Journal of Scientific & Industrial Research
Vol. 84, February 2025, pp. 231-240
DOI: 10.56042/jsir.v84i02.8882

Histopathological Studies on Liver, Kidney and Spleen of *Staphylococcus aureus* Infected BALB/c Mice

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Received 17 February 2024; revised 22 October 2024; accepted 13 November 2024

Worker bees collect resin from tree buds, sap flows and other botanical sources mix it with salivary enzymes and wax to create propolis also referred as "bee glue". They use it to seal gaps in the beehive, reinforce structural stability and protect against microorganisms. This study focuses on therapeutic effects of propolis on histology of liver, kidney and spleen of BALB/c mice infected with *Staphylococcus aureus*. In the experimental regimen, the *Staphylococcus aureus* infected mice group was compared with a normal control group and treatment groups, including those receiving propolis, antibiotics (ampicillin and amoxicillin) and a combination of propolis with antibiotics. Administration of *Staphylococcus aureus* caused notable histological changes in the organs. However, treatment with propolis extract at a dose of 250 mg/kg body weight successfully mitigated the histological alterations in liver, kidney and spleen of infected mice which demonstrate ameliorative efficacy of propolis against *Staphylococcus* mediated damage. Although propolis has been widely recognized for its antimicrobial properties, limited research has explored its histoprotective effects on internal organs in bacterial infections. Furthermore, studies investigating its synergistic potential with conventional antibiotics remain scarce. This study aims to address these gaps by assessing the impact of propolis both independently as well as in combination with antibiotics on organ histology in bacterial infections. This research provides novel insights into the protective role of propolis in mitigating *Staphylococcus aureus* induced histological damage. Additionally, it highlights the potential of propolis as an adjunct therapy to conventional antibiotics paving the way for alternative treatment strategies that could enhance antibiotic efficacy while minimizing resistance development. Future studies should focus on elucidating the precise molecular mechanisms underlying propolis's protective effects and clinical trials are also necessary to validate its therapeutic efficacy in humans.

Keywords: Ameliorative efficacy, Antibiotics, Ethanolic extract, Histopathology, Propolis

Introduction

Staphylococcus aureus, a Gram (+ve), cocci shaped bacterium is commonly found on the skin and upper respiratory tract especially anterior nares. It is the most commonly found micro-biota of human body and acts as a commensal but can also become an opportunistic pathogen by releasing virulence factors. Among them skin infections, respiratory tract infections and food poisoning are common but the bacteria can be infectious to distant organs as it can spread through bloodstream.[1] Generally during infection, an immune reaction is initiated by macrophages which via releasing cytokines activates neutrophils and leads phagocytosis i.e. the primary mode by which staph infections can be controlled by immune system. However, this tiny organisms has evolved to escape the immune reactions by multiple mechanisms such as; its capacity to hijacks host defense system, its ability to evade phagocytosis by surviving within the immune cells, by producing toxic chemicals and enzymes such as; exotoxins, toxic shock syndrome toxins, exo-enzymes and by releasing adhesions and numerous cell associated components.[2,3] Moreover, this bacterium is well known for emergence of antibiotics resistant strains and is found to be leading pathogen for death associated with antimicrobial resistance and hence the combination of all these factors makes it an opportunistic pathogen which can renders the host defenseless.[4,5] Despite much research and development, no vaccines have been approved against *S. aureus*. Thus, antibiotic resistance has encouraged the introduction of natural products such as; medicinal plants and bee engineered products as alternative therapy in addition to antibiotic therapy.[6]

Propolis is one such beehive product, collected by worker honey bees from plant exudates and mixed with beeswax and its salivary secretions. It has attracted the interest of scientists and researchers all over the world on account of its remarkable biological and pharmaceutical properties. It is a very sticky,

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resinous substance which is collected by worker honey bees and is used to seal the walls and cracks of beehive to ensure its hygienic environment.[7] It acts as a natural antibiotic/chemical weapon for honey bees protecting them from the attack of intruders like small insects and microorganisms as well as for embalming large predators such as; lizards. Thus honey bees are remarkably known for maintaining natural sterile environment by using this resinous material.[8]

Research has been done to demonstrate the antibacterial, antifungal, antiviral, antioxidant, anticancer and other biological activities of propolis.[7–10] Its antimicrobial activities are very complex and could be attributed to the synergistic activity between its phenolic acids and flavonoids. Various clinical studies are in progress to verify the ameliorative/curative and preventive potential of propolis alone as well as in combination with antibiotics to see its synergistic behavior. This study is a step to support the use of natural products as a global remedy against *S. aureus* infection in different organs such as; liver, kidney and spleen. Along with propolis, other bee products such as; bee pollen, bee venom, honey and royal jelly are being extensively exploited in this direction.

