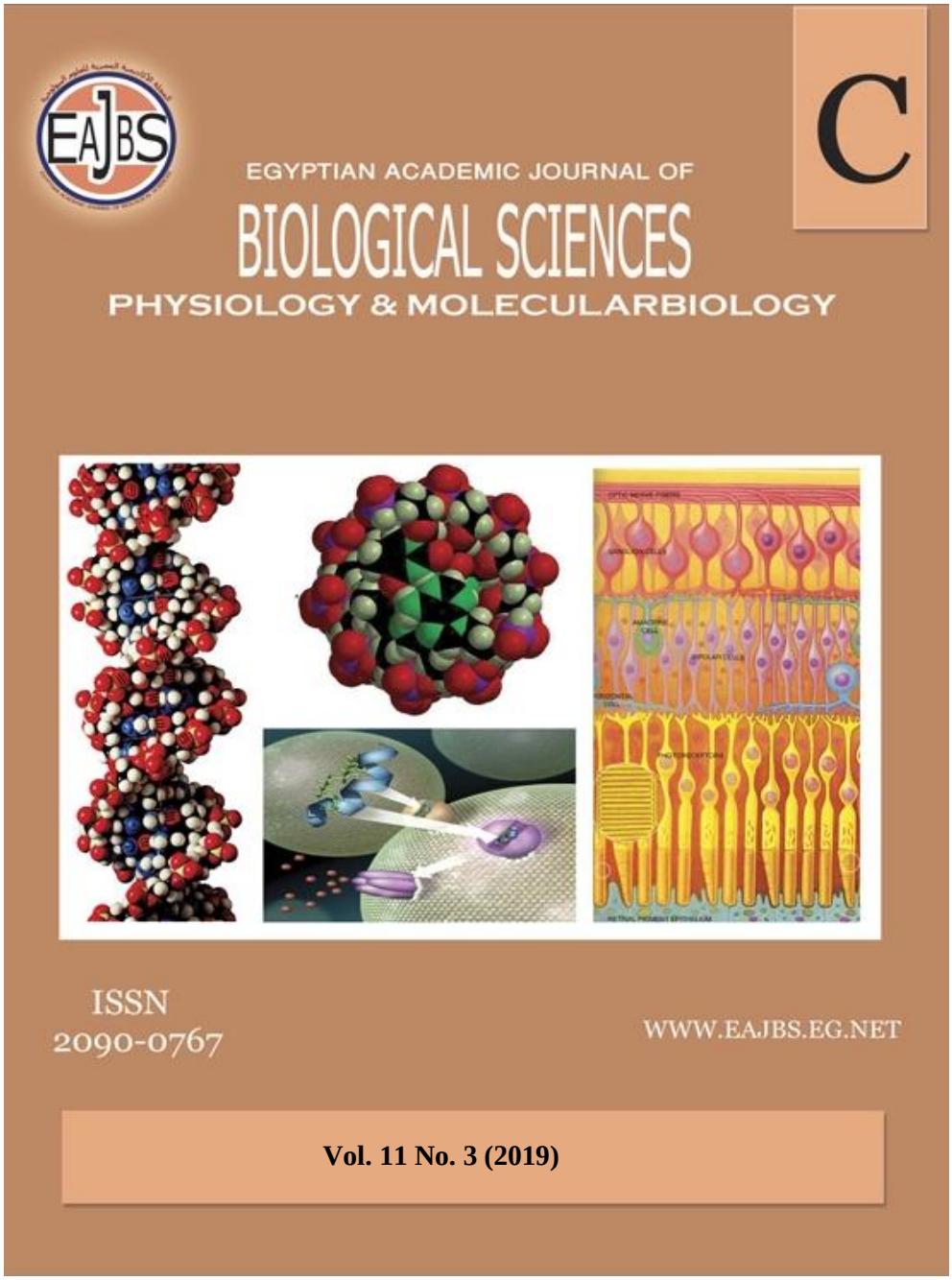

Egyptian Academic Journal of Biological Sciences is the official English language journal of the Egyptian Society for Biological Sciences, Department of Entomology, Faculty of Sciences Ain Shams University.

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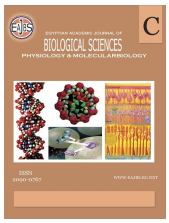
http://eajbsc.journals.ekb.eg/

Egypt. Acad. J. Biolog. Sci., 11(3): 1- 19 (2019)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology
ISSN 2090-0767
http://eajbsc.journals.ekb.eg



Propolis Extract Attenuates Sepsis-Induced Hepatotoxicity and Neurotoxicity in Male Rats

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ARTICLE INFO
Article History
Received:18/6/2019
Accepted 17/7/2019

***Keywords*:**
Sepsis; Propolis; Oxidative stress; Inflammation, Monoamines

ABSTRACT

Inflammation is an important biological event in host defenses against bacterial or viral infections. However, excessive inflammatory responses by aberrantly activated macrophages, producing excess amounts of inflammatory mediators, which disrupt immune homeostasis and result in immunopathological conditions such as sepsis. Sepsis was recognized as a systemic inflammatory response syndrome after serious infections, most commonly with bacteria which have a high mortality rate. Propolis (Prop) could be used as adjuvant therapy in the management of sepsis. The present study aimed to investigate the protective effect of Prop against sepsis-induced in rats. Evaluation of complete blood count, activity of liver function enzymes in serum; alanine and aspartate aminotransferases were recorded. Oxidant/antioxidant markers in liver tissue namely; malonaldehyde, nitrites/nitrates, glutathione disulfide, glutathione levels, catalase and superoxide dismutase activities. Also, tumor necrosis factor-alpha and prostaglandin E-2 levels in both serum and liver tissue were estimated. Furthermore, interleukin-8, cyclooxygenase-2 and inducible nitric oxide synthase genes expression were determined. Histopathological examination of liver tissue was investigated. Brain neurotransmitters; Dopamine, Norepinephrine and 5-hydroxytryptamine were also determined against sepsis-induced inflammation. The present study indicated that Prop could be an efficient protector that resets sepsis-induced severe oxidative stress, inflammation and improve the immune response of the liver as well as septic neurotoxic problems.

