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Research Article
Therapeutic Efficacy of *Anodonta cygnea* and Crayfish *Procambarus clarkii* Hemolymph Extracts on Sepsis-Induced Acute Liver Injury in Neonate Rats

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Abstract

Background and Objective: In newborns, sepsis is the third highest cause of morbidity and death. The current research intended to assess the efficacy of freshwater mussel *Anodonta cygnea* and crayfish *Procambarus clarkii* hemolymph extracts on sepsis-induced liver damage in neonatal rats. **Materials and Methods:** Twenty-four newborn rats were allocated into four groups at random, control, sepsis and septic rats treated with *A. cygnea* hemolymph or *P. clarkii* hemolymph. The researchers looked at the antiseptic mechanisms of *A. cygnea* and *P. clarkii* hemolymph extracts. **Results:** Hemolymph exhibited an *in vitro* antioxidant and antibacterial activities. The results showed that hemolymph enhanced liver function significantly, as seen by lower liver enzyme activity. Such as aminotransferases as well as the increase in protein content. Anti-inflammatory activity of hemolymph was assessed by the reduction in levels of Cytochrome C, IL-6 and PG-E_2. Moreover, the extracts may be able to reduce hepatic oxidative stress by a marked increment in CAT activity as well as a decrease of MDA level. Furthermore, hemolymph therapy helped to correct the aberrant architecture of hepatic tissues caused by polymicrobial infection. **Conclusion:** *Anodonta cygnea* and *P. clarkii* hemolymph extracts attenuated the liver injury that induced by sepsis as it improves liver biomarkers and suppresses reactive oxygen species propagation due to their antibacterial and anti-inflammatory activities.

Key words: *Procambarus clarkii*, *Anodonta cygnea*, hemolymph, sepsis, cecal slurry, oxidative stress

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sepsis is a complicated clinical illness caused by a microbe's interaction with the host. Sepsis is the Systemic Inflammatory Response Syndrome (SIRS) with evidence of infection, according to clinical definitions[1]. The SIRS is the clinical syndrome that results from a dysregulated inflammatory response to infectious and also noninfectious insult[2]. According to statistics, sepsis affects around 18 million people globally each year, with a fatality rate of approximately 30% and the incidence rises 1.5-8% yearly[3]. In fact, sepsis is the third largest cause of mortality in the neonatal period[4]. Respiratory difficulties affect up to 40% of newborns with sepsis and up to 31% of survivors have long-term disabilities[5,6]. The most commonly bacteria linked to neonatal sepsis are *Listeria* and Group B *Streptococcus*, gram-negative[7]. The etiology of neonatal sepsis is marked by an increased inflammatory response that results in single or multiorgan failure. In neonatal sepsis, an increase in proinflammatory cytokines such as TNF-α, IL-1 and IL-6 is commonly reported[8-12]. The development of sepsis-induced liver failure is attributed to oxidative damage caused by the activation of inflammatory pathways[13]. Pathogens, poisons and inflammatory mediators all cause liver damage in sepsis[14]. A homeostatic equilibrium occurs between the generations of oxygen free radicals (ROS) and their elimination by endogenous scavenging antioxidants under normal physiological circumstances[15]. Reactive oxygen species (ROS) are thought to have a role in the pathophysiology of newborn sepsis and related consequences[16]. Increase production of reactive oxygen species (ROS) causes a decrease in cellular antioxidant capacity and hepatic dysfunction[17]. As a result, decreasing oxidative stress and/or boosting tissue antioxidant capacity may be more effective treatment strategies for treating hepatic damages in sepsis.

Cecal slurry (CS) was injected intraperitoneally in animals as an experimental model of sepsis because it resembled the pathophysiology of human sepsis[18].

Unfortunately, in addition to the unfavorable side effects of some antibiotics, the recent emergence of a rising number of bacteria resistant to traditional antibiotics is causing concern[19]. As a result, in recent years, researchers' focus has shifted to marine biota, which has a wide range of bioactive chemicals[20]. Freshwater-derived products have gained popularity as a nutraceutical and functional foods, as well as a source of raw materials for the production of medications and particular health meals[21]. Supplements made from freshwater foods have recently received a lot of interest for their potential as hepatoprotective agents[21,22]. Complement, lectins, clotting factors and antimicrobial peptides are among the physiologically active chemicals found in the circulating hemolymph of marine invertebrates[23].

The freshwater mussel (*Anodonta cygnea*) is abundant in Egypt along the Nile River[24]. Many researchers reported bioactive chemicals from mollusks that have anticancer, antileukemic and antiviral properties[25]. Freshwater crayfish (*Procambarus clarkii*) has also been found across the Nile River[26]. It is a powerful antioxidant, antibacterial and anticancer agent[27]. Fahmy and Hamdi[28] suggested that *Procambarus clarkia* extract's antioxidative activity may help to alleviate liver and erythrocyte damage. The purpose of this study was to assess the antiseptic efficacy of *A. cygnea* and *P. clarkii* hemolymph on sepsis induced acute liver injury by CS in neonate rats.

MATERIALS AND METHODS

Study area: All experiments were performed at Cairo University between January and May, 2022.

Experimental animals: The experimental animals used in this study were the neonate albino rats (*Rattus norvegicus*) (5-7 days old). Pups were not identified as either male or female and they remained with their mothers throughout the experiments.

Collection of *A. cygnea* and *P. clarkii*: Freshwater *A. cygnea* and crayfish, *P. clarkii*, specimens were collected from the River Nile at Al Rawda area-Giza Governorate.

Collection of *A. cygnea* hemolymph: Hemolymph was collected from the posterior adductor muscle sinus of *Anodont*, using a sterile syringe with a 25-gauge needle[29].

Collection of *P. clarkii* hemolymph: Using a 1 mL syringe and a 23-gauge needle, the hemolymph (250-500 L) was removed from the arthrodial membrane between the coxa and the base of the cheliped[30]. The haemocyte degranulation and coagulation were avoided and the hemolymph was collected in the presence of anticoagulant citrate - EDTA solution for the collection of marine invertebrate haemolymph (100 mM glucose, 30 mM trispodium citrate, 26 mM citric acid, 510 mM NaCl and 10mM EDTA-Na, pH = 4.6) (2:1 v/v) was prepared according to Soderhlll and Smith[31]. The haemolymph was centrifuged at 2000 rpm for 15 min at 4°C to remove haemocytes. Supernatant were collected by aspiration and stored at 4°C until use. The admixture collected immediately, concentrated and lyophilized to a solid residue using a lyophilizer (LABCONCO lyophilizer, shell freeze system, USA).

Determination of antioxidant activity (Scavenging activity of DPPH radical): The free radical scavenging activities of each extract and ascorbic acid (standard antioxidant) were estimated by the DPPH (1,1-diphenyl-2picrylhydrazyl) assay[32]. The following concentrations of the *A. cygnea* and *P. clarkii* hemolymph powders were prepared, separately, in methanol 20, 40, 60, 80, 100 and 120 mg mL^{-1} and was mixed with 2 mL of DPPH (0.3 mM in methanol). Similar concentrations of ascorbic acid (vitamin C) were prepared. The certain concentrations of *A. cygnea*, *P.clarkii* or ascorbic acid and the final volume was adjusted to 4 mL with methanol. The absorbance was taken at 517 nm after 30 min of incubation in the dark at room temperature against blank (methanol). The percentage of antioxidant activity was calculated as follows:

$$\text{Antioxidant activity (\%)} = \frac{\text{Abs control-Abs sample}}{\text{Abs control}} \times 100$$

where, Abs sample was the absorbance of sample solution and Abs control was the absorbance of the DPPH solution.