Though literature about the therapeutic properties of propolis is abundant, however, there are some gaps in its background knowledge such as; the diversity of propolis types, its relation with pathogenic microbe's especially multidrug resistant ones remains to be uninvestigated so far. Moreover several types of propolis from active apicultural centers in India have been poorly studied regarding their antimicrobial potential on animal model (*in vivo* studies) especially on histopathological studies. Despite interesting results obtained from Indian propolis no significant work could be found on the testing of its efficacy and its synergistic behavior with commercial antimicrobial drugs.

In the present study histopathological analysis of different organs of all experimental groups (infected as well as treatment groups) was studied with the aim to determine the ameliorative/curative effect of honey bee propolis in combination with standard antibiotics (ampicillin and amoxicillin) against *Staphylococcus aureus* infection in BALB/c mice.

Materials & Methods

Collection and Preparation of Propolis Extract

Propolis was collected by scrapping it from the comb frames in an apiary located in Chandigarh. For extraction purpose standard protocols were followed.[7–9]

Selection of Dose of Propolis

A pilot study was performed with propolis for determining the dose and days for conducting the experimental regimen. Different doses of propolis were administrated to mice for different days. The results render it noteworthy that propolis dose of 250 mg/kg body weight was able to treat sufficient alterations caused by *S. aureus* infection and enhanced the survival percentage of infected mice.

Animal Model

The animal model used in this study was BALB/c strain of mice, obtained from central animal house, Panjab University, Chandigarh, India. The experimental regimen was according to approved guidelines of institutional ethical committee with approval no: (PU/IAEC/S/14/136), Panjab University.

Animal Sacrifice

Staphylococcus aureus infection was administrated intra-peritonially at a dosage of (0.5×10^6 CFU/ml) and the animals of infected group were sacrificed on 5^{th} day after infection. In treatment groups, propolis and standard antibiotics (ampicillin and amoxicillin) were given orally at 250 mg/kg body weight everyday for 15 days. The animals were sacrificed under sterile conditions in laminar air flow chamber on 16^{th} day.

Experimental design

For this, the animals were divided in to seven groups. Each group comprises of six animals.

G1: Control mice administered with normal saline only
G2: Mice infected with *S. aureus*
G3: *S. aureus* infected mice + propolis treatment
G4: *S. aureus* infected mice + ampicillin treatment
G5: *S. aureus* infected mice + amoxicillin treatment
G6: *S. aureus* infected mice + propolis + ampicillin treatment
G7: *S. aureus* infected mice + propolis + amoxicillin treatment

Microorganisms

S. aureus was purchased from IMTECH, 39 Chandigarh. It was maintained in suitable/respective media (agar plates at 4°C).

Histopathology

In order to study the structural changes or the manifestation of diseases caused by *S. aureus* to liver, kidney and spleen, microscopic examination were carried out. For this the tissues were dissected from normal and infected group on 5^{th} day i.e. peak day of

infection and treatment group at end of experimental regimen i.e. on 16th day. After dissecting out tissues were washed properly with normal saline and were fixed in Bouin's fixative. They were further processed for dehydration/clearing and impregnation by using automatic tissue processor and were finally embedded in paraffin blocks. About 5 µm thick transverse sections were cut by using microtome and were stained by haematoxylin and counterstained with eosin. At last, microscopic examination (light microscope) was carried out for observing histological changes observed after *S. aureus* infection and in treatment groups.[8,10]

Results & Discussion

The micro-architecture of liver, kidney and spleen was observed by performing histopathological studies. *S. aureus* was found to cause oxidative stress by surviving within phagocytic cells and propolis ameliorates the damages caused by staph infections.[11–13] Thus, in the present study, curative properties of propolis (propolis therapy), ampicillin and amoxicillin (antibiotic therapy) alone as well as in combination (combinational therapy) were evaluated in *S. aureus* infected BALB/c mice.

The clinical signs observed after *S. aureus* infection in BALB/c mice demonstrated that the infection has occurred following *S. aureus* administration in experimental animals. The symptoms were suppurative inflammation i.e. abscess formation in various tissues, leukocytosis where the white blood cell count observed is more than normal i.e. infected mice have increased levels of circulating B and T lymphocytes as well as increased levels of neutrophils and monocytes. Other clinical symptoms observed after *S. aureus* administration was skin lesions such as; boils, styes and furunculosis. These symptoms confirmed that infection has occurred following *S. aureus* administration in BALB/c mice.