INTRODUCTION

Sepsis is a major global health problem, representing a challenge for physicians all over the world. New guidelines for the management of sepsis and septic shock were developed in 2016, providing an update on this area. In Sepsis three new definitions for sepsis and septic shock were published (Cabrita *et al.*, 2018). Liver injury in sepsis occurs due to pathogens, toxins or inflammatory mediators. The injury progresses from active hepatocellular dysfunction into liver damage, then into liver failure (Dkhil *et al.*, 2018a). Pathogenesis of acute liver injury were known to involve a complex interplay of oxidative stress, apoptosis, inflammation and necrosis (Shi *et al.*, 2017). Sepsis causes various complications as cardiac dysfunction, liver disorder, kidney and brain injury as well. Septic patients with those complications suffer from brain dysfunction, known as septic encephalopathy, were reported earlier and more frequent than those in other systems (Gofton and Young, 2012). Therefore, sepsis was

considered a severe disease, as it was associated with a high mortality rate (Fang *et al.,* 2018). Sepsis was reported to trigger oxidative stress, inflammation, and cell death in the brain with clinical manifestation ranges from mild delirium to deep coma (Gofton and Young, 2012).

Natural products provide a supply for treating different types of diseases such as cancer, inflammation and liver diseases. More than half of all pharmaceutical products were discovered from natural compounds or their derivatives (Farzaei *et al.,* 2018). Prop is produced by honey bees as a result of mixing wax and botanical material, such as buds, saps and plant resins. Prop was used in traditional medicine since ancient times. It's antiviral, antioxidant, anti-inflammatory, anti-ulcer, immune-stimulating and antitumor properties. Moreover, it is now used as a natural food additive, as a functional food ingredients and as a constituent of natural cosmetics (Anđelković *et al.*, 2017). Till now, therapy for liver and brain injuries due to sepsis is lacking, as the mechanism of sepsis is complex and late therapies fail to cure the disease. The role of Prop in sepsis-induced brain injury has not been extensively investigated. Therefore, the present study aimed to explore the protective effect of Prop on sepsis-induced liver and brain injuries and whether Prop regulates COX-2/PGE2 and IL-8 mRNA expression in the liver of sepsis-induced rats *via* the COX pathway. Also, if the inflammatory changes in the liver after sepsis induction lead to the progression of the disease were ameliorated by Prop treatment and participate in protecting the cerebral cortex (CC) against sepsis-induced injury.

MATERIALS AND METHODS

Experimental Animals:

Forty healthy adult male albino rats, each weighing 150-200 g were used in the present study. Animals were brought from the laboratory animal breeding of national organization of drug control and research (NODCAR), Giza, Egypt. Rats were kept under strict hygienic conditions for acclimatization under properly controlled environmental conditions in the animal house at ambient temperature (22-25 ºC). They were kept in plastic cages with stainless steel wire lids of adequate size, allowing free spontaneous motility and were kept through the period of the experiment. Rats were fed with a standard diet with composition authorized by the Association of Official Analytical Chemist (AOAC), which consists of about 78.5% carbohydrates (including about 50% crude cellulose fibers), 15.2% protein, 3.2% lipids, 2.1% salt mixture and 1% multi vitamins. Rats were allowed to free access to food and water *ad libitum*.

Induction of Sepsis:

Sepsis was induced in rats by using cecal slurry method, prepared according to Sharma *et al.* (1997) by mixing cecal contents obtained from donor rats with 5% dextrose in water (D5W) to yield 200 mg cecal material/5 ml. The slurry was prepared fresh and used within 2 hours in which 200 mg cecal material/kg was intraperitoneally (i.p.) injected.

Propolis:

30g of Prop were ground, dissolved in 100 ml 95% ethyl alcohol for one week at room temperature and protected from light, then the ethanolic solution was filtered to eliminate wax and solid particles (Bazo *et al.,* 2002). Evaporation of ethanol took place to obtain Prop extract which was dissolved in 0.5% Carboxyl Methyl Cellulose (CMC) just before administration as 250 mg/kg/day (El Menyiy *et al.,* 2016).

Experimental Design and Animal Grouping:

Experimental animals used in this study (40 adult male albino rats) were randomly divided into four equal groups; each group consists of 10 animals as the following:

I- Control Group (CON, n= 10):

Animals of this group were received an oral administration of 0.5% CMC every day for 14 days followed by 5% dextrose (D5W) on the 15th day.

II- Sepsis Group (SEP, n= 10):

Animals were received a daily oral administration of 0.5% CMC for 14 days. Then sepsis was induced experimentally on the 15th day by using the cecal slurry method.

III- Propolis Group (PROP, n= 10):

Animals of this group were received oral administration of Prop in a dose of 250 mg⁄ kg⁄ day, daily, for 14 days. Rats were received 5% dextrose (D5W) on the 15th day.

IV- Propolis & Sepsis Group (PROP & SEP, n= 10):

Animals of this group were received a daily oral administration of Prop (250 mg⁄ kg⁄ day) for 14 days and then sepsis was induced experimentally on the 15th day by using the cecal slurry method.

Animals of all groups were decapitated on the 16th day post-treatment. Blood was collected in tubes containing ethylene diamine tetra acetic acid (EDTA), as an anticoagulant, for further biochemical investigations. Liver and cerebral cortex (CC) of each animal were quickly excised, plotted on a frozen ice plate and were kept at -80ºC for further investigations. Other liver tissue samples were preserved directly in formalin for histolopathological investigations.

Tissues Sampling Preparation:

Liver tissue samples were homogenized in phosphate buffer (pH: 7.4) then centrifuged at 3500 rpm for 5 min. Tissue supernatants were obtained and processed for the estimation of oxidative stress markers, TNF-α and PGE2 levels. Another liver tissue sample was taken from each rat and frozen at -80ºC for determination of mRNA expression of IL-8, COX-2 and iNOS using real-time PCR. The CC were taken and homogenized in iced 10% KOH with glass homogenizer, then centrifuged at 3500 rpm for 5 min. Tissue supernatants were obtained and processed for estimation of the neurotransmitters (NE, DA and 5-HT) using HPLC.

Estimation of Complete Blood Count (CBC):

The CBC was performed on Sysmex KX-21N automated hematology analyzer with a white blood cell (WBC) differential count, a peripheral smear was, also, prepared to be examined microscopically**.**

-Biochemical Analysis

Determination of Liver Function:

AST and ALT activities were estimated using a kinetic method (Bergmeyer *et al.*, 1986) according to the International Federation of Clinical Chemistry (IFCC). Kits were used from Spectrum, with Catalog No. 261005.

