

## Histopathological and Immunohistochemical Studies of Antiseptic Effect of *Sepia officinalis* Against Induced Sepsis in Male Albino Rats (*Rattus norvegicus*)

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**Abstract:** In the present study we investigated the effect of both methanolic extract and cuttlebone of *Sepia officinalis* against sepsis induced by cecal ligation and puncture CLP in rats. 48 male albino rats were divided into four groups. Group I Sham control rats. Group II CLP model (septic rats). Group III Septic rats treated with body tissue extract of sepia 200mg/kg body weight, one dose daily for seven days. Group IV Septic rats treated with cuttlebone extract 200mg/kg body weight, one dose daily for seven days. It was found that CLP models lead to histopathological changes in the lung characterized by interstitial pneumonia, the kidney showed atrophy of glomerular tuft and congestion of renal blood vessel, the testis showed degeneration of spermatogoneal cells. Group III showed mild histopathological changes. Group I and group IV rats showed no histopathological changes. The immunohistochemical staining of caspase-3 in studied organs showed strong immunopositive reaction in CLP group, mild immunopositivity in Group III, while group I and group IV rats showed no expression of caspase-3. In conclusion, the current study indicated that the body tissue extract of *Sepia* had a mild antiseptic effect against sepsis while its cuttlebone showed strong antiseptic effect.

**Key words:** Histopathology • Caspase-3 • *Sepia officinalis* • Sepsis • Cecal Ligation and Puncture

### INTRODUCTION

Sepsis is a critical condition often caused by bacterial infection and is considered a common cause of morbidity and mortality [1]. Sepsis is the biggest obstacle preventing improvement of the success rate in curing critical illnesses. It is a systemic inflammatory response syndrome (SIRS) induced by infection, is accompanied by the presence of bacteria or a highly suspicious focus of infection leading to a massive, uncontrollable release of inflammatory factors [2]. High levels of inflammatory mediators can lead to the increase of blood capillary permeability and pulmonary oedema, resulting in acute respiratory distress syndrome, multiple organ failure, high mortality and other clinical disorders requiring hospitalization. Cecum ligation and puncture CLP, is currently the most widely used animal model of sepsis [3, 4]. CLP caused an increase in renal capillary

permeability at 2 hours and a decrease in renal capillary perfusion at 4 hours in murine sepsis [5]. Microbial infections, either localized or systemic, can lead to male infertility [6]. Apoptosis is a second prominent feature of sepsis. This process is a mechanism of tightly regulated disassembly of cells caused by activation of certain specialized proteases called caspases. Parenchymal cells, including intestinal and lung epithelial cells, also have increased apoptotic cell death in animal models of sepsis [7, 8]. Caspases have been recognized to play an important role in inflammation and cell death. The role of apoptosis in the development of multiple organ dysfunction syndrome (MODS) and compensatory anti-inflammatory response syndrome (CARS) is not well established [9, 10, 11]. Cytosolic caspase-3 activation is regulated by both TNF- $\alpha$  receptor mediated extrinsic and intrinsic apoptotic cascades [12]. Additionally, *in situ* localization of cleaved caspase-3 has found some favor

for histological labeling of cells in apoptosis [12]. Apoptotic renal tubular cell death was increased significantly 6 hours after CLP [13]. Despite huge expenditure of time and resources on the evaluation of new drugs for the treatment of sepsis, little success has been achieved [14]. In recent years, human pathogenic microorganisms have developed resistance in response to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. The undesirable side effects of certain antibiotics and the emergence of previously uncommon infections has forced scientists to look for new antimicrobial substitutions from various sources such as from plant and animal origin [15]. Lately, Mohanraju *et al.* [16] reported that molluscs have good antibiotic properties. Cephalopods are important as a food resource as well as animal models in scientific investigations [17]. *Sepia officinalis* is one of the most common cephalopods [18]. The present study select cuttlefish (*Sepia officinalis*) member of class cephalopoda due to its availability and its own bioactive compounds. Good antimicrobial activity was seen in the extracts of *O. dollfusi*, *S. lessoniana* and *S. inermis* species, which indicate the presence of potent antimicrobial compounds in them [19]. Methanolic extracts of six species of cephalopods (*Sepia kobeensis*, *Sepiella inermis*, *Sepioteuthis lessoniana*, *Octopus aegina*, *Octopus aerolatus*, *Octopus dollfusi*) showed in vitro antimicrobial activity [19]. The antimicrobial activity of polysaccharides extracted from cephalopods such as *Sepia aculeata* and *Sepia brevimana* and heparin – like glycosaminoglycans (GAGs) from the cephalopod *Euprymna berryi* was reported against the human pathogenic microorganism [20, 21]. Cuttlebone has antibacterial and antifungal activity against some of the human pathogenic microorganisms [22]. Therefore, the present study aims to assess the antiseptic and antiapoptotic effect of the body tissue and cuttlebone extract of *Sepia officinalis* against induced sepsis in male albino rats, using histopathological and Immunohistochemical studies.

## MATERIALS AND METHODS

**Sample Collection:** Fresh cuttlefish (*Sepia officinalis*) were collected from Thuwal area, Saudi Arabia. The animals were transported to our laboratory in an ice box where they were dissected and the cuttlebones were collected and air dried.

**Preparation of Methanolic Extract from the Body Tissue:** The body tissues of the *Sepia officinalis* will cut into

small pieces, homogenized and extracted with methanol at room temperature for 24-48h. Then, the methanolic extract was centrifuged to collect the supernatant and concentrated under vacuum in a rotary evaporator at low temperature. This method will perform according to Ely *et al.* [23].

