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Antioxidative potential of propolis on *Staphylococcus aureus* infected BALB/c mice: A biochemical study

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Drug resistance, according to World Health Organization, is one of the most serious threats to public health. This makes antibiotics ineffective and reduces their therapeutic potential. One of the most prevalent multidrug-resistant bacteria is *Staphylococcus aureus* which is considered to be the most common pathogen and mortality factor in both hospital and non-hospital environments worldwide. Due to an unprecedented increase in reports of drug resistance in pathogens, and also due to adverse and severe side effects of drugs, there is an urgent need to redirect scientific efforts towards search for anti-oxidative natural substances and other alternative sources having therapeutic potential against microbes. Natural products such as propolis seem to exhibit most promising therapeutic potential against microorganisms. Thus, present study is focused on antioxidative potential of propolis in combination with standard antibiotics ampicillin and amoxicillin against *S. aureus* infected BALB/c mice. For this, mice were divided into seven groups, they were decapitated after suitable experimental periods, then their liver, kidney and spleen were excised from control and experimental groups, which were homogenized and then used for different biochemical estimations following the standard protocols. Results showed that *S. aureus* caused severe biochemical alterations by 5th day of infection that is, lipid peroxidation increased significantly (P <0.05), reduced glutathione level and activity of antioxidant enzymes (SOD, CAT, GPx, GR, GST) decreased significantly (P <0.05) in liver, kidney and spleen of *S. aureus* infected mice. Ethanolic extract of propolis at a dose of 250 mg/kg body weight of mice when used alone to treat *S. aureus* infection gave significantly good results by 15th day of treatment. Better results were observed when propolis was used along with antibiotics. The levels of antioxidant molecules and enzymes along with liver and kidney function enzymes were restored to near normal after 15 days of treatment. So it can be concluded that propolis along with antibiotics acts as a potent free radical scavenger and can be used as a potential therapeutic agent against staphylococcal infection.

Keywords: Ampicillin, Amoxicillin, Bee products, Kidney, Liver, Oxidative stress, Spleen

The term ‘oxidative stress’ was originally used to describe toxic effects of ionizing radiations, free radicals and most importantly harmful effects caused by reactive oxygen species (ROS) like superoxide radicals, hydrogen peroxide, hydroxyl radicals, singlet oxygen species produced as metabolic by products and potential contribution of these processes in causing several diseases and ageing[1,2]. The biological targets for these highly reactive oxygen species are DNA/RNA, proteins and lipids. However, polyunsaturated fatty acids and lipids are major targets during oxidative stress. Free radicals when produced in moderate level, cause immune dysfunctions like impairing defence against pathogenic microorganisms, but when released in excess, they can directly attack polyunsaturated fatty acids in membranes and initiate lipid peroxidation[3]. A primary effect of lipid peroxidation is decrease in membrane fluidity, which alters membrane properties and can disrupt membrane-bound proteins significantly. This effect, acts as an amplifier as more radicals are formed and polyunsaturated fatty acids are degraded to a variety of products. Unlike reactive free radicals, aldehydes are rather long lived and can diffuse from site of their origin to attack targets which are distant from initial free-radical event, acting as “second toxic messengers” of the complex chain reactions initiated. Of the different aldehydes formed during lipid peroxidation, the most extensively studied are malonaldehyde (MDA) and 4-hydroxyalkenals, in particular 4-hydroxynonenal (HNE)[4].

To counter or to protect themselves from free radicals mediated damage, living organisms have built up some mechanisms against oxidative stress with enzymes such as catalase and superoxide dismutase,

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molecules such as glutathione and small proteins like thioredoxin and glutaredoxin. It has been reported that certain natural products of plant and animal origin have inherent capacity of intercepting free radical chain reaction generated by ROS during oxidation by donating hydrogen atom. By donating hydrogen atom from phenolic hydroxyl groups, they form stable end products which stop further oxidation of lipids and polyunsaturated fatty acids[5].