Estimation of Oxidant/Antioxidant Markers:

Determination of Malonaldehyde (MDA), glutathione (GSH) and glutathione disulphide (GSSG) contents and the nitrite (NO^{2-}) and nitrate (NO^{3-}) levels were performed using HPLC. Liver samples were analyzed on an Agilent HP 1100 series HPLC apparatus (USA). The analytical column was anion exchange PRP-X100 Hamilton, 150 x 4.1 mm, 10 µm. The mobile phase was a mixture of 0.1 M NaCl-methanol, at a volume ration 45:55 respectively; the flow rate was 2 ml/min, wavelength adjusted to 230 nm.

Determination of Superoxide Dismutase and Catalase Activities:

The hepatic Superoxide Dismutase (SOD) activity was

determined spectrophotometrically according to the procedure of Nishikimi *et al.* (1972) depending on the ability of the enzyme to inhibit phenazine methosulphate mediated reaction of nitroblue tetrazolium dye, meanwhile, the hepatic Catalase (CAT) activity was determined using the method of Aebi (1984) by following the decrease in the absorbance at 240nm due to the decomposition of H_2O_2. The difference in the absorbance per unit time was a measure of the CAT activity.

Estimation of Tumor Necrosis Factor-alpha and Prostaglandin E-2:

Tumor Necrosis Factor-alpha (TNF-α) level was assayed in serum and liver homogenate using an ELISA kit purchased from Koma Biotech Company; Catalog No. K0331196. Prostaglandin E-2 (PGE-2) kit was purchased From SunLong Biotech Co., LTD. Catalog No. SL0601Ra.

Quantitative Real-Time PCR:

1.RNA Extraction:

Total RNA of hepatic tissue was isolated using Qiagen tissue extraction kit (Qiagen, USA) according to instructions of manufacture.

2.cDNA Synthesis:

Moloney murine leukemia virus (MMLV) reverse transcriptase was used for synthesis of cDNA from RNA. The total RNA (0.5–2 μg) was used for cDNA conversion using a high capacity cDNA reverse transcription kit from Fermentas (USA).

3.Real-Time qPCR:

Real-time qPCR amplification and analysis were performed using an Applied Biosystem with software version 3.1 (StepOne™, USA), using SYBR Green I. Relative values of gene expression was normalized to β-actin. Primer sequences and accession numbers of the genes are provided in Table (1).

Table (1): Forward and reverse primer sequences for iNOS, COX2, IL-8 and β-actin.

	Primer sequence	Gene bank accession number
iNOS	Forward primer 5′-GACCAGAAACTGTCTCACCTG-′3 Reverse primer5′-CGAACATCGAACGTCTCACA-′3	NM_012611.1
Cox2	Forward primer :5′-CCATGTCAAAACCGTGGTGAATG-′3 Reverse primer:5′-ATGGGAGTTGGGCAGTCATCAG-′3	NM_021838.2
IL-8	Forward primer :5′-ATGCCTCGTGCTGTCTGACC-′3 Reverse primer:5′-CCATCTTTAGGAAGACACGGGTT-′3	NM_001277073.1
β-Actin	Forward primer :5′-TATCCTGGCCTCACTGTCCA-′3 Reverse primer:5′-AACGCAGCTCAGTAACAGTC-′3	NM_031144.3

Neurochemical Studies:

1. Determination of Monoamines Concentration:

Estimation of NE, DA and 5-HT in cerebral cortex of the brain of all treated rats was carried out using HPLC. All samples were analyzed on an Agilent HP1100 series HPLC apparatus (USA). HPLC system consists of a quaternary pump, a column oven, Rheodine injector 20μ loop, UV variable wavelength detector, using a program purchased from the chemstation software. Using solid-phase extraction Chromabond column NH_2 phase Catalog No. 730031, samples were immediately extracted from the trace lipids. Each sample was injected directly into an aqua column 150 mm X 4.6 mm X 5μ C18, purchased from Phenomenex (USA) under the following condition: mobile phase: 20 mM potassium phosphate, pH 2.5, flow rate: 1.5 ml/min, UV: 280 nm. NE, DA and 5-HT were separated after a few minutes. Each monoamine position and

concentration from the sample was determined as compared to that of the standard, the content of each monoamine was expressed as µg per gram brain tissue (Pagel *et al.,* 2000).

2. Histopathological Examination:

Liver specimens were fixed in 10% neutral-buffered formal saline for 72 hours at least. Sections of 6 µm thick in the serial cut were used and stained with haematoxylin and eosin according to the method described by Bancroft and Gamble (2008).

Statistical Analysis:

Results were evaluated statistically according to statistical analysis; data were presented as mean ± SEM (standard error mean) using SPSS (Statistical Package for Social Science) version 16. Variables were statistically analyzed by one-way analysis of variance (ANOVA) test. When differences were significant, Post hoc test (LSD, Least Significant Difference) was performed to find the individual differences between groups. The statistical difference with values of $p<0.05$ considered statistically significant.

RESULTS

Effect of Sepsis and/or Prop on CBC*:*

The sepsis-induced toxicity in all animal groups was detected by measuring CBC. As illustrated in Table (2), Sepsis induction showed significant changes at $p<0.05$ as RBCs count, Hb% content, platelets and neutrophils were decreased with a percentage difference of -14.6%, -15.3%, -48.1% and -24.0%, respectively, while increased WBCs whole count with a percentage difference of 128.6% represented with increased lymphocytes with a significant change of 48.6% and in eosinophils with a significant change of 44.6%. If compared to Sepsis group, Prop & Sepsis treated rats exhibited a decrease in WBCs whole count, precisely in neutrophils, together with an increase in lymphocytes, those changes were of a significant change at $p<0.05$, which indicated the ameliorative effect of Prop.

Table (2): The Protective Effect of Propolis on Sepsis-induced Changes in Complete Blood Count (CBC) of Adult Male Albino Rats.