### Preparation of Polysaccharide Extract from Cuttlebone:

The polysaccharide extract was prepared using the method described by Okutani and Morikawa [24]. The air dried cuttlebones were pulverized and washed with acetone. The powder was extracted with hot 10mM EDTA solution and filtered using Whatman No. 1 filter paper. Then saturated barium hydroxide solution was added to the filtrate. The precipitate obtained after standing overnight was collected on Whatman No. 1 paper and washed with water. The precipitate was dissolved in 10mM EDTA solution and was dialyzed against deionized water. The dialysate was freeze-dried which was then used in the present investigation.

**Animals:** Adult male albino rats (*Rattus norvegicus*) weighing 150 -170 g were obtained from the King Fahd Research Unit in King Abdulaziz University, Jeddah, KSA. Rats were housed in polypropylene cages in an air conditioned room at a temperature of  $25 \pm 2^{\circ}\text{C}$  and under natural day and night cycle. They were fed standard chow pellets and drinking water ad libitum. The rats were kept for one week before the commencement of the experiment for acclimatization.

**Experimental Design:** 48 male albino rats were divided into four groups. Group I Sham control rats, group II CLP model (septic rats) both fed with drinking water ad libitum, while group III Septic rats treated with body tissue extract of sepia fed with cuttlefish methanolic extract at a limit test dose of 200mg/kg body weight, one dose daily for seven days. Group IV Septic rats treated with cuttlebone extract fed with polysaccharide extract at a limit test dose of 200mg/kg body weight, one dose daily for seven days. The animals will observe at 0 min, 30 min, 1, 2, 4, 6 hrs and thereafter every day for 7 days. At the end of the 7<sup>th</sup> day the animals were euthanized by ether anesthesia and dissected for examination of lung, kidney and testis for pathological changes [25, 26].

**Rat Septic Model:** Sepsis is achieved in rats by operating of the cecal ligation and puncture (CLP) process which is considered to be the most clinically relevant models of sepsis [27].

**Histopathological Examination:** Samples for histopathological examination were taken from lung, kidney and testis. Samples were fixed in 10% neutral buffer formalin solution then washed in tap water and dehydrated by different grades of alcohol and cleared by xylene then embedded in paraffin. The paraffin embedding blocks were cut at 5 µm thick. The sections were routinely stained with haematoxylin and eosin [28].

**Immunohistochemistry:** The samples embedded in paraffin were cut into 3µm thick sections and mounted on positively charged slides for caspase-3 immunohistochemistry. Sections were dewaxed, rehydrated and incubated in antigen retrieval buffer (boiling the sections at 98°C for 20 min in 10 mmol/L sodium citrate buffer), treated with 3% H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase. Primary rat-specific antibody for caspase-3 (Thermo Fisher Scientific Co., USA), was added after dilution by PBS (1:100) and incubated for 30 min. The slides were washed three times for 3 min each with PBS. Biotinylated polyvalent secondary antibody (Cat. No. 32230, Thermo Scientific Co., UK) was applied to tissue sections and co-incubated for 30 min. The slides were washed three times for 3 min each with wash buffer. The reaction was visualized by adding Metal Enhanced DAB Substrate Working Solution to the tissue and incubated 10 min. The slides were washed two times for 3 min each with wash buffer. Counterstaining was performed by adding adequate amount of haematoxylin stain to the slide to cover the entire tissue surface [29].

## RESULTS

**Mortality Description:** The mortality rate was significantly higher in CLP group than the other groups. 24 h after CLP, mortality was 16.66% of rats died and it became 8.33% after 2 days and it became 33.32% after 7 days in septic group. Mortality rate was 8.33% of rats died and it became 8.33% after 2 days and it became zero after 7 days in the septic group treated with methanolic body tissue extract. The septic group treated with cuttlebone extract showed mortality rate 8.33% after 24 h and it became zero after 2 days and 7 days. Mortality rate was zero in sham group during the experimental period (Fig. 1).

**Histopathology:** The lung of sham control group showed a normal structure of alveoli and alveolar sacs (Fig. 2A). In contrast, the Septic group showed interstitial pneumonia with edema, leucocytic infiltration,

hyperplasia, vacuolations of bronchial epithelium associated with focal desquamation of epithelial lining and hemorrhage with perivascular round cells infiltration (Fig. 2B, C and D). The septic group treated with methanolic body tissue extract of sepia showed mild interstitial pneumonia (Fig. 2E). The septic group treated with cuttlebone extract showed apparent normal pulmonary parenchyma (Fig. 2F).

The kidney of sham group showed normal glomerular structure with intact Bowman's capsule and normal renal tubular structures (Fig. 4A). The septic group showed a marked atrophy of glomerular tuft, intra-tubular protein casts, dilation and congestion of renal blood vessel with perivascular inflammatory cells infiltration and edema (Fig. 4B, C and D). The septic group treated with methanolic extract of sepia showed protein casts in the lumen of renal tubules (Fig. 4E). The septic group treated with cuttlebone extract showed normal glomerular structure with intact Bowman's capsule and normal renal tubular structures (Fig. 4F).

The testis of sham control group showed normal histological structure of active mature functioning seminiferous tubules composed of spermatogenic cells (spermatogonia, spermatocytes, spermatids) (Fig. 6A). The septic group showed small diameter of seminiferous tubules and degeneration of spermatogoneal cells (Fig. 6B). The septic group treated with methanolic extract of sepia improved histological structure of seminiferous tubules with accumulation of germ cells in the lumen (Fig. 6C). The septic group treated with cuttlebone extract showed normal structure of seminiferous tubules (Fig. 6D).