In line with the above fact, certain plant extracts and honey bee products such as propolis have been studied for their potential role in reducing oxidative stress as evidenced by previous studies on liver, kidney and heart of obese rats[6]. Propolis is complex dark brown resinous fluid collected by worker honey bees from plant exudates and is mixed with bee wax, bee pollen and hypo-pharyngeal glands secretions for use in hive as a sealant. Earlier reports on biological activity of propolis supported its antioxidative potential, which can be due to presence of pharmacologically active compounds like flavonoids, phenolic acids, their esters, caffeic acid phenylether ester (CAPE) and various aromatic compounds. Among them "CAPE" has been found to be most active in protection of tissues from oxidative stress[7]. The antioxidant properties of bioactive compounds present in propolis are supported from previous studies as it has been used in traditional medicine since ancient times in many countries[8]. Recent researches show that it possesses various biological and pharmacological activities such as antioxidative[9-11], antibacterial[10-13], antiviral & anticancer[14], antifungal & anti-inflammatory[15], therapeutic and cosmetic[16] and is also a feed additive in poultry nutrition[17].

The antioxidant and antimicrobial activities of propolis against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* were also reported by Abdullah[18]. Results obtained showed higher antibacterial activity of propolis against Gram-positive (*B. subtilis* and *S. aureus*) bacteria. The synergistic effects of propolis along with honey were also studied by Al-Waili[19] against multi drug resistant *S. aureus*, *E. coli* and *Candida albicans* isolates in single and polymicrobial cultures. The results obtained showed that it prevents growth of microorganisms in single and mixed microbial cultures and has synergistic effect when used with honey or ethyl alcohol. Results obtained also validate antimicrobial property of propolis which varies with geographical origin. Synergistic behavior of propolis along with antibiotics is also corroborated from our previous studies on BALB/c mice, where *Staphylococcal* infection caused severe biochemical and histopathological alterations[10,11].

Much research has been done to study the antioxidative properties of propolis under *in vitro* conditions. However, systematic studies on ameliorative effects of honey bee products using animal model are still lacking. The challenge to look for some alternatives for the treatment of deadly *S. aureus* infection is imperative. Here in our present studies we selected BALB/c strain of mice as animal model. The reason behind BALB/c strain of mice as most suitable lineage for experimental work is presence of mutated Nramp 1 gene which makes it susceptible to pathogenesis of disease[20].

Materials and Methods

Collection and preparation of propolis extract

Propolis of *Apis mellifera* was collected from Langstroth hives placed in the field of *Brassica campestris* in an apiary in Chandigarh (India). It was collected by scrapping it from the frames with the help of the hive tool. For extraction of propolis, the crude sample (10 g) was cut into small pieces, ground and extracted using ethanol[10,11]. The volume was made to 40 ml and was kept for 5 days with occasional shaking. It was then filtered through a Whatman No.41 filter paper and dried. Propolis so extracted was stored in a dry and cool place. The percentage yield was calculated by the formula:

$$\text{Percentage yield}\,(\%) = \frac{\text{Pure product recovered}}{\text{Crude material used}} \times 100$$

Specific dilutions were then calculated and made as and when required.

Microorganism

Staphylococcus aureus (MTCC-1144) was procured from Institute of Microbial Technology (IMTECH), Chandigarh, India. It was grown in BHI (Brain Heart Infusion) broth and maintained in BHI agar for further experiments. The organism was checked biochemically prior to storage at -30°C.

Colony forming units

Staphylococcus aureus (MTCC-1144) was grown in BHI broth at 37°C. After a period of 24 h incubation, bacterial culture was centrifuged for 10 mins and rinsed thrice with saline water. The bacterial count was then determined by plating 10 µL

each of 10-fold serial dilutions of the culture on nutrient agar plates. The plates were incubated overnight at 37°C. Following incubation, bacterial colonies were counted and colony-forming units (CFU) were calculated by our standard protocol[21].

CFU/mL= (No. of colonies × dilution factor)/ Vol. of culture plated on agar plates.

Animal model

BALB/c strain (five to six weeks old, either male or female, weighing 25-30g) of mice was used as experimental model. Mice were obtained from Central Animal House, Panjab University, Chandigarh, India and fed with a standard pellet diet (purchased from Ashirwad Industries, Kharar, Punjab) and water. Mice were kept in animal house in polypropylene cages at temperature 25±2°C under 12 hr light/dark cycle. Treatment was according to the guidelines of institutional ethical committee for the purpose of control and supervision of experiments on animals. It was approved by Institutional Animal Ethics Committee (PU/IAEC/S/14/136) of Panjab University, Chandigarh, India.