Parameters	Experimental Groups			
	CON	SEP	PROP	PROP & SEP
RBCs (10^6/cmm)	4.03 ± 0.21	3.44 ± 0.19 (-14.6%)[a]	4.19 ± 0.15 (4.0%)	4.04 ± 0.22 (0.3%)[b]
WBCs (10^3/cmm)	7.90 ± 0.68	18.04 ± 1.6 (128.6%)[a]	8.06 ± 0.60 (2.3%)	9.06 ± 0.73 (15.1%)[b]
Platelets (10^3/cmm)	441.80 ± 18.40	229.60 ± 13.00 (-48.1%)[a]	441.20 ± 16.56 (-0.2%)	351.20 ± 27.20 (-20.7%)[a,b]
Haemoglubin (g/dl)	12.18 ± 0.68	10.32 ± 0.56 (-15.3%)[a]	12.56 ± 0.46 (3.2%)	12.12 ± 0.65 (-0.5%)[b]
Neutro. (%)	65.30 ± 0.89	49.62 ± 3.42 (-24.0%)[a]	64.90 ± 1.2 (-0.6%)	58.77 ± 2.77 (-10.0%)[a,b]
lympho. (%)	30.00 ± 1.14	44.58 ± 2.00 (48.6%)[a]	30.18 ± 1.48 (0.6%)	35.58 ± 2.96 (18.6%)[a,b]
Mono (%)	4.10 ± 0. 31	4.30 ± 0.40 (4.8%)	3.90 ± 0.26 (-4.9%)	4.51 ± 0.35 (10.0%)
Eosino (%)	1.80 ± 0.26	2.60 ± 0.35 (44.4%)[a]	2.20 ± 0.26 (22.2%)	2.19 ± 0.40 (21.7%)

Data expressed as mean ±SEM

( ): % Difference with respect to control value

a: significant changes at $p<0.05$ as compared to control group.

b: significant changes at $p<0.05$ as compared to sepsis group.

Effect of Sepsis and/or Prop on ALT and AST:

As depicted in Figure (1), sepsis induction in rats showed an increased level of ALT and AST, with a significant change at $p<0.05$, if compared with the control values. Prop-treated rats increased ALT level and AST level non-significantly. Prop administration to sepsis-induced rats decreased both enzyme levels with significant changes ($p<0.05$) in ALT and AST levels as compared to SEP group.

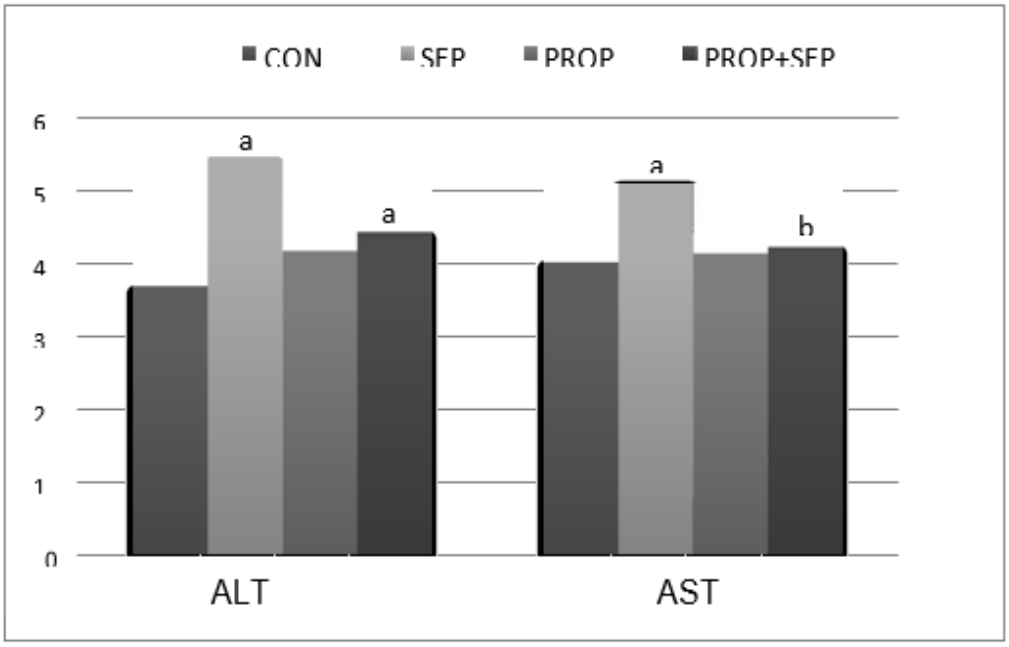

Fig. (1): The Protective Effect of Propolis on Sepsis-induced Changes in Liver Enzymes Activity (ALT & AST) of Adult Male Albino Rats.

Effect of Sepsis and/or Prop on Oxidant/Antioxidant Markers:

In the present study, sepsis induction to rats elicited sharp increases of significant change at $p<0.05$ in MDA, GSSG, $NO2^-$ and $NO3^-$ levels in livers of septic rats when compared to the control values (Table, 3). Meanwhile, the antioxidant molecule GSH showed a marked decrease in its content accompanied by sharp decreases with significant change at $p<0.05$ in CAT and SOD activities. Data concerning Prop & Sep-treated rats indicated that, though Prop treatment completely ameliorated the changes observed in sepsis-induced rats, like MDA, GSSG and $NO2^-$ levels significantly decreased with respect to the sepsis group values. In addition, Prop treatment to septic rats increased GSH level with significant change, moreover, CAT and SOD activities also, increased being of a significant change at $p<0.05$ with respect to Sep group.

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Table (3): The Protective Effect of Propolis on Sepsis-induced Changes in Oxidant and Antioxidant Markers in Liver of Adult Male Albino Rats.

Parameters	Experimental Groups			
	CON	**SEP**	**PROP**	**PROP & SEP**
NO_2 (µmol/g tissue)	0.60 ± 0.03	0.81 ± 0.05 (35.8%)[a]	0.62 ± 0.04 (3.2%)	0.65 ± 0.04 (9.4%)[b]
NO_3 (µmol/g tissue)	0.30 ± 0.02	0.48 ± 0.03 (61.3%)[a]	0.30 ± 0.01 (0.0%)	0.33 ± 0.04 (11.1%)[b]
GSH (µmol/g tissue)	31.17 ± 2.7	12.85 ± 2.3 (-58.7%)[a]	30.07 ± 3.3 (-3.3%)	20.48 ± 2.1 (-34.1%)[a,b]
GSSG (µmol/g tissue)	0.63 ± 0.05	1.20 ± 0.08 (90.7%)[a]	0.63 ± 0.04 (0.0%)	0.97 ± 0.02 (55.8%)[a,b]
MDA (nmol/g tissue)	20.05 ± 1.51	44.57 ± 3.07 (122.5%)[a]	22.91 ± 2.09 (14.1%)	30.92 ± 3.16 (54.1%)[a,b]
CAT (µmol/sec/g tissue)	137.86 ± 16.6	34.75 ± 4.8 (-74.8%)[a]	134.37 ± 12.7 (-2.5%)	72.57 ± 6.6 (-47.5%)[a,b]
SOD (U/g tissue)	116.40 ± 14.31	52.80 ± 5.09 (-54.7%)[a]	113.27 ± 14.62 (-2.7%)	88.77 ± 11.37 (-23.9%)[a,b]

Data expressed as mean ±SEM

( ): % Difference with respect to control value

a: significant changes at $p<0.05$ as compared to control group.

b: significant changes at $p<0.05$ as compared to sepsis group

Effect of Sepsis and/or Prop on Inflammatory Markers:

The present results in Table (4) showed that sepsis induction in rats induced a dramatic increase, in TNF-α and PGE2 levels in both serum and liver being of a significant change at $p<0.05$. Meanwhile, Prop treatment to septic rats exerted a strong and significant ($p<0.05$) lowering effect on TNF-α and PGE2 levels versus sepsis group but still elevated significantly than the control values.