**Immunohistochemical Staining of Caspase-3:** The immunohistochemical staining of caspase-3 was localized in the cytoplasm and the nucleus of pulmonary cells. The lung of sham control group and the septic group treated with cuttlebone extract showed no expression of caspase-3 (Fig. 3A and D). The Septic group showed strong immunopositive reaction (Fig. 3B). The septic group treated with methanolic extract of sepia showed mild immunopositive reaction (Fig. 3C).

The immunohistochemical staining of caspase-3 was localized in the nuclei of renal tubular epithelial cells. The kidney of sham control group and the septic group treated with cuttlebone extract showed no expression of caspase-3 (Fig. 5A and D). The Septic group showed strong immunopositive reaction (Fig. 5B). The septic group treated with methanolic extract of sepia showed mild immunopositive reaction (Fig. 5C).

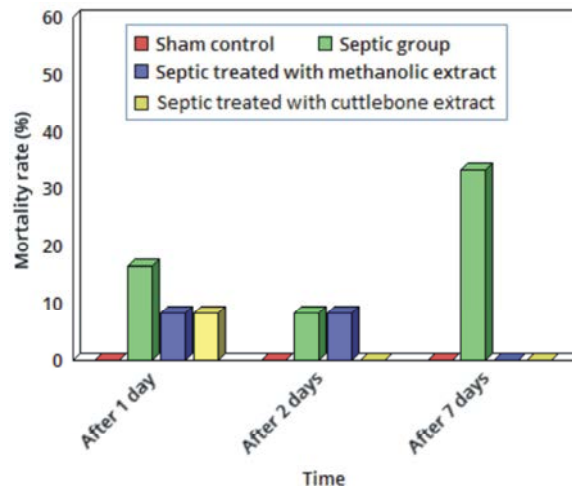


Fig. 1: Mortality rate of group I sham control rats, group II septic rats, group III septic rats treated with methanolic extract and group IV septic rats treated with cuttlebone extract of *Sepia officinalis*.

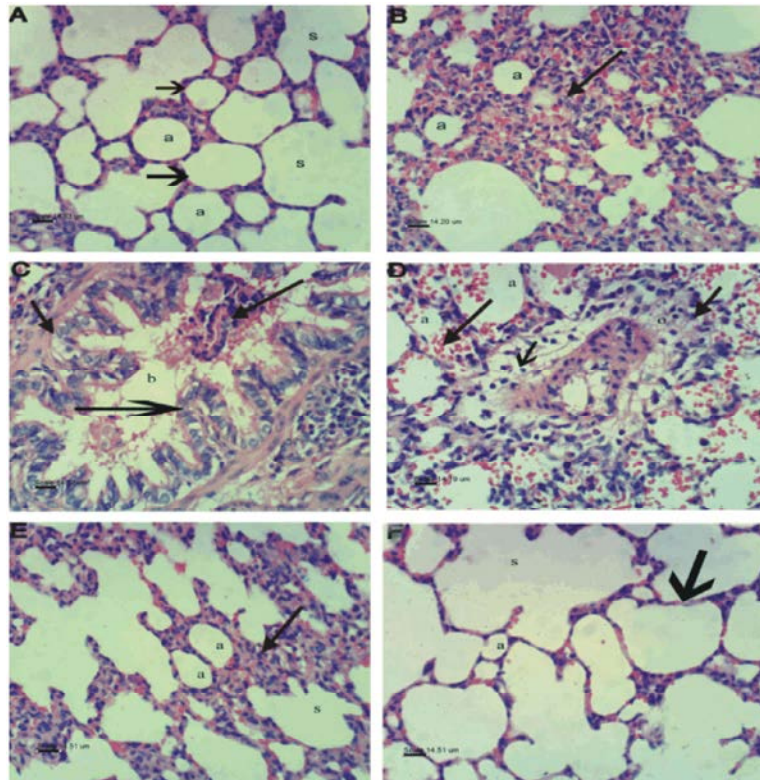


Fig. 2: Photomicrograph of rat's lung. (A) Sham control group showed normal alveolar structure (a), alveolar sacs (s) and normal thin interalveolar septum (arrows): H&E. (bar=14.83 $\mu$ m). (B) Septic group showed interstitial pneumonia with edema and leucocytic infiltration (arrow). Alveolus (a): H&E. (bar=14.20 $\mu$ m). (C) Septic group showed desquamation of superficial lining epithelium (upper long arrow), hyperplasia (lower long arrows) and vacuolations of bronchial epithelium (short arrow) associated with focal desquamation of epithelial lining. Bronchiole (b): H&E. (bar=14.52 $\mu$ m). (D) Septic group showed hemorrhage (long arrow), edema (o) with perivascular round cells infiltration (short arrows). Alveolus (a): H&E. (bar=14.19 $\mu$ m). (E) Septic group treated with body tissue extract of sepia showed mild interstitial pneumonia (arrow). Alveolus (a), alveolar sac (s): H&E. (bar=14.51 $\mu$ m). (F) Septic group treated with cuttlebone extract showed apparent normal pulmonary parenchyma and normal thin interalveolar septum (arrow). Alveolus (a), alveolar sac (s): H&E. (bar=14.51 $\mu$ m)