Antibacterial activity of *A. cygnea* and *P. clarkii* hemolymph: The spectrum of antibacterial activity was studied by using the techniques Bauer *et al.*[33]. Antibacterial and activity was expressed in terms of the diameter of zone of inhibition was measured in mm using a Vernier caliper or a ruler and recorded. *Staphylococcus aureus* ATCC 25923, *E. coli* ATCC 93111 and *P. aeruginosa* ATCC 9027 were used for testing antimicrobial activity. Lyophilized bacteria were obtained from the standard ATCC bacterial strain collection of the Microbiology Research Lab of the Faculty of Agriculture at Cairo University.

Determination of molecular weight by using SDS-PAGE: The SDS-PAGE is used to find out the molecular weight active fractions of the *A. cygnea* and *P. clarkii* hemolymph extracts. The SDS-PAGE was performed in 15% separating gels, according to the method described by Laemmli[34].

Acute toxicity study (LD_{50}): The LD_{50} of *A. cygnea* and *P. clarkii* hemolymph was determined according to the method described by Chinedu *et al.*[35]. The rats were fasted overnight then separated into four groups (2 rats/group). Different doses of the *A. cygnea* and *P. clarkii* hemolymph (10, 100, 300 and 600 mg kg^{-1}) are administered to the rats. The animals were observed for post-administration and then 10 min every 2 hrs interval for 24 hrs. The animals were monitor for any change in behaviors such as paw licking, fatigue, semi-solid stool, salivation, writhing and loss of appetite in addition to mortality. The LD_{50} calculated from the following formula:

$$LD_{50} = \frac{M_0 + M_1}{2}$$

Where:

M_0 = Highest dose of *A. cygnea* and *P. clarkii* hemolymph that gave no mortality

M_1 = Lowest dose of *A. cygnea* and *P. clarkii* hemolymph that gave mortality

Ethical consideration: Experimental protocols and procedures used in this study were approved by the Cairo University, Institutional Animal Care and Use Committee (IACUC) (Egypt) (CU/I/F/24/19). All the experimental procedures were carried out in accordance with international guidelines for the care and use of laboratory animals.

Preparation cecal slurry: The cecal slurry (CS) was prepared according to the modified methods previously described by Starr *et al.*[18]. Briefly, the adult donor rats were euthanized and the collected cecal contents were combined, weighed and mixed with sterile water at a ratio of 0.5 mL of water to100 mg of cecal content. This cecal slurry (2×CS) was sequentially filtered through two sterile meshes (first 860-mm and then 190 mm, Bellco Glass, Inc., Vineland, New Jersey). The filtered CS was then mixed with an equal volume of 30% glycerol in phosphate buffered saline (PBS), resulting in a final CS stock solution. The CS stock aliquots were stored at -80°C for use.

Induction of polymicrobial sepsis using CS: Neonatal sepsis was induced by a cecal slurry (CS) method adapted from Starr *et al.*[18]. To administer CS, newborn rats (5-7 days old) were removed from the mothers, separated into two groups, placed on a 37°C heating pad. Neonates were injected intraperitoneally with single dose of CS (100 µL of CS per neonate rat). In preliminary experiments, we examined survival after cecal slurry injection for six pups from the same mother. At 5th days, the survival rate was 100% and at 10 days, it was 50%.

Experimental design: Twenty four neonatal rats were randomly divided into 2 groups, the rat groups were divided as follow:

- **Group 1:** Control neonatal rats IP injected with 0.9% saline (6 neonates)
- **Group 2:** Septic neonatal rats (18 neonates) IP injected with CS (100 µL of CS per neonates), after 24 hrs. The septic neonates were subdivided as follow:
  - **Subgroup I:** Septic neonatal rats IP injected with 0.9% saline (6 neonates)
  - **Subgroup II:** Septic neonatal rats IP treated with *A. cygnea* hemolymph (45 mg kg^{-1}) for 2 days (6 neonates)
  - **Subgroup III:** Septic neonatal rats IP treated with *P. clarkii* hemolymph (45 mg kg^{-1}) for 2 days (6 neonates)

Animals handling: At the end experimental period (4th day), the neonate rats were euthanized by exsanguination. Blood samples collected in centrifuge tubes. Liver was removed and immediately blotted using filter paper to remove traces of blood. Part of the liver stored at -80°C for biochemical analysis. Another parts of liver were suspended in 10% formal saline for fixation preparatory to histopathological examination.

Assessment of serum liver function: The accessible kits were used for estimation of serum aminotransferase enzymes (aspartate transaminase and alanine transaminase) activities according to Reitman and Frankel[36] and total protein[37].

Assessment of oxidative stress markers: Liver tissue was homogenized (10% w/v) in an ice-cold 0.1 M Tris HCl buffer (pH 7.4) and centrifuged at 3000 rpm min^{-1} for 15 min. Using the commercial kits, the liver supernatant was used for determination of malondialdehyde (MDA)[38], glutathione reduced (GSH)[39] and catalase (CAT)[40].

Measurement of Interleukin-6 (IL-6) concentration in liver: Interleukin-6 (IL-6) was measured in liver homogenate using commercially available rat Interleukin 6 ELISA kit (Cat. No: E0135Ra, Bioassay Technology Laboratory, Shanghai, China) was used for detection according to the manufacturer's instructions.

Determination of prostaglandin E2 level in liver: Prostaglandin E2 (PGE2) in liver homogenates was quantified using the Rat Prostaglandin E2 ELISA Kit (Cat. No: E0504Ra, Bioassay Technology Laboratory, Shanghai, China) per manufacturer's instructions and expressed as (ng L^{-1}).

Quantification of Cytochrome C in liver tissue: To quantify the Cytochrome c (Cyt-C) released from mitochondria was measured with a commercially available Cytochrome c ELISA kit (Cat. No: E0627Ra, Bioassay Technology Laboratory, Shanghai, China) according to the manufacturer's instructions.

Histopathological examination: Liver tissues were fixed in 10% formal saline, embedded in paraffin and sectioned. Then, the sections stained with a hematoxylin and eosin (H&E) for histological examination using a light microscope.

Immunohistochemical examination: Immunohistochemical evaluation of Tumor Necrosis Factor-α (TNF-α) was studied. In brief, tissue sections (4-5 µm) were cut into poly l-lysin coated slides. After rehydration and heat induced epitope retrieval, slides were washed then incubated with primary antibodies (Mouse monoclonal Anti-TNF-α, Santa Cruz Biotechnology Inc., Dallas, Texas, USA) at a dilution of 1:200 overnight at 4°C. Then tissue sections were incubated with HRP labeled goat anti-mouse secondary antibody (Abcam, Cambridge, UK) for 2 hrs followed by detection step using DAB-Substrate kit (Thermo Scientific). Positive staining was quantified as area percent using Cell Sens dimensions (Olympus software). Negative control slides were obtained by deletion of the primary antibody.