Histopathology of Kidney, Liver and Spleen

Kidney

It is very important to study the effect of microbial infections on cyto-architecture of kidney, as they are vital organs responsible for selective re-absorption and blood filteration. They also regulate blood pH & blood volume, aid in blood cells formation and also maintaining homeostasis.

The histological studies observed for normal mice kidney (G1) revealed typical organization consisting of outer cortex, inner medulla, (Fig. 1; Plate 1) and a single papilla. Nephrons are the basic functional unit of kidney which comprises "Renal corpuscle" (glomerulus *i.e.* "a capillary bed" surrounded by the Bowman's capsule) and "Renal tubules" (proximal convoluted tubule, distal convoluted tubule, descending and ascending limbs of loop of Henle). Bowman's capsule comprising a visceral layer of epithelial cells called podocytes forms the distended end of the renal tubule. A bunch of capillaries invaginates into the Bowman's capsule and forms a network called glomerulus. It contains specialized intra-glomerular pericytes called Mesanglial Cells (MC) that function as structural support for the capillary network. This organization of the renal corpuscle was clear and intact in the sections of normal kidney studied during present investigation (Fig. 1; Plate1).

The *S. aureus* infected (G2) kidney on the other hand revealed severe damage and disorganization of structure. Glomerular constriction and necrotic changes were observed in the glomeruli. Extensive damage to epithelium, convoluted tubule and renal capsule was evident.

The observed necrotic changes leads renal failure which can be authenticated from kidney functions tests from previous studies of the authors.[7] Further, there was abnormal proliferation of MC between loops which led to congestion. Shrinkage of renal corpuscles and vacuolation of tubules was observed (Fig. 1; Plate 2). Mesangial space was observed to be increased in sections of infected kidney under higher magnification. These observed alterations in *S. aureus* infection were in line with the findings of Mahitha *et al.*[14] who observed $AlCl_3$ mediated histopathological changes in the renal tissue.

After propolis treatment (G3), the morphology of brush border cells was maintained as in the normal group, though renal capsule was still ruptured and vacuolated (Fig. 1; Plate 3). These observations were also supported by previous studies.[15–19] where propolis was found to be source of antioxidant, and plays important role in preventing tissue damage caused by microorganisms and apoptosis.

In ampicillin treated group (Fig. 1; Plate 4) and amoxicillin treated group (Fig. 1; Plate 5) cortex and medulla were more clearly distinguishable as compared to the propolis treated group (Fig. 1; Plate 3). Further in G4 and G5, there was intact capsule and well defined mesangial space in the renal corpuscle. There was constriction of the MC around the

Bowman's capsule and regular morphology of the PCTs, DCTs and CTs as observed. Amoxicillin treatment was found to be more effective than ampicillin in restoring the histology.[7] Further, regular kidney morphology with intact renal capsule, clearly distinguishable cortex and medulla, reduction in mesanglial spaces, increased MC proliferation, regular PCTs DCTc morphology and glomerular constrictions similar to G1 were observed in G6 & G7 i.e. propolis and antibiotics (ampicillin and amoxicillin) treatment groups (Fig. 1; Plate, 6 & 7). Observations revealed that propolis along with