Table (4): The Protective Effect of Propolis on Sepsis-induced Changes in TNF-α and PGE2 Levels in Both Serum and Liver of Adult Male Albino Rats

Parameters	Experimental Groups			
	CON	**SEP**	**PROP**	**PROP & SEP**
TNF-α in serum (pg/ml)	115.73 ± 20.2	650.38 ± 35.0 (461.5%)[a]	116.27 ± 18.7 (0.3%)	187.07 ± 41.2 (61.1%)[a,b]
TNF-α in liver (pg/g tissue)	4.07 ± 0.52	26.73 ± 2.36 (556.8%)[a]	2.23 ± 0.34 (-44.7%)	15.80 ± 1.73 (289.2%)[a,b]
PGE2 in serum (pg/ml)	337.20 ± 30.0	490.58 ± 47.0 (45.5%)[a]	330.69 ± 29.0 (-1.9%)	392.95 ± 38.0 (16.5%)[a,b]
PGE2 in liver (pg/g tissue)	250.33 ± 31.0	1968.53 ± 98.7 (686.4%)[a]	241.48 ± 20.6 (-3.8%)	1553.35 ± 96.8 (519.9%)[a,b]

Data expressed as mean ±SEM

( ): % Difference with respect to control value

a: significant changes at $p<0.05$ as compared to control group.

b: significant changes at $p<0.05$ as compared to sepsis group.

Constant with the biochemical findings, sepsis induction increased the expression of IL-8, COX-2 (mediates inflammation through the production of prostaglandins) and iNOS in liver tissue of septic rats as illustrated in Table (5). Meanwhile, Prop & Sep group showed a sharp and significant inhibitory effect at $p<0.05$ on liver IL-8, COX-2 and iNOS mRNA expression with respect to Sep-induced rats.

Table (5): The Protective Effect of Propolis on Sepsis-induced Changes in IL-8, COX-2 and iNOS Genes Expression in Liver Tissue of Adult Male Albino Rats.

Parameters	Experimental Groups			
	CON	**SEP**	**PROP**	**PROP & SEP**
IL-8	1.01 ± 0.004	8.55 ± 0.729 (739.7%)[a]	1.05 ± 0.022 (4.0%)	5.26 ± 0.336 (417.7%)[a,b]
COX-2	1.00 ± 0.001	14.20 ± 0.133 (1318.7%)[a]	1.04 ± 0.018 (4.0%)	3.55 ± 0.155 (253.5%)[a,b]
iNOS	1.02 ± 0.007	18.70 ± 0.795 (1735.7%)[a]	1.04 ± 0.013 (2.0%)	9.45 ± 0.331 (828.4%)[a,b]

Data expressed as mean ±SEM
( ): % Difference with respect to control value
a: significant changes at $p<0.05$ as compared to control group.
b: significant changes at $p<0.05$ as compared to sepsis group.

Effect of Sepsis and/or Prop on Monoamine Levels in Cerebral Cortex of Rats:

The present results in Table (6) indicated that sepsis significantly ($p<0.05$) decreased the levels of NE, DA and 5-HT in the CC with a percentage difference of -39.0%, -23.7% and -38.8%, respectively as compared to control values. Meanwhile, Prop & Sep treated rats showed a significant increase ($p<0.05$) in their CC levels of NE and 5- HT, when compared to Sep group indicating the ameliorative and protective role of Prop on brain neurotransmitter during sepsis.

Table (6): The Protective Effect of Propolis on Sepsis-induced Changes in NE, DA and 5-HT levels in Cerebral Cortex of Adult Male Albino Rats.

Parameters	Experimental Groups			
	CON	**SEP**	**PROP**	**PROP & SEP**
NE (µg/g tissue)	0.86 ± 0.013	0.53 ± 0.016 (-39.0%)[a]	0.85 ± 0.042 (-1.2%)	0.70 ± 0.020 (-18.2%)[a,b]
DA (µg/g tissue)	1.84 ± 0.023	1.41 ± 0.047 (-23.7%)[a]	1.835 ± 0.031 (-0.9%)	1.71 ± 0.031 (-7.4%)[b]
5-HT (µg/g tissue)	0.67 ± 0.008	0.41 ± 0.014 (-38.8%)[a]	0.63 ± 0.015 (-6.9%)	0.47 ± 0.014 (-29.4%)[a,b]

Data expressed as mean ±SEM
( ): % Difference with respect to control value
a: significant changes at $p<0.05$ as compared to control group.
b: significant changes at $p<0.05$ as compared to sepsis group.

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Histopathological Examination:

Histopathological examination of liver tissue was undertaken to monitor the changes in liver architecture in septic rats, Prop group and Prop & Sep treated rats group. Liver injury in sepsis was confirmed by the histological changes. Sepsis induction in rats caused hydropic changes in the cytoplasm of most hepatocytes. Many nuclei showed karyolysis and karyorrhexis. Fibrosis with cellular infiltration was also observed around blood vessels focal aggregation of inflammatory cells and dilatation with congestion of blood vessels in liver of sepsis-induced rats (Figure, 2). Prop treatment alone showed a quite normal structure of liver tissue of Prop-treated rats group (Figure, 2). Prop treatment to sepsis-induced rats was found to reduce the cellular changes and to a lesser extent the cellular infiltration and fibrosis in liver tissue of Prop & Sep treated rats. Also, Prop treatment to septic rats was found to slightly reduce the dilatation and congestion of blood vessels. Hepatocytes showed healthy nuclei although some hydropic changes were still observed (Figure, 2).