Statistical analysis: All data were expressed as Mean±Standard Error of the Mean (SEM). The comparisons within groups were evaluated utilizing One-way Analysis of Variance (ANOVA) with Duncan's *post hoc* Test was used to compare the group means and $p<0.05$ was considered statistically significant. The SPSS statistical software package for Windows (version 21.0) was used for the statistical analysis.

RESULTS

In spite of, none of rats died after oral administration of *A. cygnea* and *P. clarkii* hemolymph extracts, an evidence of toxicity was noticed at dose 600. Therefore LD_{50} of the extracts was 450 mg kg^{-1}.

Free radical scavenging activity of *A. cygnea* and *P. clarkii* hemolymph extracts: The results of DPPH scavenging activity of *A. cygnea* and *P. clarkii* hemolymph and vitamin C are shown in the Table 1. The obtained results showed that *A. cygnea* antioxidant activity increased in a dose dependent manner ranging from 12-22.8%. *Procambarus clarkii*, on the other hand, caused dose-dependent suppression of DPPH radicals (ranging from 20.3-48.2%).

Table 1: Percent inhibition of DPPH by *A. cygnea* and *P. clarkii* hemolymph and vitamin C

	Inhibition of DPPH (%)		
Concentration (mg mL^{-1})	*A. cygnea*	*P. clarkii*	Vitamin C
20	12.0	20.3	95.10
40	16.3	23.4	95.30
60	20.3	27.5	95.50
80	22.8	48.2	95.50

DPPH: 1,1-Diphenyl-2-picrylhydrazyl

Table 2: Antibacterial activities of *A. cygnea* and *P. clarkii* hemolymph against different gram-positive and gram-negative bacteria

	Zone of inhibition (mm)								
	Hemolymph								
	A. cygnea			*P. clarkii*			Positive reference standard		
Pathogens									
E. coli O:157 (ATCC93111)	24	23	23	25	25	25	20	20	20
S. aureus (ATCC 25923)	22	22	20	-	-	-	30	30	30
P. aeruginosa (ATCC9027)	14	14	14	9	9	9	21	21	21

Antibacterial activity of *A. cygnea* and *P. clarkii* hemolymph: The *A. cygnea* extract has stronger bactericidal efficacy against both gram-positive and gram-negative bacteria than the *P. clarkii* extract, as shown in Table 2.

Qualitative profile of total proteins by SDS-PAGE: The results of SDS-PAGE experiments for qualitative characterization of hemolymph total proteins in *A. cygnea* and *P. clarkii* was shown in Fig. 1. The quantity of protein in the *A. cygnea* and *P. clarkii* hemolymph extract was determined to be 100/mg of the sample. The electrophoretic profile of the samples revealed the existence of tiny to high molecular weight protein, with some of them being unique. Under non-reducing circumstances, 14 bands in *P. clarkii* hemolymph and 5 bands in *A. cygnea* hemolymph were visible. Crude protein of *A. cygnea* hemolymph yielded 14 bands ranging from 20-196 KDa with well-defined bands at 20, 20, 21, 23, 25, 32, 37, 41, 48, 59, 107, 152, 154 and 196 KDa and in *P. clarkii* hemolymph was showed 5 bands ranging from 37-196 KDa with will defined bands at 37, 55, 75, 145 and 196 KDa.

Serum liver functions biomarkers: The significant ($p<0.05$) rise in serum alanine transaminase and aspartate transaminase activity, as shown in Table 3, indicated that CS caused hepatic damage, while total proteins level non-significantly changed ($p>0.05$) in septic rats in comparison with the control group. In comparison to the CS group, *A. cygnea* and *P. clarkii hemolymph* injection substantially lowered the examined hepatic enzyme activity ($p<0.05$).

Liver oxidative stress biomarkers: Table 4 showed that the CS group had a significant rise ($p<0.05$) in liver MDA levels and significant declines in both GSH and CAT levels when compared to the control group. When compared to the CS groups, treatment with *A. cygnea* and *P. clarkii* hemolymph generated a significant ($p<0.05$) drop in liver MDA and a significant ($p<0.05$) rise in liver CAT activity, as well as non-significantly modified ($p>0.05$) GSH levels.

Anti-inflammatory markers: In comparison to the control group, CS-induced sepsis resulted in a significant rise ($p<0.05$) in PGE2, IL-6 and Cyt-C levels. Treatment with *A. cygnea* and *P. clarkii* hemolymph significantly decrease ($p<0.05$) the PGE2, IL-6 and Cyt-C levels, as compared to the untreated CS-septic rats (Table 5).

Histopathology of liver: Control group (Fig. 2a) showed normal architecture of the liver. The hepatocytes were arranged in hepatic cords radiating from the central veins to the portal triads. In contrast, the CS group (Fig. 2b-c) showed several histopathological alterations in the liver tissues. The liver exhibited vacuolation (V) of the hepatocytes associated with scattered necrosis (N). The portal area was expanded by moderate numbers of mononuclear cells infiltration (I). The liver of treated group with *A. cygena* (Fig. 2d) showed moderate vacuolation of the hepatocytes. The treated group with *P. clarkii* (Fig. 2e-f) showed few scattered areas of vacuolation in the hepatic parenchyma. The injury score of the liver showed a significant difference in the CS group compared to the two treated groups (Fig. 2g).

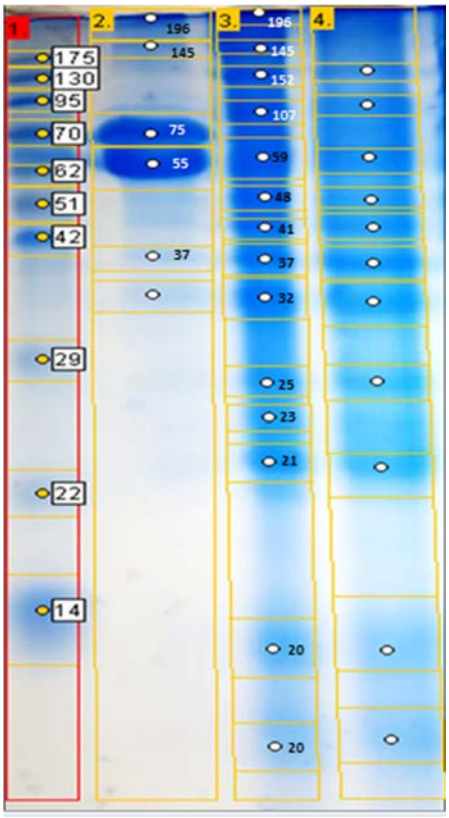

Fig. 1: Electrophoretic profile of the hemolymph extract was analyzed using 15% SDS-PAGE. Gel was stained with Coomassie brilliant blue

Lane 1: Molecular weight of protein and Lane 2: Hemolymph of *P. clarkii* and Lane 3: Hemolymph of *A. cygnea*

Table 3: Effect of *A. cygnea* and *P. clarkii* on serum liver biomarkers analysis of CS-septic rats