Experimental Design: Eight animals were taken for each group as detailed below

For the experimental design the animals were segregated into seven groups, each group comprising of eight mice, that is, Group 1: Control mice administered with normal saline only (negative control); Group 2: Mice infected with *S. aureus* (0.2 mL once, intra-peritoneal injection of 5×10^6 CFU/mL) *i.e*. Positive control group; Group 3: Mice infected with *S. aureus* and given propolis extract (250 mg/kg body weight) every day for 15 days; Group 4: Mice infected with *S. aureus* and given antibiotic (ampicillin: 250 mg/kg body weight) everyday for 15 days; Group 5: Mice infected with *S. aureus* and given antibiotic (amoxicillin: 250 mg/kg body weight) everyday for 15 days; Group 6: Mice infected with *S. aureus* and given ampicillin and propolis extract, dosages as above, with a difference of 2 h, everyday for 15 days; Group 7: Mice infected with *S. aureus* and given amoxicillin and propolis extract, dosages as above, with a difference of 2 h, everyday for 15 days.

Separation, homogenization of tissues and Biochemical studies

S. aureus infected mice were sacrificed on 5th day as this was the peak day of infection. Animals of the other groups included in this study were sacrificed immediately after 15th day, by decapitation. Liver, Kidney and spleen tissues were excised from mice of different experimental groups, washed with cold normal saline, homogenized in ice-cold buffer containing 0.25 M sucrose, 1mM EDTA and 1mM Tris-HCl, pH 7.4. This homogenate was used for LPO and GSH estimation directly and was centrifuged at 1000 rpm for 30 min at 4°C. Supernatant was used for further biochemical estimation of GST, SOD, CAT GPx and GR.

Bacterial load

Bacterial loads were determined in liver, spleen and kidney of experimental mice by following standard protocols[21].

Assay for liver and kidney function tests

Mice from all the groups were sacrificed and blood was collected from jugular vein in eppendorf tubes. Blood was kept for 20 min at room temperature and then centrifuged at 3000 rpm for 30 min. The collected colorless serum was then used for biochemical assay for liver function *viz*. SGOT, SGPT, alkaline phosphatases, bilirubin and biochemical assays of kidney function *viz*. urea, uric acid and creatinine, using kits from Reckon Diagnostics Pvt. Limited, India.

Statistical analysis

Data were expressed as mean ± standard deviation (SD) and the statistical significance of the inter group difference of biochemical parameters and bacterial count was evaluated by one way analysis of variance (ANOVA) using SPSS software version 20. Further, data was analyzed by Scheffe post-hoc analysis with Least Square Difference. A value of $P < 0.05$ was considered to indicate a significant difference and $P \leq 0.01$ highly significant difference between groups. All experiments were repeated thrice.

Results

The present observations were an attempt to test propolis, a natural honey bee product, for its antioxidative effect alone as well as in combination with antibiotics (ampicillin and amoxicillin) against *S. aureus*. Propolis and antibiotics concentration used was decided on the basis of best results obtained in our earlier studies[10,11].

Yield of propolis extract

Crude propolis contains alcohol soluble resins, wax and insoluble material as analyzed in our lab through GC MS method[22]. Details of analysis revealed that

propolis contained Flavonoids (Isoquinoline, 4',5,7 Trihydroxyflavanone, 4H-1-Benzopyran4one), Acids (5,8,11Eicosatriynoic acid, 5,8,11 Eicosatriynoic acid, Cinnamic acid, 15oxapentacyclo[12.6.0.0(1,6). 0(2,18).0(8,13)]icos a8(13),9,11triene5 carboxylic acid), Sugars (D-(-)- Fructopyranose, D-(-)-Fructofuranose, D –(-)-Tagatofuranose), Ketones (1Tetralone, Propanone and some others). So, ethanol was used as principal solvent for extracting out the bioactive constituents from it. Observed extracted weight of propolis and the percentage yield was found to be 6.089 g and 53.01%, respectively.

Survival percentage

For observing the survival of animals, eight mice were taken in each group at the start of experimental regimen. The experiment was done in triplicate. Mean survival and survival percentage is presented in (Table 1).

Bacterial load in different organs

The present studies were conducted for a period of 15 days. The bacterial count was observed to be very high on 5th day of infection. Therefore, the infected animals (without treatment) were killed on that day. The bacterial load in blood was observed to be 8.98 ±0.23 log CFU/mL after *S. aureus* infection. Propolis and antibiotics treatment alone as well as in combinations led to significant reduction in bacterial count. Bacterial load in case of liver, kidney and spleen also showed significant reduction (P <0.050) after treatment with propolis and antibiotics alone as well as in their combination at the end of the experimental regimen (Table 2).