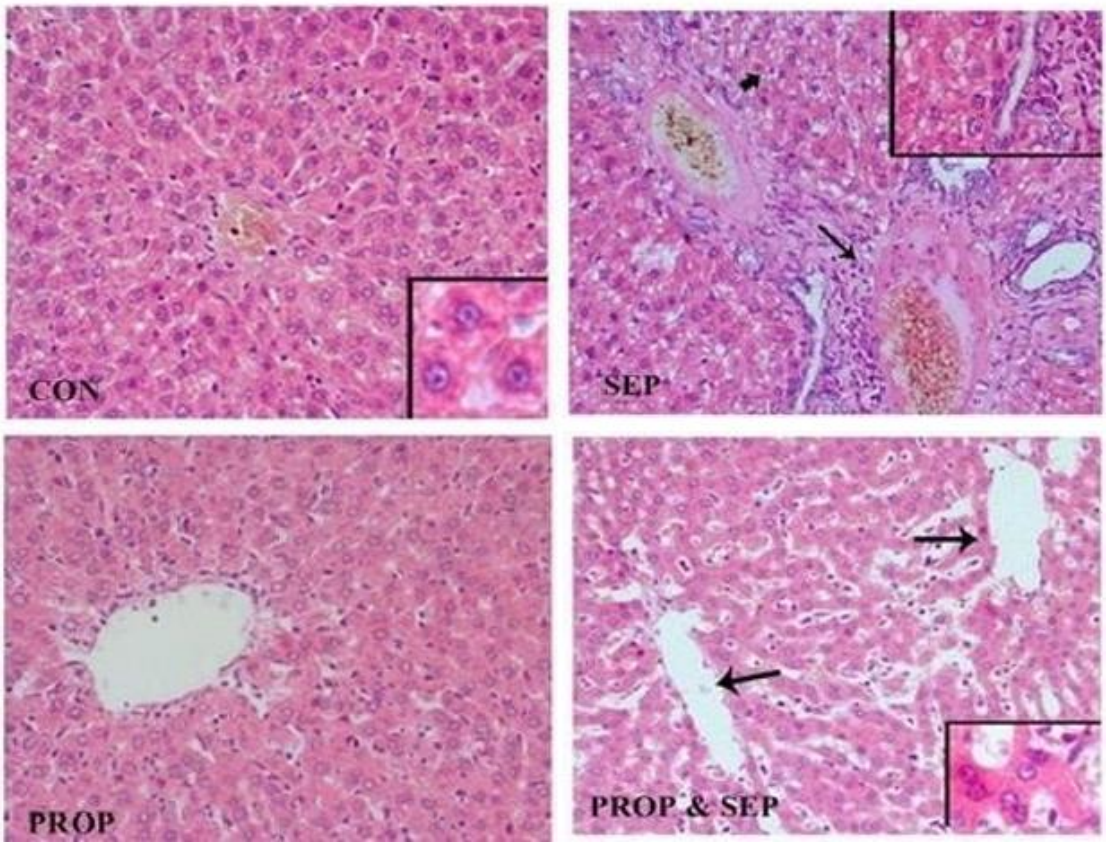

Fig. (2): The Protective Effect of Propolis on Liver Tissue against Sepsis- Induced Experimentally in Male Albino Rats

DISCUSSION

Though numerous studies were conducted to investigate the pathogenesis and therapy of sepsis, it remained a major cause of mortality, as it is still nonspecific and no approved drugs specifically target sepsis (Zhao *et al.*, 2016). Oxidative stress was considered a key causing factor of liver damage induced by many agents, including viral infections, drugs, alcohol, environmental pollutants and

dietary components, which ultimately resulted in progression of liver injury leading to liver fibrosis and cirrhosis (Farzaei *et al.*, 2018). The imbalance between the generation and degradation of ROS caused oxidative stress and eventually the generation of free radicals and cellular damage (Farzaei *et al.*, 2018). Severe sepsis was associated with systemic inflammatory response syndrome which was characterized by species active oxygen (ROS) overproduction and increased levels of proinflammatory cytokines, which contribute individually or in combination with the recruitment of leukocyte and subsequent organ damage (Liu *et al.*, 2015).

The present results indicated liver injury in rats after sepsis induction, which is obvious by significant increases of ALT and AST levels, and by increasing markers of oxidative stress in liver as; MDA, GSSG, $NO2^-$ $NO3^-$ while decreasing the antioxidant GSH content, along with the antioxidant enzymes CAT and SOD activities. The result of the present work indicated that the antioxidant defense systems, including the non-enzymatic antioxidant molecule (GSH) and enzymatic activities such as SOD and CAT play an important role in preventing liver damage as reported by (Almeer *et al.*, 2018). Prop, in the present study, ameliorated their levels, if compared to sepsis-induced rats group. Furthermore, severe pathological damages such as sinusoidal dilatation, congestion of central vein, lipid accumulation and lymphocyte infiltration were evident in the liver after sepsis induction in the present work. The present results concerning sepsis oxidative stress and inflammation were in agreement with (Chen *et al.*, 2017 and Surewaard *et al.*, 2018). In sepsis, the overproduction of oxidative stress and inflammatory cytokines were two clinical hallmarks indicating host immune defense against infection (Schuler *et al.*, 2018). Subsequently, oxidative stress and proinflammatory cytokines lead to tissue damage and multiple organ failure (Wu *et al.*, 2018).

Results of the present study demonstrated that sepsis induction decreased RBC's count, Hb% content, platelets and neutrophils with a marked increase in WBC's count especially; lymphocytes and eosinophils, compared to control group. As documented earlier, severe sepsis was characterized by white blood cell count abnormality, fever, and presumed infection and high heart rate (Studnek *et al.*, 2012). Sepsis, also, triggered the production of a diverse array of cytokines; pro-inflammatory (for controlling infection and their excessive production may lead to tissue and organ injury) and anti-inflammatory (critical in regulating the overall immune response and establishing homeostasis). Therefore, dysregulation of both, pro-inflammatory and anti-inflammatory could also trigger pathogenesis. The obtained results showed that Prop significantly reduced the destructive effect of sepsis on CBC, where Prop intake significantly increased the low levels of Hb, platelets and RBC's due to sepsis induction, also, decreased the elevated count of WBC's and lymphocytes in blood. These results were in agreement with Batista *et al.* (2015) who indicated that treatment with Prop influenced leukocyte count, as it triggered leukocytosis with lymphocytosis as they indicated that Prop was beneficial in decreasing the time of infected wound healing (Batista *et al.*, 2015).