		CS		
Variable	Control	Vehicle	*A. cygnea*	*P. clarkii*
AST (U mL^{-1})	7.40±0.51^{a}	11.80±0.52^{c}	9.80±0.20^{b}	10.11±0.26^{b}
ALT (U mL^{-1})	9.00±0.55^{a}	18.20±0.86^{c}	11.18±0.32^{b}	13.90±0.78^{b}
Total protein (g dL^{-1})	3.79±0.26^{a}	3.12±0.29^{a}	3.53±0.17^{a}	3.40±0.12^{a}

Values are Mean±SEM (n = 6) and values with different superscript letters are significantly different (p<0.05)

Table 4: Effect of *A. cygnea* and *P. clarkii* on liver oxidative stress biomarkers of CS-septic rats

		CS		
Variable	Control	Vehicle	*A. cygnea*	*P. clarkii*
MDA (nmol g^{-1} tissue)	6.90±0.28^{a}	11.31±0.67^{c}	7.94±0.31^{ab}	8.96±0.81^{b}
GSH (mg g^{-1} tissue)	8.40±0.89^{b}	4.62±0.43^{a}	5.63±0.41^{a}	5.31±0.61^{a}
CAT (U g^{-1} tissue)	2.71±0.24^{c}	1.48±0.16^{a}	2.13±0.22^{b}	2.02±0.17^{b}

Values are Mean±SEM (n = 6) and values with different superscript letters are significantly different (p<0.05)

Table 5: Effect of *A. cygnea* and *P. clarkii* on some liver inflammatory marker of CS-septic rats

		CS		
Variable	Control	Vehicle	*A. cygnea*	*P. clarkii*
Cytochrome c (ng mL^{-1})	2.81± 0.09^{a}	3.65±0.08^{c}	3.31±0.05^{b}	3.24±0.04^{b}
IL- 6 (ng mL^{-1})	2.76±0.30^{a}	3.67±0.13^{c}	3.37±0.06^{b}	3.33±0.05^{b}
PG-E2(ng mL^{-1})	2.84±0.10^{a}	3.69±0.10^{c}	3.28±0.18^{b}	3.15±0.12^{b}

Values are Mean±SEM (n = 6) and values with different superscript letters are significantly different (p<0.05)

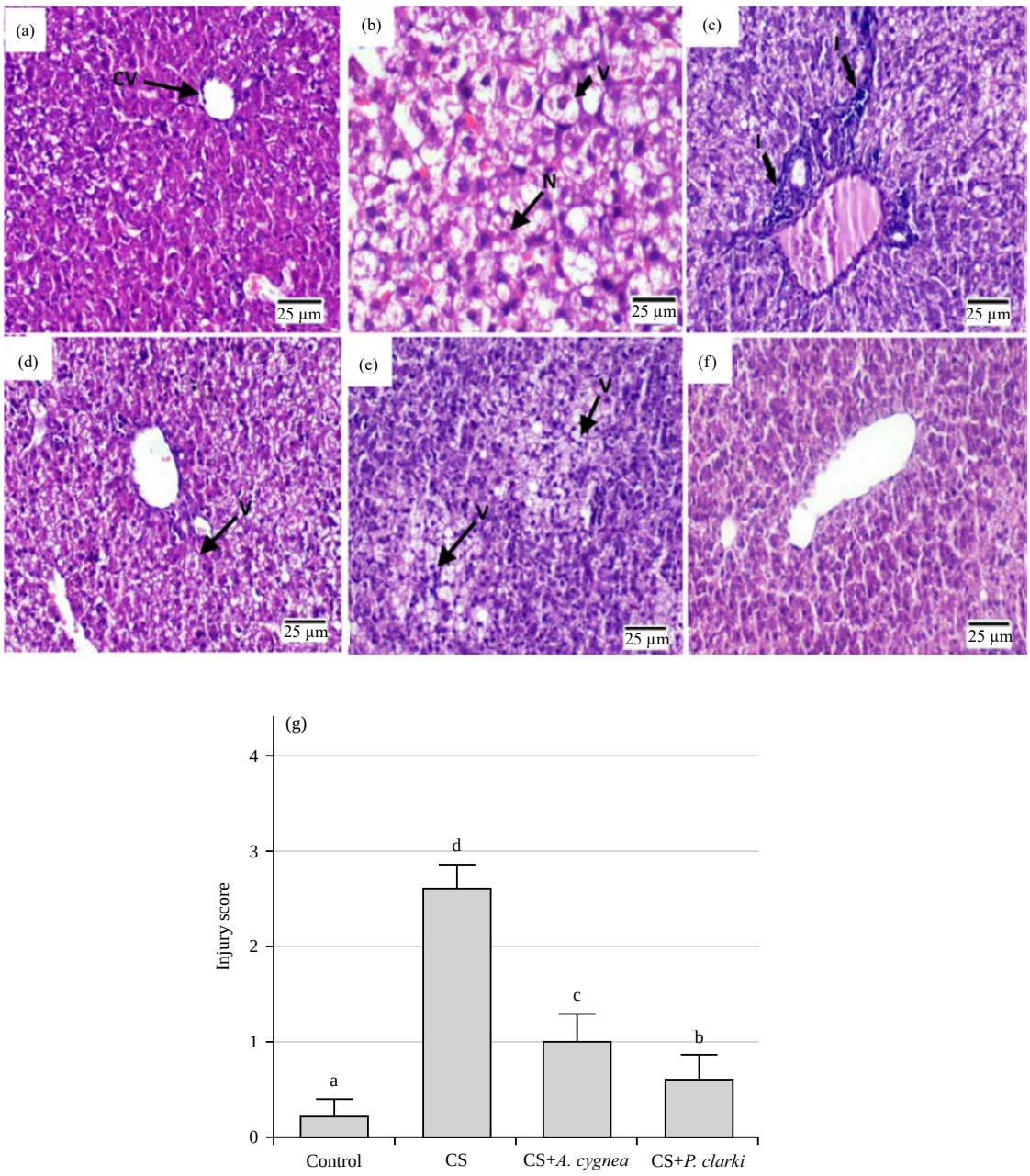

Fig. 2(a-g): Photomicrographs of liver of the control and treated groups stained by hematoxylin and eosin (H&E), (a) Liver section of control group, (b-c) Liver section of CS group, (d) Liver of *A. cygnea* group and (e-f) Liver of *P. clarkii* group
CV: Central vein, V: Vacuolation, N: Necrosis and I: Mononuclear cells infiltration

Immunohistochemistry of liver: The immune labeling of the TNF-α marker showed a normal lower level of positive expression in the hepatic tissues (Fig. 3a). However, the CS group showed marked diffuse expression of the hepatocytes (Fig. 3b). The *A. cygena* group showed a weak positive reaction in the hepatic tissue with widespread negative areas (Fig. 3c). The *P. clarkii* group exhibited a lower level of TNF-α expression in the liver tissue (Fig. 3d) compared to the previously treated and CS groups. The area percent of the TNF-α showed a significant difference in the CS group compared to the two treated groups in the liver. No significant difference was detected in the liver tissue of *A. cygena* in comparison with the CS+*P. clarkii* group (Fig. 3e).