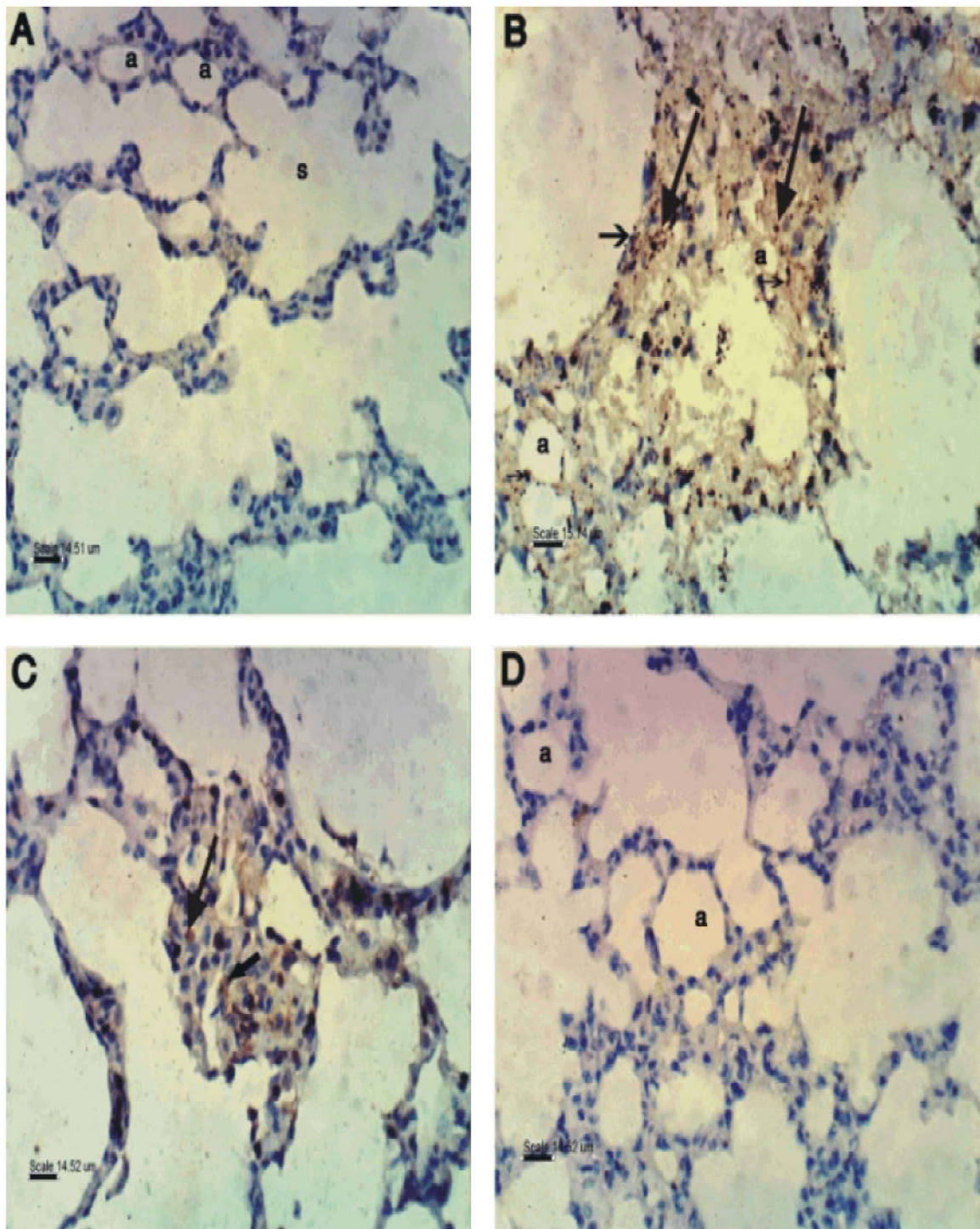


Fig. 3: Photomicrograph of immunohistochemical staining of caspase-3 in rat's lung. (A) Sham control group showed no expression of caspase. Alveolus (a), Alveolar sacs (s): (bar=14.51μm). (B) Septic group showed strong immunopositivity in pneumocytes and round cells (arrows). Alveolus (a): (bar=15.14μm). (C) Septic group treated with body tissue extract of sepia showed mild immunopositivity in pneumocytes and round cells (arrows): (bar=14.52μm). (D) Septic group treated with cuttlebone extract showed no expression of caspase. alveolus (a): (bar=14.52μm)