Table 1 — Survival percentage (8 mice were taken in each group at start of experiment)

Experimental Groups	BALB/c mice: At start of experiment (1st Day)	BALB/c mice: At end of experiment (15th Day)	Survival percentage observed at the end of experiment (15th Day)
Gp.1	8±0	8.00±001	100%
Gp.2	8±0	1.07±0.022	12.5%
Gp.3	8±0	5.34±0.161	62.5%
Gp.4	8±0	4.78±0.210	50%
Gp.5	8±0	4.599±0.181	56.25%
Gp.6	8±0	7.12±0.192	87%
Gp.7	8±0	7.79±0.211	96.25%

Data was expressed as Mean± SD

Body weight

The body weight is an important parameter for general health of an organism. In present studies it was found to be decreased after *S. aureus* infection (Gp.2) as compared to the normal mice (Gp.1). The decrease was from 26.88±0.46g to 19.76±0.31g and this was found to be statistically significant (P ≤0.0001). The *S. aureus* infected+ propolis treated group (Gp.3) and positive control groups (Gp.4 and Gp.5) showed significant increase in the body weight as compared to *S. aureus* infected (Gp.2) group. The *S. aureus* infected+ propolis+ ampicillin (Gp.6) and *S. aureus* infected+ propolis+ amoxicillin (Gp.7) treated groups restored the values to near normal. This increase in body weight observed after combination therapy was found to be statistically highly significant (P ≤0.0001) as compared to *S. aureus* infected (Gp.2) group. The administration of propolis, antibiotics alone and their combination with propolis revealed their therapeutic potential in restoring weight of *S. aureus* infected mice (Fig. 1).

Biochemical studies

For evaluating biochemical parameters, *S. aureus* infected mice were sacrificed on 5th day as this was the peak day of infection while other groups were sacrificed immediately after 15th day by decapitation. Liver, kidney and spleen were excised from mice of different experimental groups. The homogenate was used directly for estimation of LPO and GSH (Figs 2A & B). It was then centrifuged and the supernatant obtained was used for the estimation of enzymatic activities of SOD (Fig. 2C), CAT (Fig. 2D), GST (Fig. 2E), GR (Fig. F) and GPx (Fig. G).

Table 2 — Bacterial load in liver, kidney and spleen of different groups (log cfu/gm).

	Liver		Spleen		Kidney	
Groups	5th Day	15th Day	5th Day	15th Day	5th Day	15th Day
GP.1	0±00	0±00	0±00	0±00	0±00	0±00
GP.2	8.26±0.23	0±00	8.01±0.12	0±00	5.62±0.23	0±00
GP.3	7.89±0.13	6.30±0.12	6.23±0.19	6.01±0.11	4.23±0.11	3.28±0.25
GP.4	1.23±0.42	1.20±0.33	2.15±0.31	1.82±0.23	1.02±0.67	0.82±0.51
GP.5	0.89±0.46	0.42±0.32	1.55±0.23	1.00±0.21	1.00±0.71	0.62±0.43
GP.6	6.54±0.54	5.49±0.28	6.03±0.18	5.80±0.33	3.89±0.28	2.62±0.67
GP.7	6.00±0.38	5.99±0.22	5.67±0.19	3.89±0.54	3.24±0.38	1.89±0.49

Data was expressed as Mean± SD

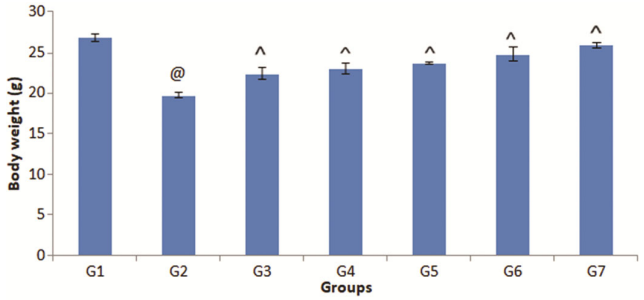

Fig. 1 — Histogram showing effect on body weight of *S. aureus* infected mice and treatment with propolis, ampicillin and amoxicillin alone and in combination. Data is expressed as mean ± SD. N *vs* I (@: $P \leq 0.0001$, &: $P \leq 0.001$, $: $P \leq 0.05$), I *vs* Treated groups (^: $P \leq 0.0001$, #: $P \leq 0.001$, *: $P \leq 0.05$), I+ propolis *vs* other treated groups (@: $P \leq 0.0001$, $: $P \leq 0.001$)