Liver plays a central role in the systemic response to critical illness, through both; the clearance of pathogenic microorganisms and toxins from the circulation, and the acute phase reaction and release of liver-derived cytokines, inflammatory mediators, and coagulation cascade components (Dkhil

et al., 2018a). Multiple types of liver cells play a role in bacterial phagocytosis and clearance (Protzer *et al.*, 2012). Kupffer cells, liver sinusoidal endothelial cells, and stellate cells, were the first line of defense against blood-borne bacteria in the liver, therefore, protecting the liver and the whole body. The hepatic reticuloendothelial system was found to trap and eliminate, efficiently. Bacteria and its soluble products were cleared by Kupffer cells by exhibiting a pronounced endocytic and phagocytic activity (Yan and Li, 2014). Through the cooperation of Kupffer cells with platelets and neutrophils, clearance of bacteria from the bloodstream was carried out as indicated by (Wong *et al.,* 2013). Following an attack by harmful bacteria and/or endotoxins, Kupffer cells were found to increase the rate of pro-inflammatory mediators release as well as secondary mediators of tissue injury, NO and ROS (Koo *et al.,* 1999). The liver was reported to act as a source of inflammatory mediators in sepsis, as Kupffer cells were responsible for inflammatory cytokines production in early sepsis and for mediating sepsis-induced liver injury (Yan and Li, 2014).

The oxidant/antioxidant status in the present work was accompanied by increased TNF-α, PGE2 levels in serum and liver tissue homogenate with increased expression of IL-8, COX-2 and iNOS mRNA in liver of sepsis-induced rats group. Results of the present work, were in agreement with Tveteraas *et al.* (2012) who indicated an increase in COX-2, IL-8 and PGE2 levels after sepsis induction, contributing to acute and chronic inflammation, oxidative stress, bacterial or viral infection, and cancer.

Data of the present work indicated that oral administration of Prop could protect liver and preserve NO^{2-} and NO^{3-} level from elevation during sepsis induction. These results were in agreement with (Kismet *et al.,* 2008, Enis Yonar *et al.,* 2011, Mujica *et al.,* 2017). In addition, Prop was also indicated to keep GSH content even during sepsis induction, these results were in agreement with Enis Yonar *et al.* (2011), where they showed that, Pre-oral treatment with Prop attenuated oxidative stress by markedly decreasing MDA level in tissues. In addition, Prop was documented to increase the reduced level of glutathione, also, CAT, SOD, and glutathione peroxidase activities (Enis Yonar *et al.,* 2011). Moreover, El-Guendouz *et al.* (2017) indicated that in rats given Prop, the livers of rats receiving Prop showed less lipid. Furthermore, Mujica *et al.,* (2017) also, supported the role of Prop in diverse chronic disease, through different mechanisms such as; the increase in high-density lipoproteins-cholesterol, and its antioxidant effect due to enhanced GSH and by decreasing MDA and markers of oxidative stress. Present results pointed out that the treatment of Prop provided protection against liver damage and modulated the toxic effects of sepsis. In agreement with the present work, (Kismet *et al.* (2017), Tzankova *et al.* (2019) also, documented the protective effect of Prop on liver.

Inflammation was considered as an adaptive physiological response induced by deleterious circumstances including infection and tissue injuries and was reported to be the product of complex series of responses triggered by the immune system (Dkhil *et al.,* 2018a). Hyperinflammation was reported to have an early phase, followed or overlapped by a prolonged state of immune-suppression (Germain, 2012), referred to as sepsis-induced immunoparalysis (Hotchkiss and Nicholson, 2006), which was characterized by impaired innate and adaptive immune responses, thus playing a pivotal role in the pathogenesis of tissue damage, multiple organ failure, and finally death (Hotchkiss and

Nicholson, 2006). The liver-mediated immune response to sepsis was found to act as a double-edged sword: it clears bacteria and toxins but caused inflammation, immunosuppression and organ damage (Dkhil *et al.,* 2018a). During sepsis, various pathogens and damage-associated molecular patterns cause activation, of platelets (produced from bone marrow megakaryocytes as anucleate cells), playing a role in regulation of inflammatory response (Dkhil *et al.,* 2018b). The pro-inflammatory factors in platelets granules are released into the surrounding environment or transferred to plasma membrane such as; interleukins, monocyte chemo-attractant protein, platelet factor, for activating more remote platelets and immune cells, therefore, playing a deleterious role in the dissemination of coagulopathy and inflammatory responses in sepsis (Woth *et al.,* 2012).

Neutrophils, the chemotactic factors in the site of infection, are the first line of defense against bacterial and fungal pathogens, which recruit to the site of infection. Chemokine IL-8 played a major role in neutrophils activation, influencing the chemotaxis of immune cells (an inflammatory mediator in response to viral or bacterial pathogen), as potential biological marker in fibrosis and ALT levels (Lee *et al.,* 2011), also, in tissue repair mechanisms such as angiogenesis and cell proliferation. IL-8 can be up-regulated at the transcriptional level, on receiving inflammatory stimuli, in many different cell types; fibroblasts, monocytes, and hepatocytes, for protection of cells from the effects of inflammatory stimuli (Mukaida, 2000). Moreover, Hu *et al.* (2016) indicated a marked increased serum IL-8 levels in patients with sepsis.

The present study indicated that oral administration of Prop for two weeks before sepsis induction attenuated the deteriorating effect of sepsis on ALT and AST as liver function enzymes. These results are in agreement with (González *et al.,* 1995, Sahu *et al.,* 2018, Tsuchiya *et al.,* 2018). Moreover, Sahin and Ozturk (2018) found that addition of Prop extract, in diet, improved immune status and antioxidant activity as well as, enhanced Ca^{2+} absorption. The anti-oxidizing properties of Prop were found to improve lipid metabolism, liver morphological structure, and biological functions.

TNF-α, a pro-inflammatory cytokine released by macrophages, monocytes, T-lymphocytes, and other cells, and also, synthesized in many tissues playing important and critical roles in several biological processes, as host resistance to infection and inflammatory responses. Both TNF-α and IL-8, showed increased levels, in response to pathogen infection, in sera and livers of sepsis-induced rats. With the abnormal accumulation of neutrophil in capillary beds, especially those in liver sinusoids, leading to microvascular occlusion, impaired recruitment of neutrophils to the infectious foci, and damaged neutrophil migration could result in tissue ischemia and subsequently multiple organ failure (Dkhil *et al.,* 2018a).

Data of the present work indicated that oral administration of Prop could protect liver therefore, maintaining TNF-α within the normal values during sepsis induction in rats. These results were in agreement with (Kismet *et al.,* 2008, Funakoshi-Tago *et al.,* 2016) who indicated that Prop could have hepatoprotective effects against sepsis *in vitro*, which could be, partly, mediated via antioxidant effects (Nakajima *et al.,* 2007). Moreover, (Doğanyiğit *et al.,* 2013) indicated the hepatoprotective role of Prop with improved TNF-α level and reduced stress-induced by sepsis.