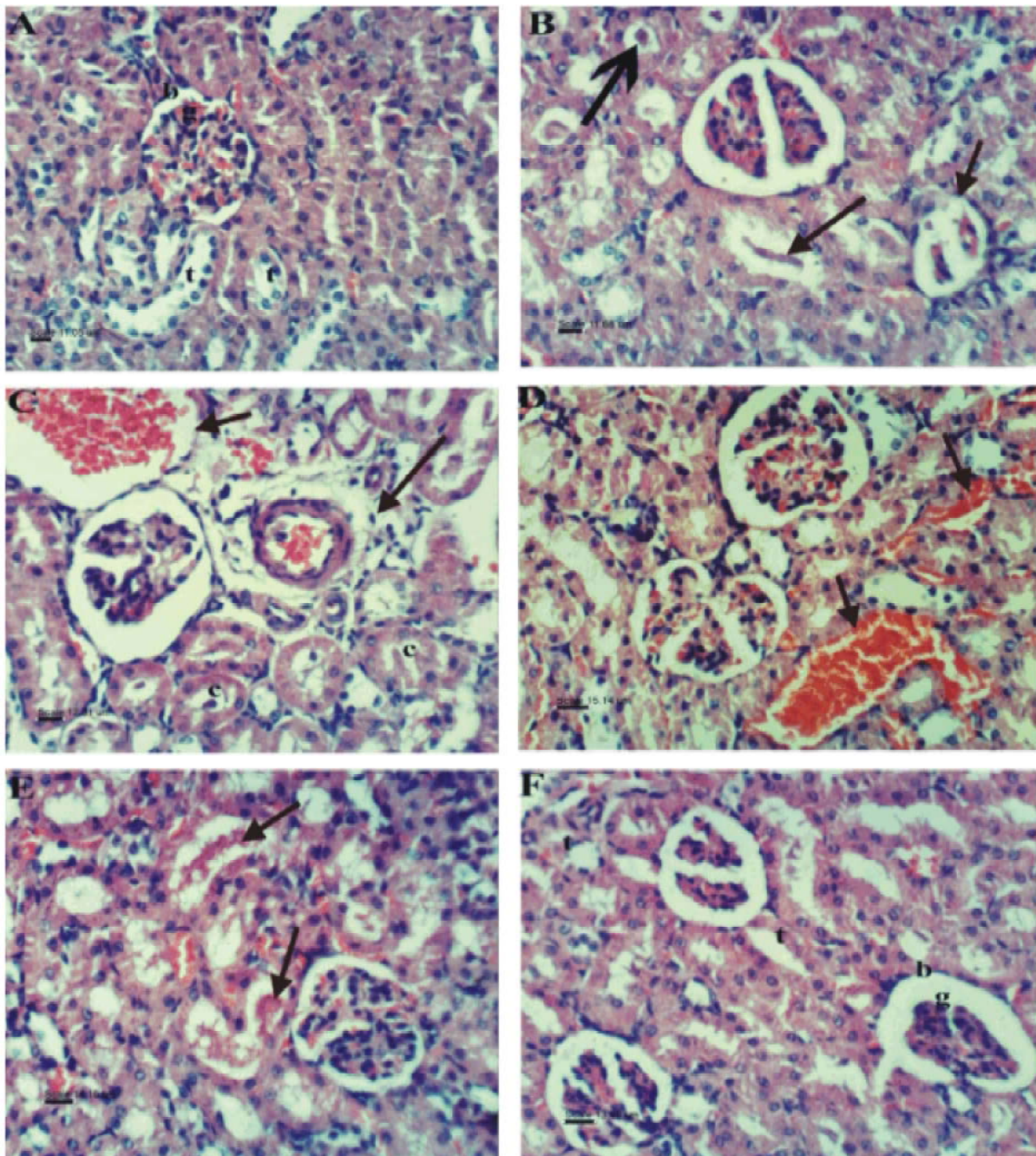


Fig. 4: Photomicrograph of rat's kidney. (A) Sham control group showed normal glomerular structure (g) with intact Boman's capsule (b) and normal renal tubular structures (t): H&E. (bar=11.05 $\mu$ m). (B) Septic group showed atrophy of glomerular tuft (short arrow) and intra-tubular protein casts (long arrows): H&E. (bar=11.68 $\mu$ m). (C) Septic group showed dilation and congestion of renal blood vessel(short arrow) with perivascular inflammatory cells infiltration and edema (long arrow) and intra-tubular protein cast (c): H&E. (bar=12.31 $\mu$ m). (D) Septic group showed sever congestion of renal blood vessel(arrows): H&E. (bar=15.14 $\mu$ m). (E) Septic group treated with body tissue extract of sepia showed protein casts in the lumen of renal tubules (arrows): H&E. (bar=14.19 $\mu$ m). (f) Septic group treated with cuttlebone extract showed normal glomerular structure (g) with intact Boman's capsule (b) and normal renal tubular structures (t): H&E. (bar=13.69 $\mu$ m)