Lipid peroxidation (LPO) in liver, kidney and spleen

Lipid peroxidation is primary measure of oxidative damage in tissues and organs. It is oxidative degradation of lipids where free radicals steal electrons from lipids and disturb the integrity and functioning of cell membrane resulting in its degradation. The chemical products of this oxidation are known as lipid peroxides or lipid oxidation products. The end products of lipid peroxidation are reactive aldehydes such as malondialdehyde (MDA) and 4 hydroxynonenal (HNE) which act as second messenger of free radicals mediated oxidative stress. During present study, level of lipid peroxides was assayed by measuring the end product malondialdehyde (MDA) in liver, kidney and spleen, of all experimental groups. It was observed that the levels of LPO increased significantly in liver, kidney and spleen of *S. aureus* infected mice (Fig. 2A). After treatment with propolis and antibiotics alone (250 mg/kg/bw/day for 15 days) there was significant reduction in lipid peroxidation as compared to infected group but the level was still higher than normal. When the combination therapy (propolis+antibiotics) was used there was significant ($P \leq 0.0001$) reduction in LPO as compared to the infected group (Fig. 2A). In Gp.7 (propolis+amoxicillin) levels of lipid peroxide were restored to normal level, suggesting the effectiveness of this combination in liver, kidney and spleen.

Glutathione (GSH) in liver, kidney and spleen

Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. Increased oxidized glutathione over reduced glutathione indicates oxidative stress in the body. Reduced glutathione (GSH) is simply the stable and active form required for healthy system. The oxidized glutathione is converted back to its reduced form by an antioxidant enzyme called glutathione reductase (GR). In present studies levels of GSH decreased significantly ($P \leq 0.0001$) in liver, kidney and spleen of *S. aureus* infected mice indicating oxidative stress (Fig. 2B). There was no significant change observed after propolis treatment (Gp.3), while both ampicillin and amoxicillin (Gp.4 & Gp.5) showed significant increase in GSH levels as compared to propolis treatment alone. In groups 6 and 7 significant ($P \leq 0.0001$) increase in levels of reduced glutathione was found and the levels were restored to near normal in liver, kidney and spleen of *S. aureus* infected mice as compared to control group (Fig. 2B).

Antioxidant enzymes: **(GST, SOD, CAT GPx and GR) in liver, kidney and spleen**

Oxidative stress plays a major role in pathogenesis of many disorders, so antioxidative enzymes play important role in mitigating harmful effects caused by free radicals generated through metabolic activities or microbial infection. One of the curative approach through which these disorders can be prevented is to increase the levels of antioxidant enzymes (GST, SOD, CAT GPx and GR) in the body by intake of dietary supplements rich in antioxidants and regular exercise[23]. In present studies *S. aureus* infection caused significant reduction in the activities of these enzymes in liver, kidney and spleen of experimental mice (Figs 2C-G). On propolis and antibiotics treatment (Gp.3, 4 & 5) there was significant increase in these enzymatic activities. Propolis when used along with ampicillin (Gp.6) and amoxicillin (Gp.7) led to restoration of the enzymatic activities in liver, kidney and spleen of mice; this authenticated therapeutic efficacy of the combinational treatment (Figs 2C-G).

Liver and kidney function tests

For assessing liver and kidney functioning, levels and activities of various enzymes/ molecules were estimated in serum of different experimental groups. For evaluating liver functioning, estimation of serum glutamate pyruvate transaminase (SGPT)/ Alanine aminotransferase (ALT), serum glutamate oxaloacetate transaminase (SGOT)/ Aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin was done in serum samples of all experimental groups using commercially available kits. The levels of all these parameters were increased in *S. aureus* infected mice as compared to normal