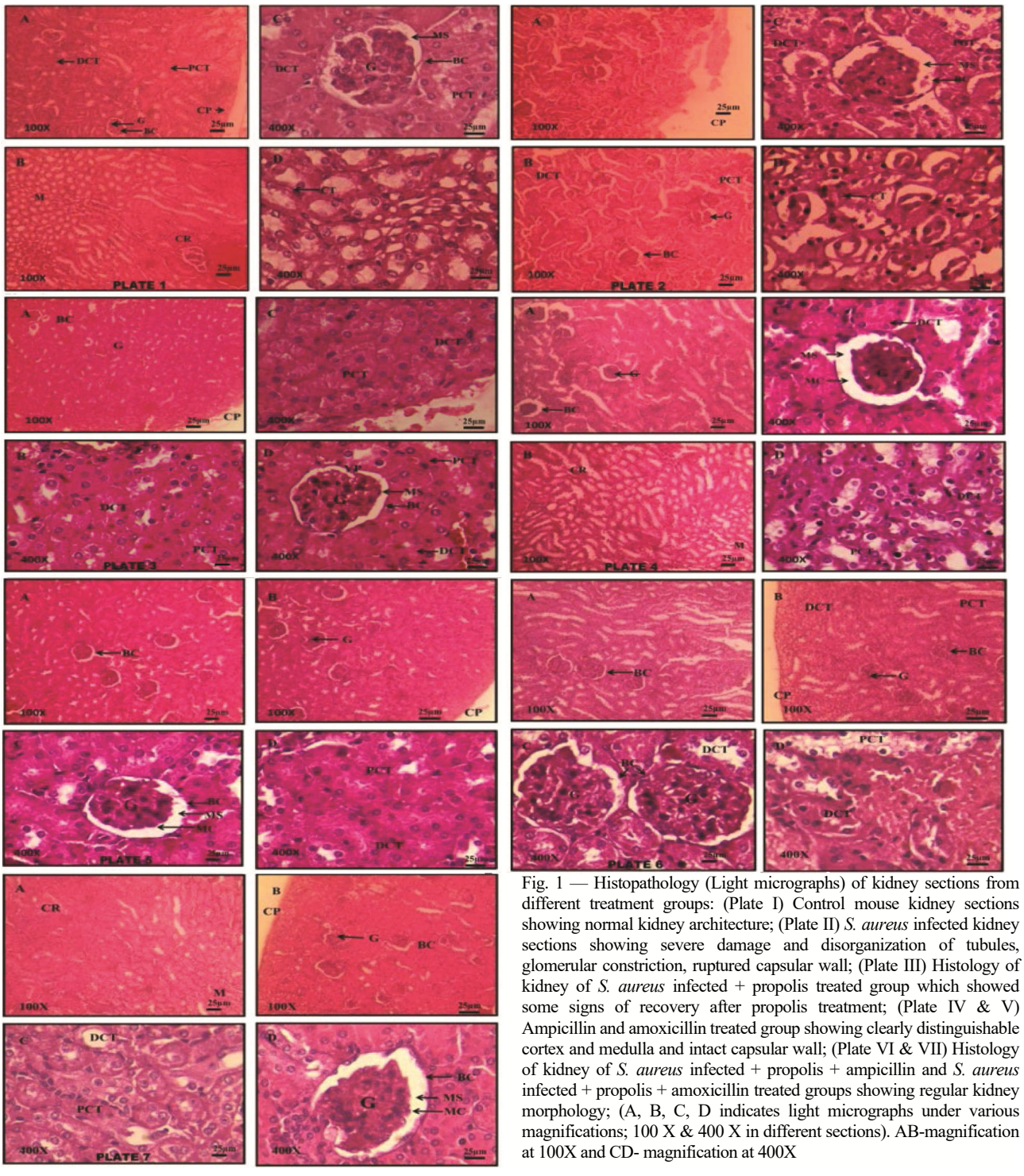

Fig. 1 — Histopathology (Light micrographs) of kidney sections from different treatment groups: (Plate I) Control mouse kidney sections showing normal kidney architecture; (Plate II) *S. aureus* infected kidney sections showing severe damage and disorganization of tubules, glomerular constriction, ruptured capsular wall; (Plate III) Histology of kidney of *S. aureus* infected + propolis treated group which showed some signs of recovery after propolis treatment; (Plate IV & V) Ampicillin and amoxicillin treated group showing clearly distinguishable cortex and medulla and intact capsular wall; (Plate VI & VII) Histology of kidney of *S. aureus* infected + propolis + ampicillin and *S. aureus* infected + propolis + amoxicillin treated groups showing regular kidney morphology; (A, B, C, D indicates light micrographs under various magnifications; 100 X & 400 X in different sections). AB-magnification at 100X and CD- magnification at 400X

antibiotics treatment showed higher ameliorative/ curative properties as compared to individual propolis/antibiotic treatment in restoring the normal kidney morphology.

Liver

Liver plays very important role in detoxification, metabolism and transformations. Hence this organ is vulnerable to microbial infections and drug induced injuries or toxicity caused by drugs/ medicines.[20]

The histology of normal liver revealed a huge network of hepatic cords with large polygonal cells called hepatocytes arranged in single-cell thick plates separated by vascular sinusoids (Fig. 2; Plate 1). The sinusoids showed typical fenestrated endothelial cells and Kupffer cells which constituted about 70–80% of macrophages of the reticulo-endothelial system responsible for phagocytosis. The hepatocytes along with vascular channels formed organized micro structures constituting the structural and functional unit of liver. There were innumerable liver lobules which were hexagonal structures consisting of a central vein surrounded by radiating hepatocyte plates. The portal tracts could be seen as triangular or round structures which enclosed portal veins, terminal branches of hepatic artery and bile ducts embedded in fibrous connective tissue. Except for a few lymphocytes no significant population of any inflammatory cells were present here. Especially no plasma cells, eosinophils, or neutrophils were seen.