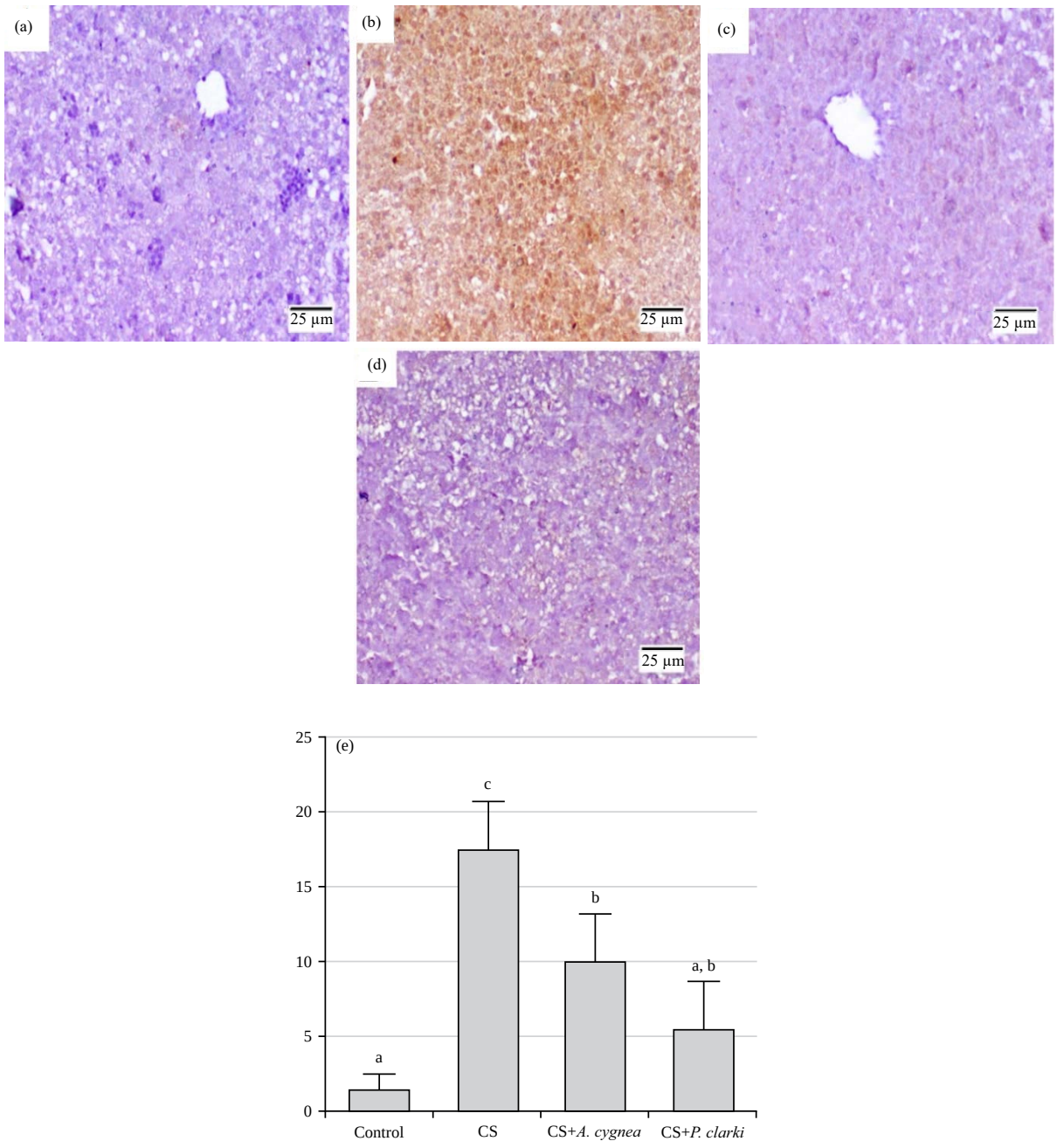

Fig. 3(a-e): Representative images of high and low expression of TNF-a the hepatic tissues (IHC), (a) Liver of rat, control group, showing negative expression of TNF-α marker, (b) Liver of rat, CS group, showing excessive expression of TNF-α marker in the hepatic tissue, (c) Liver of rat (*A. cygena* group), showing negative expression of TNF-α marker in hepatocytes, (d) Liver of rat (*P. clarkii* group), showing weak expression of TNF-α marker in hepatocytes and (e) Expression of TNF-α marker in liver tissue of different experimental groups

Different letters (a-c) above the error bar indicate statistically significant differences at $p \leq 0.05$

DISCUSSION

Neonatal sepsis is a highly inflammatory illness that leads to septic shock and numerous organ dysfunctions as a result of the inflammatory system's uncontrolled activation in response to infections. Because of their immature immune systems, newborns are more susceptible to serious bacterial infections[41,42].

The most fatal consequence in the clinical course of sepsis, multi organ failure, is thought to be initiated in large part by the liver. The hepatic circulation is entered by microbes and their virulence factors, which then activate sinusoidal endothelial cells and Kupffer cells and cause them to generate proinflammatory mediators such TNF, IL-1 and IL-6, reactive oxygen species (ROS) and eicosanoids. These mediators not only destroy bacteria, but also harm the parenchymal cells of the liver, causing structural and functional damage[43]. The liver enzyme activity of untreated septic rats significantly increased, demonstrating that the CS process significantly damaged hepatocellular tissue. An ongoing study suggests that the overproduction of proinflammatory cytokines in response to polymicrobial illness may result in hepatic damage[44].

The current study found that inducing sepsis raised the interleukin-6 (IL-6) and Tumour Necrosis Factor (TNF) in the liver, which might be attributable to KCs' primary defense against portal bacteremia and endotoxemia. In response to excessive lipopolysaccharide (LPS) stimulation, KCs release cytokines, ROS and NO, which damage hepatocytes and endothelial cells[45]. In addition, various cytokines produced by monocytes and macrophages, such as IL-6 and TNF-α are known to be activated by infection in the early neonatal period[46,47]. These findings are supported by the Immunohistochemical examination which clarified that CS had effect on the septic rat liver and it is caused excessive expression of TNF-α marker in the hepatic tissue.

The PGE2 is one of the most prevalent PGs generated in the body, has been studied extensively in animal species and has a wide range of biological actions[48]. In present study demonstrated that sepsis induction increased PGE 2 level in liver tissues which were in agreement with Tveteraas *et al.*[49] who discovered a rise in COX-2 and PGE2 levels, which may contribute to acute and chronic inflammation, oxidative stress, bacterial or viral infection and malignancy following sepsis.

The production of reactive oxygen species (ROS) activated signalling pathways that led to the production of pro-inflammatory mediators and chemokines. In turn, they were create additional ROS and inflammatory prostaglandins in the injured locations, resulting in oxidative stress-induced inflammation[50]. The ROS have been proposed as a biological mechanism underpinning liver damage in sepsis[13]. The imbalance between the production and breakdown of ROS resulted in oxidative stress, which led to the production of free radicals and cellular damage[51]. The shock associated with newborn sepsis is influenced by lipid peroxidation, which contributes to the high morbidity and mortality of neonatal sepsis[52].