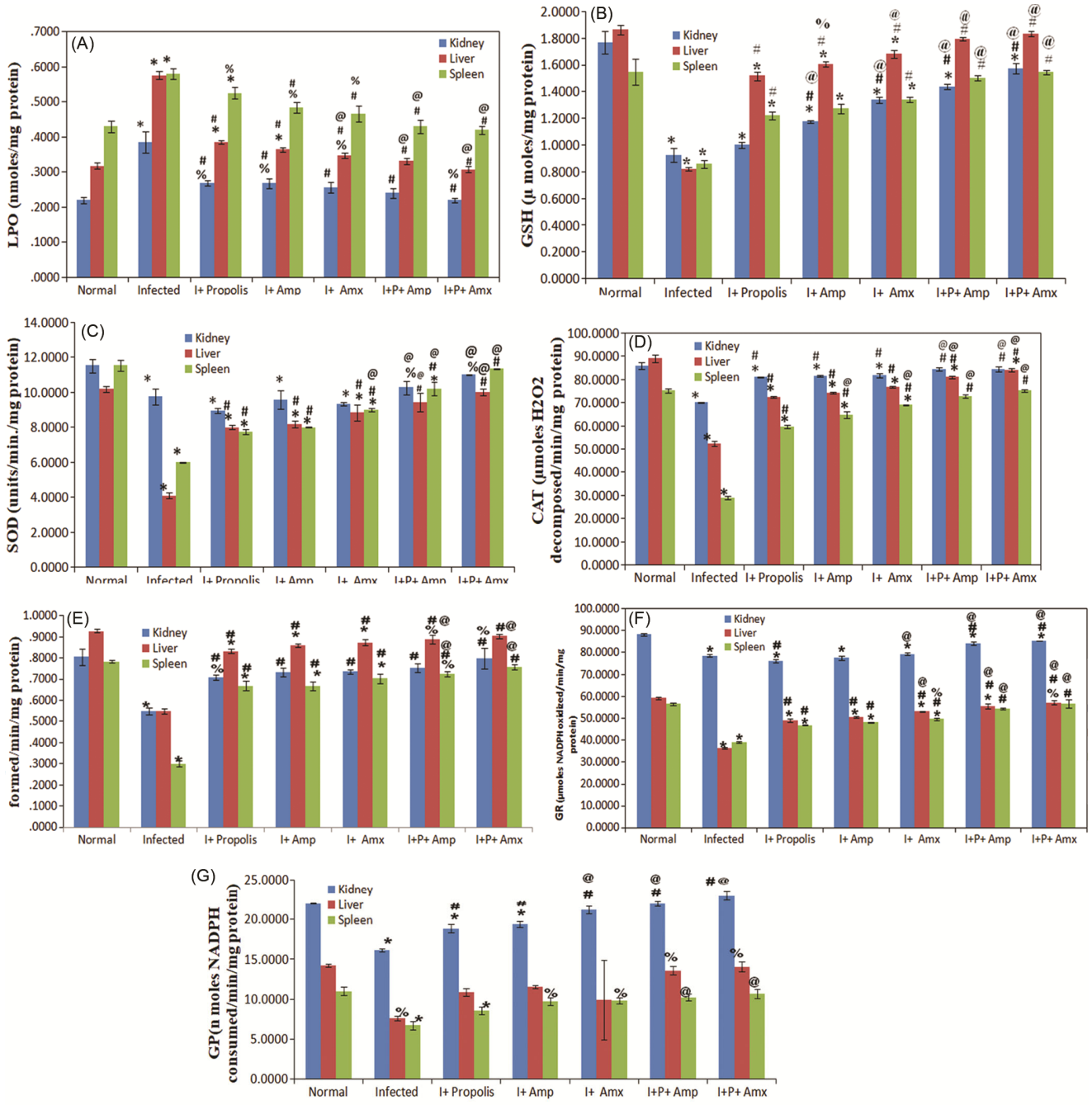

Fig. 2 — Histogram showing effect on (A) LPO; (B) levels of GSH (reduced glutathione); (C) SOD (Superoxide dismutase) activity in kidney, liver and spleen; (D) CAT (Catalase); (E) GST (Glutathione S Transferase) activity; (F) GR (Glutathione reductase) activity in kidney, liver and spleen; and (G) GP (Glutathione peroxidase) activity in kidney, liver and spleen of *S. aureus* infected mice and treatment with propolis, ampicillin and amoxicillin alone and in combination. Data is expressed as mean ± SD. N *vs* I (@: $P \leq 0.0001$, &: $P \leq 0.001$, $: $P \leq 0.05$), I *vs* Treated groups (^: $P \leq 0.0001$, #: $P \leq 0.001$, *: $P \leq 0.05$), I+ propolis *vs* other treated groups (@: $P \leq 0.0001$, $: $P \leq 0.001$)

mice (Table 3). Similarly, in case of kidney function test the levels of urea, uric acid and creatinine were observed to be increased in *S. aureus* infected mice as compared to the normal group (Table 4). After treatment with propolis and antibiotics (ampicillin and amoxicillin) alone, significant reduction was observed in levels and activities of both liver and kidney function parameters. In combination therapy (propolis+antibiotics), the values were restored to near normal showing synergistic efficacy of the combination.