The *S. aureus* infected liver showed prominent alterations like presence of scattered, small nodular abscesses composed of degenerated hepatocytes which were infiltrated with polymorphonuclear cells (Fig. 2; Plate 2). The infiltration of plasma cells, eosinophils, neutrophils and lymphocytes were also observed. Inflammation of tissues and thrombosis along with hepatitis was seen after the infection. The Kupffer cell hyperplasia, acute liver necrosis, portal triaditis and enlargement or vacuolization of sinusoids was the main alteration found in the *S. aureus* infected liver.

Previous studies also reported that infected liver showed continuously increased formation of granulomatous lesions and formation of polymer-phonuclear cells with variable degree of necrosis and authenticate. Above mentioned necrotic changes seen in the present study might be responsible for the liver failure as indicated by liver function tests. It was also corroborated from previous studies that after microbial infection the hepatic plates were structurally disintegrated; showed dilated sinusoids and increased intracellular gaps. It has been suggested that variable degree of necrosis and initiation of tissue death/apoptosis could be induced by the activation of caspases.[21,22] The generation of reactive oxygen species is an important mediator of tissue injury and the enhanced production of the oxidants was responsible for damage during the infection. Imbalance between free radicals and the natural antioxidants caused oxidative stress and hence tissue injury.[23] In the previous studies it has been revealed that the damage during infection was due to the toxins produced by the bacterium that caused proliferation of reticuloendothelial cells which have malignant properties.[24] This led to formation of irregular shaped phagocytic cells which caused focal lesions in the organ by filling the lymph spaces and vessels around portal vein in liver

In present investigation histology of *S. aureus* infected and propolis treated liver (Fig. 2; Plate 3) revealed improvement in the cyto-architecture of liver. The treatment was able to ameliorate symptoms of *S. aureus* infection to some extent. At higher magnification partially recovered hepatocytes and portal vein were found. Partial infiltration of polymorphonuclear cells with lesser infiltration of plasma cells and vacuolization was observed. Slight inflammation of tissues was, however, observed as compared to the control group (G1). Rana *et al.*[24] reported that propolis treated group restored the normal histology of liver with regular polyhedral hepatocytes radiating outwards from the central vein. The Kupffer cells hyperplasia decreased and no infiltration of polymorphonuclear cells was observed in mice after propolis treatment at different doses in their study. El- Mawla *et al.*[25] also reported that with the help of propolis multifocal necrotic changes in liver were completely reversed showing its effectiveness. Jabir and Hadi[26] also evaluated the protective efficacy of ethanolic extract of propolis against the oxidative stress and hepatotoxicity induced by CCl_4 in white male rabbits. The modulatory potential of propolis was confirmed by improvement in both biochemical and histoarchitectural parameters after propolis treatment.

Saddiq and Elhalwagy[27] also explored the anti-staphylococcal potential of Saudi Arabia propolis. Their studies revealed; *S. aureus* mediated injury in hepatocytes, elevated liver function enzymes/biomarks as well as increased inflammation

and angiogenesis markers. They also revealed increased oxidative stress i.e. increased lipid peroxidation, accompanied by decreased activity of antioxidant enzymes as well as induced DNA damage. Their investigation demonstrated the fact that aqueous extract of propolis improved the *S. aureus* mediated alterations in hepatocytes and also decreased lipid Peroxidation which further led to increase in the activity of anti-oxidative enzymes.

In ampicillin treated group (Fig. 2; Plate 4) and amoxicillin treated group (Fig. 2; Plate 5) during the present study, normal architecture of the central vein