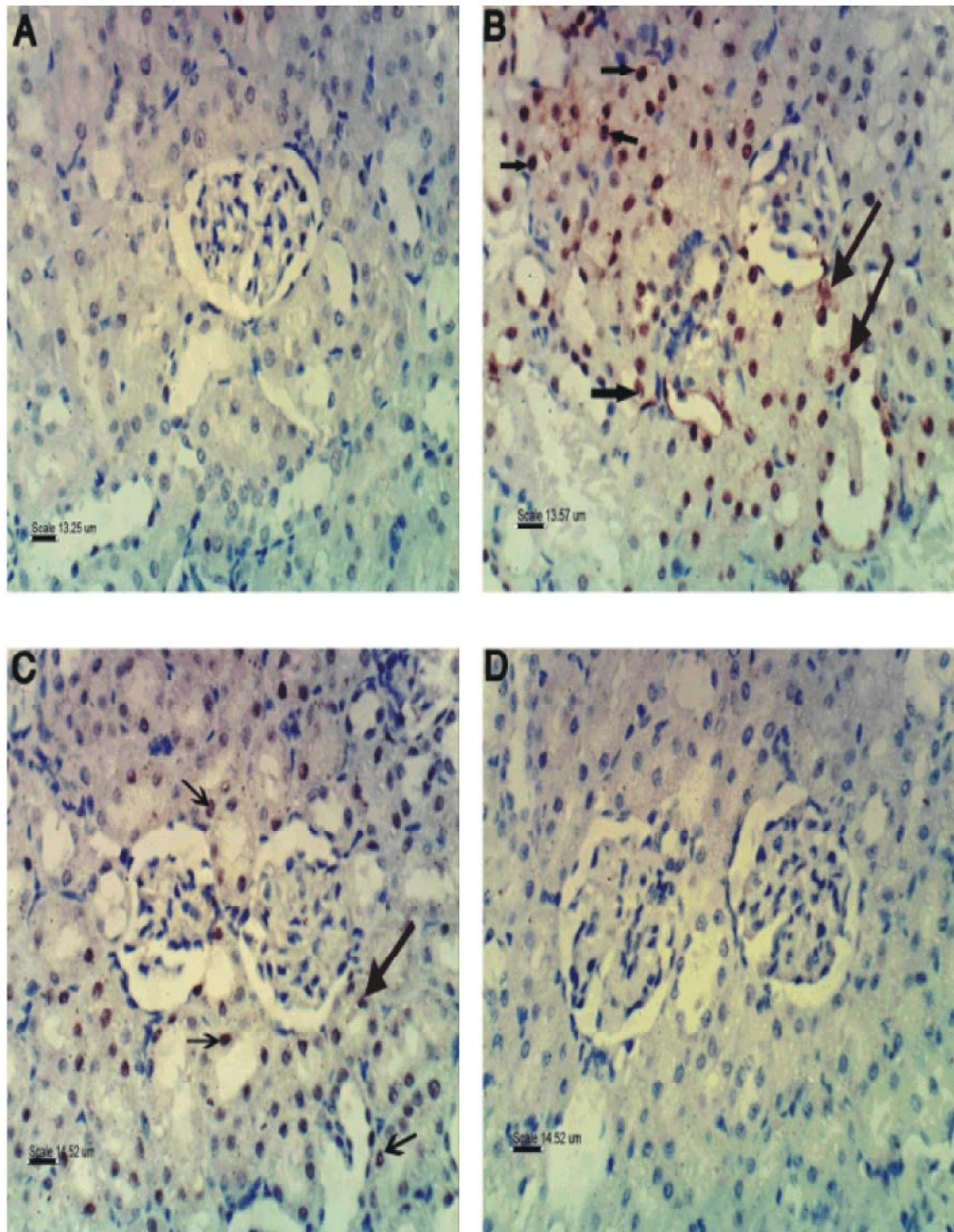


Fig. 5: Photomicrograph of immunohistochemical staining of caspase-3 in rat's kidney. (A) Sham control group showed no expression of caspase: (bar=13.25μm). (B) Septic group showed strong immunopositivity in the nuclei of renal tubular epithelial cells (arrows): (bar=13.57μm). (C) Septic group treated with body tissue extract of sepia showed mild immunopositivity (arrows): (bar=14.52μm). (D) Septic group treated with cuttlebone extract showed no expression of caspase: (bar=14.52μm)



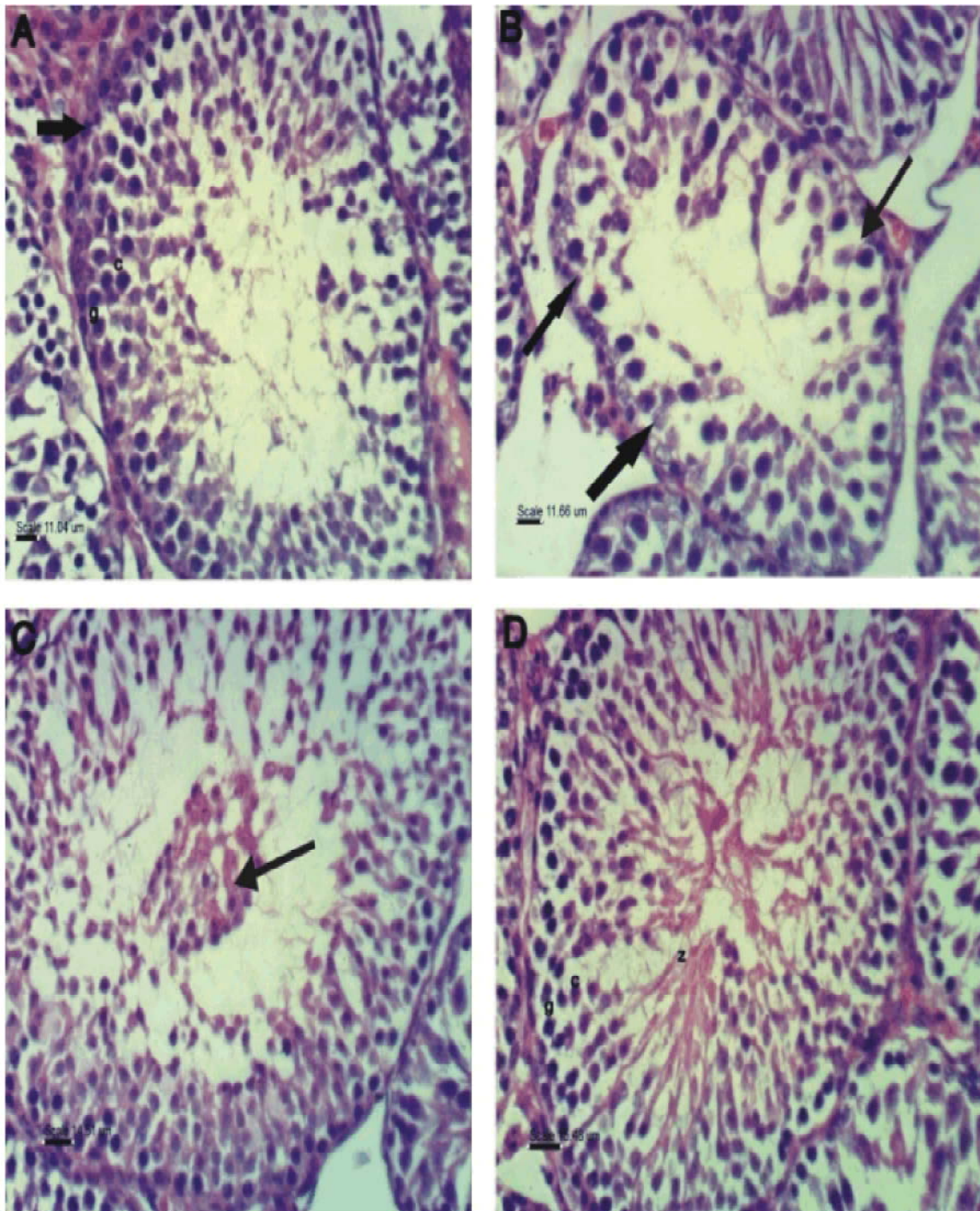


Fig. 6: Photomicrograph of rat's testis. (A) Sham control group showed normal histological structure of active mature functioning seminiferous tubules (arrow) composed of spermatogonia (g) followed by spermatocytes (c): H&E. (bar=11.04 $\mu$ m). (B) Septic group showed small diameter of seminiferous tubules and degeneration of spermatogoneal cells (arrows): H&E. (bar=11.66 $\mu$ m). (C) Septic group treated with body tissue extract of sepia showed desquamated germ cells and accumulated in the lumen (arrow): H&E. (bar=14.51 $\mu$ m). (D) Septic group treated with cuttlebone extract showed normal structure of active mature functioning seminiferous tubules composed of spermatogonia (g) followed by spermatocytes (c) and spermatozoa (z): H&E. (bar=15.48 $\mu$ m)