The current findings showed that sepsis induction caused liver damage in rats, as evidenced by substantial increases in ALT and AST activities, as well as increased indicators of oxidative stress in the liver, such as MDA content and decrease antioxidant GSH content and CAT activity. The findings of this study show that antioxidant defence systems, such as non-enzymatic antioxidant molecule (GSH) and enzymatic activity such as CAT, are significant in reducing liver damage as reported by Mohamed *et al.*[53]. The accumulation of reactive oxygen and nitrogen species in hepatic tissue might explain the rise in hepatic MDA levels[54,55].

The ROS generation by hepatic mitochondria has been demonstrated to cause mitochondrial oxidative damage, malfunction, edema and Cytochrome c (Cyt c) release in a rat model of sepsis[56]. Jiang *et al.*[57] demonstrated that sepsis induction increased expression of Cyt C in liver tissues. This study confirms these results in liver tissues of CS group. The neonatal sepsis redox cycle' causes oxidative stress and a drop in ATP content in mitochondria, both of which can lead to mitochondrial permeability transition pore opening, organelle swelling and Cyt C release[58]. Cytochrome c can bind to Apoptotic Protease Activating Factor 1 (APAF-1) in the cytoplasm and activate caspase-9, which then activates caspases-3 and -7, resulting in apoptosis[59], which confirmed by the histopathological investigation of liver sections.

The current study's findings clearly shown that the *A. cygnea* hemolymph had higher antibacterial activity against human pathogens (*S. aureus*, *E. coli* and *P. aeruginosa*) than the *P. clarkii* hemolymph, which had intermediate antibacterial activity. This high activity of *A. cygnea* may be due to cationic and amphipathic characteristics of antimicrobial peptides (AMP) in bivalves, which connect to the bacteria's membrane, causing permanent damage[60,61]. The presence of lysozyme, an enzyme that catalyses the degradation of the peptidoglycan cell wall, may explain *A. cygnea's* increased antibacterial activity in this investigation[62]. Anti-lipopolysaccharide factors (ALFs) from arthropods such as *P. clarkia* have been found and described, including their activities of binding and neutralising LPS, binding to bacteria and even antimicrobial and antiviral activity[63]. In addition, *P. clarkii's* antibacterial effect may also be linked to its flavonoid content[27].

The antioxidant and prooxidant effects of the hemolymph of *A. cygnea* and *P. clarkii* were investigated in this work. Antioxidant testing might be based on the measurement of free radical scavenging potency or the evaluation of lipid peroxidation. The data recorded from this study showed that, the *A. cygnea* and *P. clarkii* hemolymph exhibited radical scavenging activity. The hemolymph of *A. cygnea* and *P. clarkii* had a high antioxidant activity, indicating that they might be a rich source of natural antioxidants.

Interestingly, the treatment with *A. cygnea* and *P. clarkii* after the CS procedure resulted in a considerable improvement in liver function indicators, indicating its capacity to reduce hepatic damage. *Anodonta cygnea* and *Procambarus clarkii* hepatoprotective properties may be due to its antioxidant potency, which aids in the recovery of liver damage by boosting antioxidative defense systems[27]. The results showed that both extracts attenuate septic shock as evidenced by a significant reduction in MDA and an increase in GSH levels and CAT activity, indicating their role in quenching reactive intermediates and radical species produced during oxidative stress. These findings were backed up by histopathological analysis, which revealed that *A. cygnea* and *P. clarkii* therapy protected the septic rat liver.

Furthermore, Fahmy and Hamdi[28] shown that *P. clarkii* extract possesses antioxidant activity due to its high taurine, glutamic acid, cysteine and glycine content (the amino acids components of GSH). Many compounds, such as alkaloids, carotenoids, steroids, phenolics and terpenoids, were discovered to be the most prevalent classes of bioactive chemicals in mollusks and may be associated to the hepatoprotective activity of *A. cygnea* hemolymph[64-66].

The present investigation found that *A. cygnea* and *P. clarkii* extracts attenuated TNF-α, IL6 and PGE2 levels in the livers of Aseptic rats. This might be due to Polyunsaturated Fatty Acids (PUFAs) in *A. cygnea*, which have been demonstrated to have anti-inflammatory characteristics[67-69]. *Procambarus clarkii* extract, on the other hand, contains anti-lipopolysaccharide factors (ALFs), which bind to and neutralize lipopolysaccharides (LPS)[63].

The main findings of current study were that both hemolymph injections resulted in a substantial improvement in liver function indicators in neonatal rats following CS, as well as a reduction in mitochondrial dysfunction (assessed from Cyt C release into the cytoplasm). Herein the current findings showed that the hemolymph of *A. cygnea* and *P. clarkii* protects effect against hepatic oxidation, lipid peroxidation, inflammation and liver dysfunction caused by sepsis.

CONCLUSION

Anodonta cygnea and *P. clarkii* hemolymph extracts attenuated the liver injury that induced by sepsis as it improves liver biomarkers, suppresses reactive oxygen species propagation due to its antibacterial and anti-inflammatory activities.

SIGNIFICANCE STATEMENT

Anodonta cygnea and *P. clarkii* hemolymph have antibacterial, antioxidant and anti-inflammatory activities. The main findings of current study were that both hemolymph injections resulted in a substantial improvement in liver function indicators in neonatal rats following CS, as well as a reduction in mitochondrial dysfunction (assessed from Cyt C release into the cytoplasm).

REFERENCES

1. Lyn-Kew, K. and T.J. Standiford, 2008. Immunosuppression in sepsis. Curr. Pharm. Des., 14: 1870-1881.
2. Klouche, K., J.P. Cristol, J. Devin, V. Gilles and N. Kuster *et al*., 2016. Diagnostic and prognostic value of soluble CD14 subtype (Presepsin) for sepsis and community-acquired pneumonia in ICU patients. Ann. Intensive Care, Vol. 6. 10.1186/s13613-016-0160-6.
3. Martin, G.S., 2012. Sepsis, severe sepsis and septic shock: Changes in incidence, pathogens and outcomes. Expert Rev. Anti-infect. Ther., 10: 701-706.
4. Yost, C.C., M.J. Cody, E.S. Harris, N.L. Thornton and A.M. McInturff *et al*., 2009. Impaired neutrophil extracellular trap (NET) formation: A novel innate immune deficiency of human neonates. Blood, 113: 6419-6427.
5. Weiss, S.L., J.C. Fitzgerald, J. Pappachan, D. Wheeler and J.C. Jaramillo-Bustamante *et al*., 2015. Global epidemiology of pediatric severe sepsis: The sepsis prevalence, outcomes, and therapies study. Am. J. Respir. Crit. Care Med., 191: 1147-1157.
6. Liu, L., H.L. Johnson, S. Cousens, J. Perin and S. Scott *et al*., 2012. Global, regional and national causes of child mortality: An updated systematic analysis for 2010 with time trends since 2000. Lancet, 379: 2151-2161.
7. Wattal, C. and J.K. Oberoi, 2011. Neonatal sepsis. Indian J. Pediatr., 78: 473-474.
8. Leviton, A., T.M. O'Shea, F.J. Bednarek, E.N. Allred, R.N. Fichorova and O. Dammann, 2012. Systemic responses of preterm newborns with presumed or documented bacteraemia. Acta Paediatr., 101: 355-359.
9. Reinhart, K., M. Bauer, N.C. Riedemann and C.S. Hartog, 2012. New approaches to sepsis: Molecular diagnostics and biomarkers. Clin. Microbiol. Rev., 25: 609-634.
10. Remick, D.G., 2007. Pathophysiology of sepsis. Am. J. Pathol., 170: 1435-1444.
11. He, Y., W.X. Du, H.Y. Jiang, Q. Ai, J. Feng, Z. Liu and J.L. Yu, 2017. Multiplex cytokine profiling identifies interleukin-27 as a novel biomarker for neonatal early onset sepsis. Shock, 47: 140-147.
12. Khaertynov, K.S., S.V. Boichuk, S.F. Khaiboullina, V.A. Anokhin and A.A. Andreeva *et al*., 2017. Comparative assessment of cytokine pattern in early and late onset of neonatal sepsis. J. Immunol. Res., Vol. 2017. 10.1155/2017/8601063.