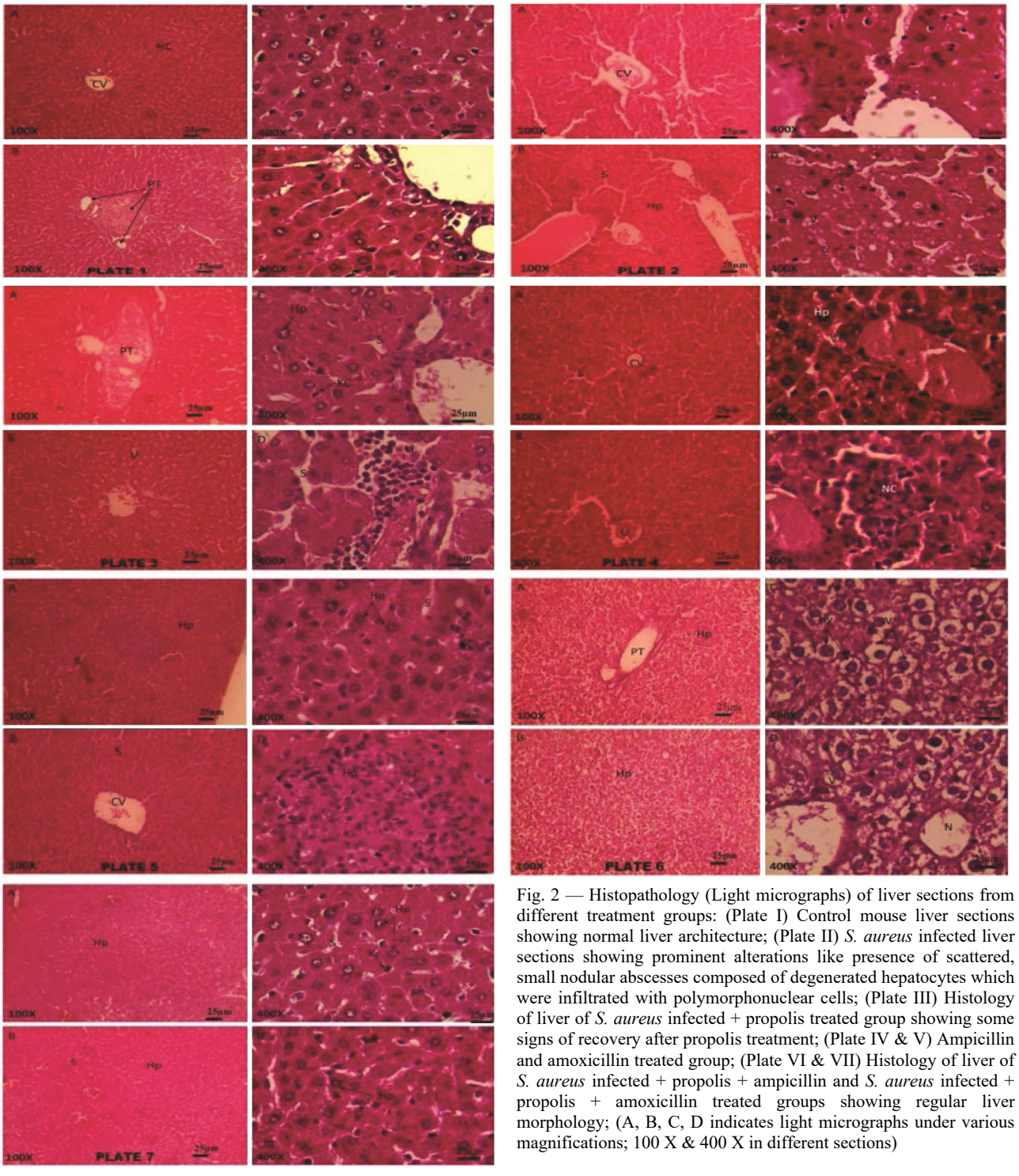

Fig. 2 — Histopathology (Light micrographs) of liver sections from different treatment groups: (Plate I) Control mouse liver sections showing normal liver architecture; (Plate II) *S. aureus* infected liver sections showing prominent alterations like presence of scattered, small nodular abscesses composed of degenerated hepatocytes which were infiltrated with polymorphonuclear cells; (Plate III) Histology of liver of *S. aureus* infected + propolis treated group showing some signs of recovery after propolis treatment; (Plate IV & V) Ampicillin and amoxicillin treated group; (Plate VI & VII) Histology of liver of *S. aureus* infected + propolis + ampicillin and *S. aureus* infected + propolis + amoxicillin treated groups showing regular liver morphology; (A, B, C, D indicates light micrographs under various magnifications; 100 X & 400 X in different sections)

with well defined sinusoids was observed. There was only mild infiltration of mononuclear cells. No signs of necrosis were observed in amoxicillin treated group as compared to the ampicillin (G4) and propolis alone (G3) treated groups. This demonstrated that amoxicillin treatment was more effective than ampicillin or propolis in restoring liver histology.

Sections of *S. aureus* infected + propolis + ampicillin treated (Fig. 2; Plate 6) and *S. aureus* infected + propolis + amoxicillin treated (Fig. 2; Plate 7) liver exhibited regular liver morphology with no patch of necrosis and normal architecture of central vein with well defined sinusoids and distinct Kupffer cells. Al-Waili *et al.*[28] observed that propolis prevented the growth of microorganisms in single and mixed microbial cultures and had synergistic effect when used with honey or ethyl alcohol. Rana *et al.*[24] also reported the synergistic behavior of bee product propolis in combination with antibiotic cefixime against *Salmonella enteric* in an *in vitro* study. In G6 and G7, binucleated hepatocytes with negligible infiltration of lymphocytes and polymorpho-nuclear cells and well defined Kupffer cells were observed which demonstrated the synergistic behavior shown by propolis with antibiotics used in the present study. Hence it is concluded that propolis with ampicillin and amoxicillin showed higher therapeutic efficacy as compared to propolis and antibiotic treatment alone.