Inflammation was associated with altered signaling pathways, resulting in increased levels of inflammatory

markers. COX-2, PGE2 and IL-8 mRNA were, also, up-regulated by sepsis induction as depicted from the present results. TNF-α was found to stimulate the acute phase reaction, inducing apoptotic cell death, and inhibiting tumorigenesis and viral replication. Regulation of IL-8 was found to be ruled by many transcription factors, such as; TNF-α, nuclear-factor kappa B (NF-κB), and IL-6 (Xie *et al.*, 2010). Moreover, IL-8 was reported to up-regulate some tumor genes, such as COX-2 (rate-limiting enzyme, involved in inflammation, cellular proliferation, anti-apoptosis activity, and tumorigenesis), lipooxygenase-5, and phospholipase A2, thus promoting the development of cancer (Vendramini-Costa and Carvalho, 2012). Cytokine production in sepsis is affected by toll receptors signaling (TLRs) signaling, as those receptors were found to activate platelets to release TNF-α as an immune-modulatory agent, promoting neutrophils and endothelial cells activation.

Moreover, lipopolysaccharides binding to TLRs leads to their activation, which in turn activates c-Jun N-terminal kinase, NF-κB and AP-1, causing binding to the promoters of inflammatory cytokines, leading to massive cytokine production in sepsis (Wen *et al.*, 2010). So, the inappropriate activated platelets were major contributors in the initiation of disseminated intravascular coagulation leading to the platelet adhesion thus reducing oxygen supply and enhancing inflammatory cytokine networks (Tyml, 2011).

Moreover, studies have documented the anti-inflammatory effect of Prop, as it suppressed prostaglandins and leukotrienes generation by inhibiting the expression and activities of cyclooxygenases (COX-1 and COX-2) and lipoxygenases (LOX), retarding the gene expression of iNOS, blocking TNF-α that mediated NF-κB activation, and reducing immune response in T cells (Rossi *et al.*, 2002, Paulino *et al.*, 2008 and Farooqui and Farooqui, 2012). Those reports were in agreement with the present results which proved that, Prop maintained the level of PGs within the normal range even after sepsis induction.

The present study indicated that, Prop significantly decreased and attenuated elevated TNF-α and PGE2 levels in both serum and liver of Prop & Sep-treated rats group and likely, COX-2, iNOS and IL-8 mRNA expression in their livers. Sahu *et al.*, (2018) indicated that Prop, *in vitro*, inhibited COX-2 expression and production of prostanoids in cancer cells. Prop also inhibited LPS-induced COX-2 expression in the mouse macrophage cell line. Herein, the present results demonstrated the protective effect of Prop against sepsis-induced hepatic oxidation, lipid peroxidation, inflammation, and liver dysfunction. The present results demonstrated the protective effect of Prop against sepsis-induced hepatic oxidation, lipid peroxidation, inflammation, and liver dysfunction.

Linking liver injury to the brain, in fact, the brain was found to remove or inactivate, potentially, the damaging agents or tissues and was indicated to response to injury, infection, or disease through neuroinflammation. Glias of the CNS, and lymphocytes, monocytes, and macrophages of the hematopoietic system, mediate this inflammatory response. As over-activation of the brain has severe detrimental consequences including depressive-like behavior. Major depressive disorders showed alterations in immunological markers, with increased pro-inflammatory cytokines levels. Moreover, chronic inflammation changes brain structure and synaptic plasticity leading to neurodegeneration, coupled with a

reduction in neuroprotection, leading to dementia, particularly in older people (Souza-Fonseca-Guimaraes *et al.,* 2013).

The present results indicated that sepsis-induction decreased DA, NE and 5-HT levels in CC of Sepsis-induced rats. Moreover, Deng *et al.,* (2013) indicated that enhanced inflammatory markers level from peripheral immune cells and serum proteins may enter the central nervous system through blood-brain barrier (BBB) leakage, as endothelial cells injury as well as astrocytes lead to BBB disruption, therefore, triggering the leakage of immune cells and inflammatory mediators which are in turn enhance the inflammatory responses leading to aggravated brain injury. Patients with sepsis suffering from brain injury recorded high mortality rate as sepsis triggers cell death, due to the overproduction of pro-inflammatory cytokines, ROS production and mitochondrial dysfunction, leading to organ damage (Srinivasan *et al.,* 2010).

As a result of elevated levels of pro-inflammatory cytokines, the "sickness behavior" symptoms were recorded including; depression, reduction in locomotor activity, anhedonia, anorexia and cognitive disturbances. A delayed and progressive loss in dopaminergic neurons in the substantia nigra was also observed due to increased pro-inflammatory response (Walker *et al.,* 2013). Changes in behavior similar to depression, major depression, and neurodegeneration could arise from unregulated inflammation and high levels of pro-inflammatory cytokines, while attenuation of inflammatory response was found to reduce the depressive symptoms (Capuron and Miller, 2004). Moreover, Walker *et al.* (2013) proposed that inflammation may be a common mediator of observed death conditions due to depression and chronic pain. Furthermore, O'sullivan *et al.* (2009) suggested the suppression of neuroinflammation by NE and those NE uptake inhibitors‟ were therapeutically efficient in treating depression may be partially related to this mechanism.

The present study proved the correlation between Prop uptake and amelioration of brain neurotransmitters. This was in agreement with Diab *et al.* (2012), Mahmoud and El-Yamany (2012) and Durdagi *et al.* (2018), who demonstrated the protective effect of Prop against oxidative stress, through improving and regulating the neurotransmitters secretion within the central nervous system. Lima Cavendish *et al.,* (2015) indicated the anti-inflammatory activities of red Prop, containing formononetin showed anti-inflammatory and antioxidant activities by reducing IL-1β and NF-κB levels, *in vitro*, and protecting neurons and lung tissue *in vivo*, by decreasing TNF-α and IL-6 levels and improving SOD activity (Talas *et al.,* 2012 and Lima Cavendish *et al.,* 2015).

Conclusion

Prop extract was evident to be useful in the early protection against sepsis, by protecting liver tissues from injuries, inflammation, congestion and also, damage. The present findings provide evidence for the ability of Prop extract to maintain brain neurotransmitters and liver function enzymes level through attenuation of oxidative stress and inflammatory markers, thus protecting both tissues.

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