes in mice. *Brain Behav Immun*. 2008; **22**(4):573-89. doi: 10.1016/j.bbi.2007.12.001.
22. Aziz Ibrahim IA, Kamisah Y, Nafeeza MI, Nur Azlina MF. The effects of palm vitamin E on stress hormone levels and gastric lesions in stress-induced rats. *Arch Med Sci*. 2012; **8**(1):22-9. doi: 10.5114/aoms.2012.27276.
23. Vedantam D, Poman DS, Motwani L, Asif N, Patel A, Anne KK. Stress-Induced Hyperglycemia: Consequences and Management. *Cureus*. 2022; **14**(7): e26714. doi: 10.7759/cureus.26714.
24. Bossù P, Cutuli D, Palladino I, Caporali P, Angelucci F, Laricchiuta D, et al. A single intraperitoneal injection of endotoxin in rats induces long-lasting modifications in behavior and brain protein levels of TNF-α and IL-18. *J Neuroinflammation*. 2012; **9**:101. doi: 10.1186/1742-2094-9-101.
25. Sultana DR, Shahin AD, Md Jawadul H. Measurement of oxidative stress and total antioxidant capacity in hyperthyroid patients following treatment with carbimazole and antioxidant. *Heliyon*. 2021; **8**(1): e08651. doi:10.1016/j.heliyon.2021.e08651.
26. Swarnkar S, Tyagi E, Agrawal R, Singh MP, Nath C. A comparative study on oxidative stress induced by LPS and rotenone in homogenates of rat brain regions. *Environ Toxicol Pharmacol*. 2009; **27**(2):219-24. doi: 10.1016/j.etap.2008.10.003.
27. Zhang T, Lu X, Li J, Chidiac P, Sims SM, Feng O. Inhibition of Na/K-ATPase promotes myocardial tumor necrosis factor-alpha protein expression and cardiac dysfunction via calcium/mTOR signaling in endotoxemia. *Basic Res Cardiol*. 2012; **107**:254. doi:10.1007/s00395-012-0254-8.
28. Feingold KR, Grunfeld C. Effect of Inflammation and Infection on Lipids and Lipoproteins. (Updated 2025 Jun 18). In: Feingold KR, Ahmed SF, Anawalt B, et al., editors. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000. Available from: https://www.ncbi.nlm.nih.gov/books/NBK326741/
29. Cai L, Li R, Wu QQ, Wu TN. Effect of hesperidin on behavior and HPA axis of rat model of chronic stress-induced depression. *Zhongguo Zhong Yao Za Zhi*. 2013; **38**(2):229-233.
30. Abdel-Wahab WM. Protective effect of thymoquinone on sodium fluoride-induced hepato- toxicity and oxidative stress in rats. *J. Basic. Appl.Zool*. 2013; **66**: 263-270.
31. Fararh KM, Shimizu Y, Shiina T, Nikami H, Ghanem MM, Takewaki T. Thymoquinone reduces hepatic glucose production in diabetic hamsters. *Res Vet Sci*. 2005; **79**(3):219-223. doi: 10.1016/j.rvsc.2005.01.001.
32. Alam MF, Khan G, Safhi MM, Alshahrani S, Siddiqui R, Sivagurunathan Moni S, Anwer T. Thymoquinone Ameliorates Doxorubicin-Induced Cardiotoxicity in Swiss Albino Mice by Modulating Oxidative Damage and Cellular Inflammation. *Cardiol Res Pract*. 2018; **2018**:1483041. doi: 10.1155/2018/1483041.
33. Abdel-Raheem IT, Abdel-Ghany AA. Hesperidin alleviates doxorubicin-induced cardiotoxicity in rats. *J. Egypt Natl. Canc. Inst*. 2009; **21**(2):175–184.
34. Rosa AC, Corsi D, Cavi N, Bruni N, Dosio F. Superoxide Dismutase Administration: A Review of Proposed Human Uses. *Molecules*. 2021; **26**(7):1844. doi: 10.3390/molecules26071844.
35. Zakaryan HH, Sarukhanyan FP, Hunanyan OV, Barkhudaryan NH. Hemorphins exhibit protective effect on superoxide dismutase activity during endotoxin-induced stress in rats. *Med. Sci. Arm*. 2013; **53** (1): 20-27. doi: oai:arar.sci.am:93250.
36. Farkhondeh T, Samarghandian S, Shahri AMP, Samini F. The Neuroprotective Effects of Thymoquinone: A Review. *Dose Response*. 2018; **16**(2):1559325818761455. doi: 10.1177/1559325818761455.
37. Li X, Huang W, Tan R, Xu C, Chen X, Li S, et al. The benefits of hesperidin in central nervous system disorders, based on the neuroprotective effect. *Biomedicine & Pharmacotherapy* 2023; **159**:114222. https://doi.org/10.1016/j.biopha.2023.114222
38. Isaev NK, Genrikhs EE, Stelmashook EV. Antioxidant Thymoquinone and its Potential in the Treatment of Neurological Diseases. *Antioxidants (Basel)*. 2023, **12**(2), 433; doi:10.3390/antiox12020433
39. Al-Oanzi ZH, Alenazy FO, Alhassan HH, El-Aassar MR, Alzarea AI, Alzarea SI, et al. Effects of Thymoquinone Alone or in Combination with Losartan on the Cardiotoxicity Caused by Oxidative Stress and Inflammation in Hypercholesterolemia. *J Cardiovasc Dev Dis*. 2022; **9**(12):428. doi: 10.3390/jcdd9120428.
40. Wang R, Han Q, Fan J, Xu Z, Liu W, Liu D, et al. Sepsis-Induced Endothelial Barrier Dysfunction: Mechanisms, Pathology, and Therapeutic Advances. *Research*. 2025; **8**:0997. doi:10.34133/research.0997.
41. Duarte S, Arango D, Parihar A, Hamel P, Yasmeen R, Doseff AI. Apigenin protects endothelial cells from lipopolysaccharide (LPS)-induced inflammation by decreasing caspase-3 activation and modulating mitochondrial function. *Int J Mol Sci*. 2013;**14**(9):17664-17679. doi: 10.3390/ijms140917664.