13. Andrades, M., C. Ritter, M.R. de Oliveira, E.L. Streck, J.C.F. Moreira and F. Dal-Pizzol, 2011. Antioxidant treatment reverses organ failure in rat model of sepsis: Role of antioxidant enzymes imbalance, neutrophil infiltration and oxidative stress. J. Surg. Res., 167: e307-e313.
14. Dkhil, M.A., S. Al-Quraishy and A.E. Abdel Moneim, 2018. *Ziziphus spina-christi* leaf extract pretreatment inhibits liver and spleen injury in a mouse model of sepsis via anti-oxidant and anti-inflammatory effects. Inflammopharmacology, 26: 779-791.
15. Gutteridge, J.M.C. and J. Mitchell, 1999. Redox imbalance in the critically ill. Br. Med. Bull., 55: 49-75.
16. Varsila, E., M. Hallman and S. Andersson, 1994. Free-radical-induced lipid peroxidation during the early neonatal period. Acta Paediatr., 83: 692-695.
17. Crimi, E., V. Sica, A.S. Slutsky, H. Zhang, S. Williams-Ignarro, L.J. Ignarro and C. Napoli, 2006. Role of oxidative stress in experimental sepsis and multisystem organ dysfunction. Free Radical Res., 40: 665-672.
18. Starr, M.E., A.M. Steele, M. Saito, B.J. Hacker, B.M. Evers and H. Saito, 2014. A new cecal slurry preparation protocol with improved long-term reproducibility for animal models of sepsis. PLoS ONE, Vol. 9. 10.1371/journal.pone.0115705.
19. Mancuso, G., A. Midiri, E. Gerace and C. Biondo, 2021. Bacterial antibiotic resistance: The most critical pathogens. Pathogens, Vol. 10. 10.3390/pathogens10101310.
20. Ali, S.S., N. Medda, S.M. Dutta, R. Patra and S. Maiti, 2020. Protection against mitochondrial oxidative-stress by flesh-extract of edible freshwater snail *Bellamya bengalensis* prevents arsenic induced DNA and tissue damage. Anti-Cancer Agents Med. Chem., 20: 1266-1273.
21. Chakraborty, M., S. Bhattacharya, P. Bhattacharjee, R. Das and R. Mishra, 2010. Prevention of the progression of adjuvant induced arthritis by oral supplementation of Indian fresh water mussel (*Lamellidens marginalis*) aqueous extract in experimental rats. J. Ethnopharmacol., 132: 316-320.
22. Chijimatsu, T., I. Tatsuguchi, K. Abe, H. Oda and S. Mochizuki, 2008. A freshwater clam (*Corbicula fluminea*) extract improves cholesterol metabolism in rats fed on a high-cholesterol diet. Biosci. Biotechnol. Biochem., 72: 2566-2571.
23. Tincu, J.A. and S.W. Taylor, 2004. Antimicrobial peptides from marine invertebrates. Antimicrob. Agents Chemother., 48: 3645-3654.
24. Moloukhia, H. and S. Sleem, 2011. Bioaccumulation, fate and toxicity of two heavy metals common in industrial wastes in two aquatic molluscs. J. Am. Sci., 7: 459-464.
25. Anand, P.T. and J.K.P. Edward, 2002. Antimicrobial activity in the tissue extracts of five species of cowries *Cyprea* sp. (Mollusca: Gastropoda) and an ascidian *Didemnum psammathodes* (Tunicata: Didemnidae). Indian J. Mar. Sci., 31: 239-242.
26. Elmossalami, M.K. and M.T. Emara, 1999. Safety and quality of fresh water crayfish *Procambarus clarkii* in the river Nile. Nahrung, 43: 126-128.
27. Elkhodary, G., D.M. Beltagy, N.M. Samak, K.K. Abdul-Aziz and M.H. Mona, 2017. Assessment of antioxidant, antimicrobial and anticancer activities of carotenoid extracted from *Erugosquilla massavensis* and *Procambarus clarkii* exoskeletons. Int. J. Cancer Biomed. Res., 1: 11-20.
28. Fahmy, S.R. and S.A.H. Hamdi, 2011. Antioxidant effect of the Egyptian freshwater *Procambarus clarkii* extract in rat liver and erythrocytes. Afr. J. Pharm. Pharmacol., 5: 776-785.
29. Lowe, D.M. and R.K. Pipe, 1994. Contaminant-induced lysosomal membrane damage in marine mussel digestive cells: An *in vitro* study. Aquat. Toxicol., 30: 357-365.
30. Nakatsuji, T. and H. Sonobe, 2003. Measurement of molt-inhibiting hormone titer in hemolymph of the American crayfish, *Procambarus clarkii*, by time-resolved fluoroimmunoassay. Zool. Sci., 20: 999-1001.
31. Söderhäll, K. and V.J. Smith, 1983. Separation of the haemocyte populations of CarcinusMaenas and other marine decapods, and prophenoloxidase distribution. Dev. Comp. Immunol., 7: 229-239.
32. Sanchez-Moreno, C., J.A. Larrauri and F. Saura-Calixto, 1998. A procedure to measure the antiradical efficiency of polyphenols. J. Sci. Food Agric., 76: 270-276.
33. Bauer, A.W., W.M.M. Kirby, J.C. Sherris and M. Turck, 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol., 45: 493-496.
34. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
35. Chinedu, E., D. Arome and F.S. Ameh, 2013. A new method for determining acute toxicity in animal models. Toxicol. Int., 20: 224-226.
36. Reitman, S. and S. Frankel, 1957. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am. J. Clin. Pathol., 28: 56-63.
37. Inagawa, A., M. Kimura and N. Uehara, 2022. Total protein assay by PCA-based RGB-spectrum conversion methods with smartphone-acquired digital images. Anal. Sci., 38: 869-880.
38. Ohkawa, H., N. Ohishi and K. Yagi, 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem., 95: 351-358.
39. Cappiello, M., E. Peroni, A. Lepore, R. Moschini, A.D. Corso, F. Balestri and U. Mura, 2013. Rapid colorimetric determination of reduced and oxidized glutathione using an end point coupled enzymatic assay. Anal. Bioanal. Chem., 405: 1779-1785.
40. Aebi, H., 1984. Catalase *in vitro*. In: Methods in Enzymology, Academic Press, Cambridge, Massachusetts, ISBN: 9780121820053, pp: 121-126.
41. Cuenca, A.G., J.L. Wynn, L.L. Moldawer and O. Levy, 2013. Role of innate immunity in neonatal infection. Am. J. Perinatol., 30: 105-112.