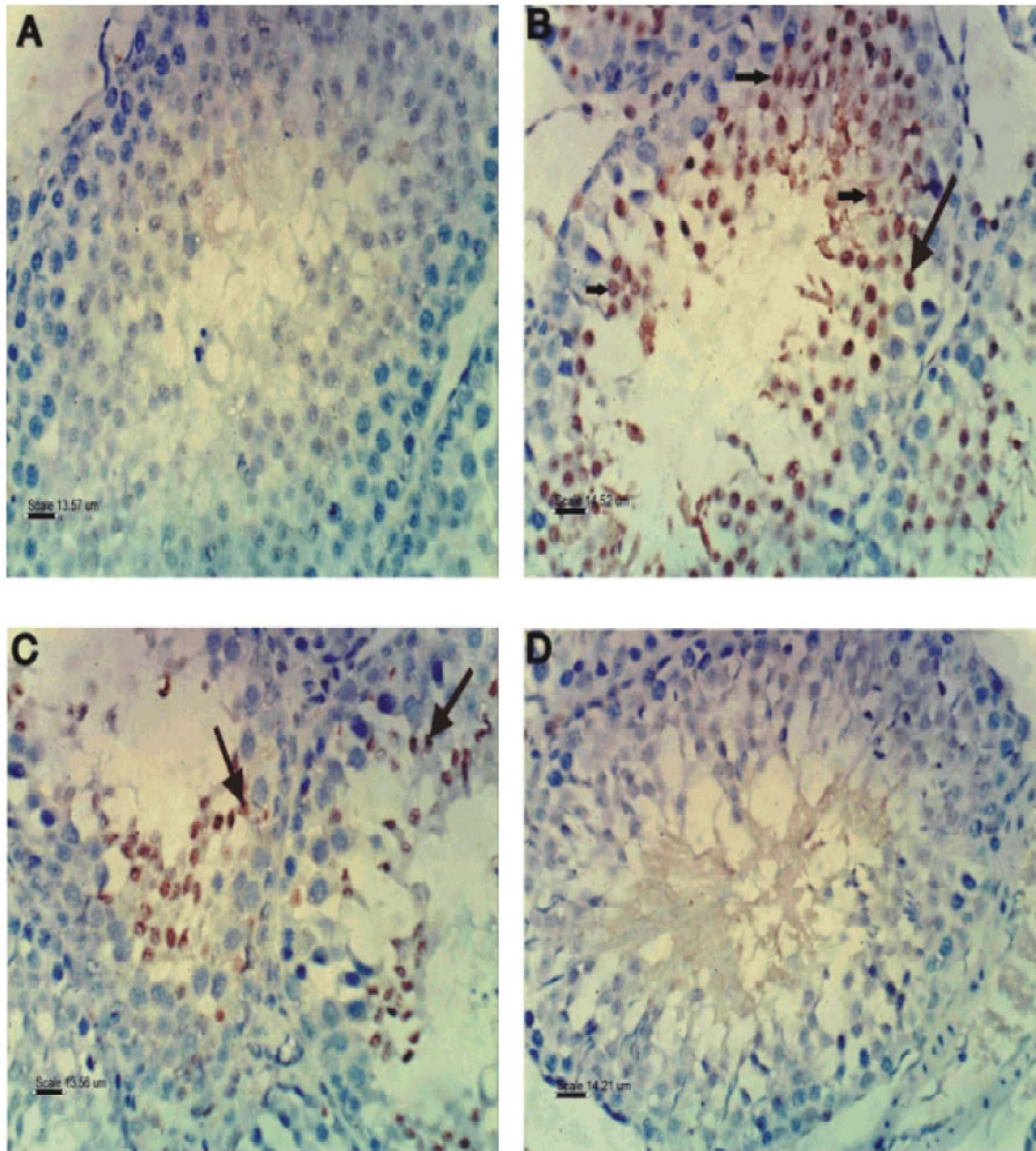


Fig. 7: Photomicrograph of immunohistochemical staining of caspase-3 in rat's testis. (A) Sham control group showed no expression of caspase: (bar=13.57µm). (B) Septic group showed strong immunopositive reaction in the nuclei of spermatogenic cells (arrows): (bar=14.52µm). (C) Septic group treated with body tissue extract of sepia showed mild immunopositive reaction (arrows): (bar=13.56µm). (D) Septic group treated with cuttlebone extract showed no expression of caspase: (bar=14.21µm).

The immunohistochemical staining of caspase-3 was localized in the nuclei of spermatogenic cells. The testis of sham control group and the septic group treated with cuttlebone extract showed no expression of caspase-3 (Fig. 7A and D). The septic group treated with body

tissue extract of sepia showed mild immunopositivity (Fig. 7C). Compared to The septic group treated with methanolic extract of sepia the septic group showed a significant increase in the number of caspase-3 positive cells observed in the seminiferous epithelium (Fig. 7B).