Spleen

It is a small organ situated above the stomach inside left rib cage and is a part of lymphatic system (largest secondary lymphoid organ) which is associated with generation of immune reactions. The main role it imparts to our body is blood purification. For immunity generation it makes immune cells/ white blood cells.

Its histology/microscopic observations revealed two important compartments: red pulp and white pulp responsible for these functions (Fig. 3; Plate 1). The red pulp is responsible for filtering blood from foreign material and also filters worn out/damaged red blood corpuscles. It also acts as a storage site for RBCs and platelets.[29,30] It was also observed to be site of hematopoiesis in some mammals like rodents. The white pulp region of spleen contains about 25% of body's immune cells (granulocytes as well as agranulocytes) hence known as the largest secondary lymphoid organ.[31,32] Histological studies revealed further three sub-compartments of white pulp such as: periarteriolar lymphoid sheath, follicles, and the marginal zone which is responsible for separating the red and white pulp. The functional role attributed to this compartment is screening and filtering blood from foreign pathogens.[31,33]

In present study the histology of *S. aureus* infected (Fig. 3; Plate 2) spleen revealed severe alteration in normal architecture. The red and white pulps were found to be degenerated and were not clearly distinguishable. Moreover, due to infiltration, the marginal zones were enlarged and numbers of follicles were increased. Most obvious damage caused by bacterial infection was ruptured capsular valve (Fig. 3; Plate 2).

Teixeira *et al.*[12] investigated *S. aureus* infection and capacity of animals to respond to this organism's systemic infection during autogenous transplantation. Results revealed higher number of colony forming units in splenectomized mice which henceforth proved that splenectomy is detrimental to immune responses and can be reestablished by autogenous implantation of the spleen.

On the other hand, the treatment group (propolis & antibiotics) alone showed recovery to some extent, while in combinational therapy (propolis along with antibiotics) recovery to tissue damage was observed in the form of clearly distinguishable, regenerated red and white pulp with decreased number of follicles and clear marginal zones along with intact capsular wall. In propolis alone treated group, the capsular wall was regenerated but internal damage caused by *S. aureus* was not ameliorated, the red and white pulp were still not clearly distinguishable and due to infiltration of white blood cells in red pulp enlargement of red pulp along with disturbed marginal zone was observed (Fig. 3; Plate 3). The antibiotics treatment group (Fig. 3; Plate 4 & Plate 5) ameliorated the spleen architecture to some extent with separate red and white pulp, intact capsular wall. The combinational treatment groups (Fig. 3; Plate 6 & Plate 7) restore the normal histology of spleen proving the synergistic behavior of propolis with antibiotics (ampicillin and amoxicillin). The results of current study were supported by Chakraborty *et. al*.[34] They also observed synergistic behavior between propolis and antibiotics bacterial infection.