42. Wynn, J.L., P.O. Scumpia, R.D. Winfield, M.J. Delano and K. Kelly-Scumpia *et al*., 2008. Defective innate immunity predisposes murine neonates to poor sepsis outcome but is reversed by TLR agonists. Blood, 112: 1750-1758.
43. Ring, A. and W. Stremmel, 2000. The hepatic microvascular responses to sepsis. Semin Thromb. Hemostasis, 26: 589-594.
44. Abraham, E. and M. Singer, 2007. Mechanisms of sepsis-induced organ dysfunction. Crit. Care Med., 35: 2408-2416.
45. Kolios, G., V. Valatas, P. Manousou, C. Xidakis, G. Notas and E. Kouroumalis, 2008. Nitric oxide and MCP-1 regulation in LPS activated rat Kupffer cells. Mol. Cell Biochem., 319: 91-98.
46. Li, Y., Y. Wang, X. Ding, B. Duan, L. Li and X. Wang, 2016. Serum levels of TNF-α and IL-6 are associated with pregnancy-induced hypertension. Reprod. Sci., 23: 1402-1408.
47. Berner, R., P. Welter and M. Brandis, 2002. Cytokine expression of cord and adult blood mononuclear cells in response to *Streptococcus agalactiae*. Pediatr. Res., 51: 304-309.
48. Legler, D.F., M. Bruckner, E. Uetz-von Allmen and P. Krause, 2010. Prostaglandin E2 at new glance: Novel insights in functional diversity offer therapeutic chances. Int. J. Biochem. Cell Biol., 42: 198-201.
49. Tveteraas, I.H., K.M. Müller, M. Aasrum, J. Ødegård and O. Dajani *et al*., 2012. Mechanisms involved in PGE2-induced transactivation of the epidermal growth factor receptor in MH1C1 hepatocarcinoma cells. J. Exp. Clin. Cancer Res., Vol. 31. 10.1186/1756-9966-31-72.
50. Ryan, K.A., M.F. Smith Jr., M.K. Sanders and P.B. Ernst, 2004. Reactive oxygen and nitrogen species differentially regulate toll-like receptor 4-mediated activation of NF-κB and interleukin-8 expression. Infect. Immun., 72: 2123-2130.
51. Farzaei, M.H., M. Zobeiri, F. Parvizi, F.F. El-Senduny and I. Marmouzi *et al*., 2018. Curcumin in liver diseases: A systematic review of the cellular mechanisms of oxidative stress and clinical perspective. Nutrients, Vol. 10. 10.3390/nu10070855.
52. Majeed, M.N. and A. Turki, 2011. Measurement of serum malondialdehyde (MDA) levels as a marker of lipid peroxidation in neonatal sepsis. Univ. Thi-Qar Med. J., 5: 9-17.
53. Mohamed, A.S., S.A. Sadek, S.S. Hassanein and A.M. Soliman, 2019. Hepatoprotective effect of echinochrome pigment in septic rats. J. Surg. Res., 234: 317-324.
54. Zhou, R., R. Zhou, J. Zhuang, Z. Zong and X. Zhang *et al*., 2016. Interaction of atmospheric-pressure air microplasmas with amino acids as fundamental processes in aqueous solution. PLoS ONE, Vol. 11. 10.1371/journal.pone.0155584.
55. Çağlıkülekci, M., C. Pata, D.D. Apa, M. Dirlik and L. Tamer *et al*., 2004. The effect of *N*-acetylcysteine (NAC) on liver and renal tissue inducible nitric oxide synthase (iNOS) and tissue lipid peroxidation in obstructive jaundice stimulated by lipopolysaccharide (LPS). Pharmacol. Res., 49: 227-238.
56. Zapelini, P.H., G.T. Rezin, M.R. Cardoso, C. Ritter and F. Klamt *et al*., 2008. Antioxidant treatment reverses mitochondrial dysfunction in a sepsis animal model. Mitochondrion, 8: 211-218.
57. Jiang, Z., X. Li, Z. Lin, J. Chen, X. Guan and M. Chen, 2015. Ethyl pyruvate reduces hepatic mitochondrial swelling and dysfunction in a rat model of sepsis. Int. J. Clin. Exp. Pathol., 8: 7774-7785.
58. Crouser, E.D., 2004. Mitochondrial dysfunction in septic shock and multiple organ dysfunction syndrome. Mitochondrion, 4: 729-741.
59. Kroemer, G. and J.C. Reed, 2000. Mitochondrial control of cell death. Nat. Med., 6: 513-519.
60. Allam, B. and D. Raftos, 2015. Immune responses to infectious diseases in bivalves. J. Invertebr. Pathol., 131: 121-136.
61. Brogden, K.A., 2005. Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol., 3: 238-250.
62. Kádár, E., J. Salánki, R. Jugdaohsingh, J.J. Powell, C.R. McCrohan and K.N. White, 2001. Avoidance responses to aluminium in the freshwater bivalve *Anodonta cygnea*. Aquat. Toxicol., 55: 137-148.
63. Sun, C., W.T. Xu, H.W. Zhang, L.P. Dong, T. Zhang, X.F. Zhao and J.X. Wang, 2011. An anti-lipopolysaccharide factor from red swamp crayfish, *Procambarus clarkii*, exhibited antimicrobial activities *in vitro* and *in vivo*. Fish Shellfish Immunol., 30: 295-303.
64. Baunbæk, D., N. Trinkler, Y. Ferandin, O. Lozach and P. Ploypradith *et al*., 2008. Anticancer alkaloid Lamellarins inhibit protein kinases. Mar. Drugs, 6: 514-527.
65. Chattopadhyay, S.K. and G. Pattenden, 2000. A total synthesis of the unique tris-oxazole macrolide ulapualide a produced by the marine nudibranch *Hexabranchus sanguineus*. J. Chem. Soc. Perkin Trans. 1, 2000: 2429-2454.
66. Pettit, G.R., Y. Tang and J.C. Knight, 2005. Antineoplastic agents. 545. isolation and structure of turbostatins 1-4 from the Asian Marine Mollusk *Turbo stenogyrus*. J. Nat. Prod., 68: 974-978.
67. Köhler-Forsberg, O. and M.E. Benros, 2018. Efficacy of Anti-Inflammatory Treatment in Depression. In: Inflammation and Immunity in Depression: Basic Science and Clinical Applications, Baune, B.T. (Ed.), Academic Press, United States, ISBN: 978-0-12-811073-7, pp: 525-538.
68. Fokina, N., O. Vasil'eva, I. Sukhovskaya and S. Kurpe, 2020. Cd and Ni modulate fatty acid composition and oxidative status in the freshwater mussel *Anodonta cygnea*. Toxicol. Environ. Health Sci., 12: 169-176.
69. Chakraborty, K., S.J. Chakkalakal and D. Joseph, 2014. Response of pro-inflammatory prostaglandin contents in anti-inflammatory supplements from green mussel *Perna viridis* L. in a time-dependent accelerated shelf-life study. J. Funct. Foods, 7: 527-540.