## DISCUSSION

The present study demonstrated by histopathological and immunohistochemical examinations, that sepia tissue extract and polysaccharides attenuate sepsis-induced lung, kidney and testis injuries in the rat model of sepsis.

We have used surgical operation similar to that described by [27] as a sepsis model by cecal ligation and puncture (CLP), which led to persistent fecal leakage from the cecum into the abdomen, thus providing a polymicrobial source of infection in rats and sudden entrance of large numbers of pathogens and its toxins into the peritoneal cavity [30]. The host response towards these invading pathogens is characterized by systemic pro-inflammatory response that is primarily mediated by cytokines, which can lead to fatal multiorgan failure and septic shock [31]. The histopathological lesions, that were observed in the Lung, kidney and testis in rats with sepsis are due to severe bacteraemia and septicaemia that result from rapid transfer of septic pathogens and its toxins from the peritoneal cavity into the systemic circulation. Gram negative aerobes and Facultative anaerobes such as *E. coli* which considers the most common pathogen agents that identified in circulation in man and laboratory animals [32, 33].

The lung is one of the most common organs affected in sepsis and cellular infiltration, together with the release of proinflammatory mediators, leads to the development of acute lung injury, characterized by oedema, hemorrhage, destruction of alveolar wall with severe infiltration of inflammatory cells and these results agreed with [34, 35, 36]. Loss of endothelial barrier integrity results in increased capillary permeability and the production of interstitial and alveolar edema [37]. It is now widely accepted that the formation of inflammatory mediators plays an important role in the pathophysiology of inflammation in acute lung injury [38]. Apoptotic cells and bodies are recognized by specific surface receptors of tissue macrophages and digested [39]. Based on these data, we could conclude that the increased mononuclear cell infiltration in lung tissue in sepsis group compared with the other groups in the present study may be involved in sepsis induced tissue inflammation and high apoptotic activity. Another recent area of interest in the pathogenesis of lung injury is the role of reactive oxygen species. Apart from direct cytotoxic effects, reactive oxygen species have important effects on the inflammatory response mediated via changes in oxidant/antioxidant balance [40].

During sepsis the most common injury to the kidney is atrophy of glomerular tuft, intra-tubular protein cast, dilation and congestion of renal blood vessel with perivascular inflammatory cells infiltration and these results agreed with [39, 41, 33]. Activated macrophages produce oxygen radicals are responsible for cellular lipid peroxidation, protein oxidation and mitochondrial impairment function, which cause further damage to tissues and can induce cell death [42]. Also, Wang *et al.* [5] demonstrated that CLP caused an increase in renal capillary permeability. The cuttlebone extract showed normal glomerular structure with intact Bowman's capsule and normal renal tubular structures. These results were supported by the findings of Subhapradha *et al.* [43] that cuttlebone extract of *Sepia aculeate* showed good antioxidant activity.

The testis of sham and cuttlebone extract treated groups showed normal histological structure of active mature functioning seminiferous tubules, the septic group treated with methanolic extract of sepia improved histological structure of seminiferous tubules, while the septic group showed degeneration of spermatogoneal cells [44]. Also, it has been documented that ROS overproduction associated with inflammatory reactions may be primarily caused by pathological bacterial strains that colonize or infect the reproductive tract [45]. Caspases inactivate proteins that protect living cells from apoptosis and they contribute to cell death by direct disassembly of cell structures [46]. Cytosolic caspase-3 activation is regulated by both TNF- $\alpha$  receptor mediated extrinsic and intrinsic apoptotic cascades (12). In the different pathways inducing apoptosis, caspase-3 appears to play a central role as most pathways result in the activation of caspase-3 [47]. Additionally, *in situ* localization of cleaved caspase-3 has found some favor for histological labeling of cells in apoptosis [12, 33]. Based on these data, we used caspase-3 immunohistochemical staining for determining the apoptosis in pulmonary, renal and testicular tissues. The cytoplasm and the nucleus of pulmonary and round cells in the lung of the Septic group showed strong caspase-3 immunopositive reaction, this result agreed with Ozdulger *et al.* [26], they demonstrated septic lung injury through strong caspase-3 immunopositive reaction in rats. The nuclei of renal tubular epithelial cells of the Septic group showed strong immunopositive reaction, agreed with Messaris *et al.* [13] they investigated the apoptotic death of renal tubular cells in rat CLP model of sepsis and Koca *et al.* [33] they observed high apoptotic activity and renal tissue inflammation in septic induced



rats. The nuclei of spermatogenic cells of the Septic group showed strong caspase-3 immunopositivity, agreed with Karaca *et al.* [48], they observed a significant increase in the number of caspase-3– positive cells in the seminiferous epithelium of the streptozotocin-induced diabetic rats and Lee *et al.* [49] in testicular germ cells of varicocela induced rats.

In lung, kidney and testis from CLP rats caspase-3 positive staining cells were decreased sharply, providing the protective effect of methanol extract treatment on cell apoptosis mediated by sepsis. While, the septic group treated with cuttlebone extract showed no expression of caspase-3 in studied organs, providing the significant antiapoptotic effect of cuttlebone extract. Our results might support earlier studies that the polysaccharide extract from *Sepia aculeate* cuttlebone showed good antioxidant and antiradical activities, so the polysaccharide may be used as a source of natural antioxidant or ingredient in the pharmaceutical industries [43]. The methanolic extract of *Sepia* showed antibacterial and antifungal activity [19, 16].

In conclusion, the present study showed the significant antiseptic and antiapoptotic effect of the cuttlebone of *Sepia* and mild effect of the methanolic extract against induced sepsis in rats. In our opinion, *Sepia officinalis* is a good food source being important in the food menu.

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