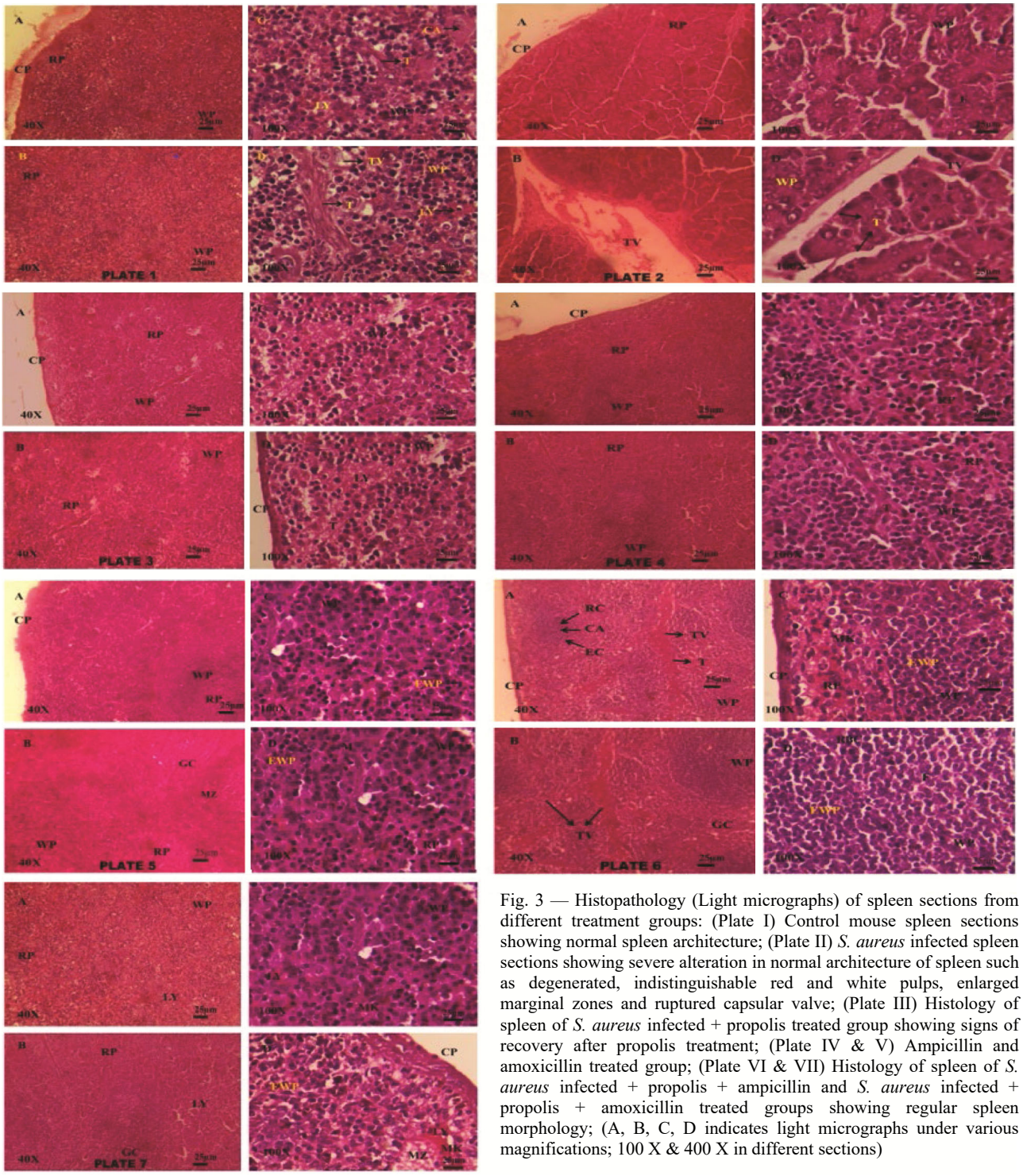

Fig. 3 — Histopathology (Light micrographs) of spleen sections from different treatment groups: (Plate I) Control mouse spleen sections showing normal spleen architecture; (Plate II) *S. aureus* infected spleen sections showing severe alteration in normal architecture of spleen such as degenerated, indistinguishable red and white pulps, enlarged marginal zones and ruptured capsular valve; (Plate III) Histology of spleen of *S. aureus* infected + propolis treated group showing signs of recovery after propolis treatment; (Plate IV & V) Ampicillin and amoxicillin treated group; (Plate VI & VII) Histology of spleen of *S. aureus* infected + propolis + ampicillin and *S. aureus* infected + propolis + amoxicillin treated groups showing regular spleen morphology; (A, B, C, D indicates light micrographs under various magnifications; 100 X & 400 X in different sections)

Conclusions

Present study revealed sharp histological alterations in liver, kidney and spleen of *S. aureus* infected mice. Propolis alone as well as in combination with antibiotics significantly countermands the histological alterations and proved its ameliorative/curative effect. Among tissues maximum damage was found to be observed in liver, as it is the main metabolic organ, then spleen, which is a filtering device for blood purification from foreign pathogens. The study revealed that combination therapy i.e. propolis along with ampicillin and amoxicillin had highest ameliorative activity and

restored histology to near normal. The obtained results are encouraging stating propolis to be used not only in traditional, complementary and alternative medicine but also as a relevant protagonist in clinical treatments. Although clinical trials for combinational therapies still need to be carried out with diversity of propolis types, its relation with multidrug resistant microbes and also the standardization of its chemical constituents responsible for therapeutic properties need to be evaluated. Moreover, further studies are required to be conducted on mechanism of propolis as protective agent in *S. aureus* infection.

Acknowledgement

The author would like to thank dept of Biosciences (UIBT), Chandigarh University, Punjab and dept of Zoology, Punjab University, Chandigarh for providing research facilities for the accomplishment of this work.

Conflict of Interest

Author declare no conflict of interest

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