Table 3 — Results of liver function tests on *S. aureus* infected BALB/c mice

Liver Function Test	Gp.1	Gp.2	Gp.3	Gp.4	Gp.5	Gp.6	Gp.7
SGPT (IU/L)	23.098±0.69	138.77±1.15*	40.308±0.703$^{*\#}$	37.316±0.543$^{*\#}$	35.474±0.518$^{*\#\%}$	29.208±0.204$^{*\#@}$	24.612±0.198$^{\#@}$
SGOT (IU/L.)	25.266±0.504	94.162±0.753*	36.676±0.625$^{*\#}$	31.780±0.195$^{*\#@}$	28.77±0.43$^{\%\#@}$	28.368±0.263$^{\%\#@}$	26.300±0.198$^{\#@}$
ALP (KA units)	7.912±0.221	25.614±0.308*	14.316±0.238$^{*\#}$	11.352±0.304$^{*\#@}$	10.368±0.206$^{*\#@}$	8.716±0.144$^{\#@}$	8.018±0.126$^{\#@}$
Bilirubin (mg/ml.)	0.670±0.009	1.494±0.028*	0.894±0.103$^{*\#}$	0.800±0.010$^{*\#@}$	0.740±0.007$^{\#@}$	0.696±0.006$^{\#@}$	0.648±0.159$^{\#@}$

All the values are expressed as mean ± S.D. N *vs* I (*: $P \leq 0.0001$, &: $P \leq 0.001$), I *vs* Treated groups (#: $P \leq 0.0001$, %: $P \leq 0.001$), I+ propolis *vs* other treated groups (@: $P \leq 0.0001$, $: $P \leq 0.001$)

Table 4 — Results of kidney function tests on *S. aureus* infected BALB/c mice

Kidney Function Test	Gp.1	Gp.2	Gp.3	Gp.4	Gp.5	Gp.6	Gp.7
Urea (mg/dl)	46.328±0.707	85.818±1.508*	57.110±0.9729$^{*\#}$	55.55±0.471$^{*\#}$	55.324±0.819$^{*\%}$	48.104±0.508$^{\#@}$	44.568±0.576$^{\#@}$
Uric Acid (mg/dl)	4.09± 0.0925	8.96±0.386*	5.88±0.163$^{\#\%}$	5.132±0.083$^{\#}$	4.62±0.199$^{\#}$	3.952±0.361$^{\#\%}$	3.386±0.197$^{\#@}$
Creatinine (mg/dL)	0.436±0.0156	0.838±0.017*	0.554±0.01$^{\#\%}$	0.486±0.009$^{\#}$	0.4500±0.020$^{\#\%}$	0.4380±0.033$^{\#\%}$	0.420±0.011$^{\#\%}$

All the values are expressed as mean±S.D. N *vs* I (*: $P \leq 0.0001$, &: $P \leq 0.001$), I *vs* Treated groups (#: $P \leq 0.0001$, %: $P \leq 0.001$), I+ propolis *vs* other treated groups (@: $P \leq 0.0001$, $: $P \leq 0.001$)

Discussion

Over the last few years, there has been renewed increase in interest in the antimicrobial activity of natural products and among them; propolis seems to exhibit the most promising therapeutic potential. Propolis, a natural bee product, is a sticky resinous substance gathered by worker honey bees from buds and bark of trees[24,25]. Generally, it is composed of 50-60% resin and balsam, 30–40% wax and fatty acids, 5–10% essential and aromatic oils, 5% pollen and approximately 5% other substances, including amino acids, micronutrients and vitamins like B1, B2, pyridoxine, vitamin C and E[26]. It shows several health benefits and activities against many human diseases due to its various pharmacological and biological activities which are related to its chemical composition[27,28] thus attracting attention from scientists and researchers. There are several research papers on *in vitro* studies regarding characterization and understanding of its biological activities but still, there is lack of information regarding its therapeutic and clinical efficacy. Hence the present studies were undertaken on a mice model.

Survival of the organisms

Survival is the most important factor in any experimental protocol. In the present studies 62.5% survival was observed in propolis treated group as compared to the infected group, where survival was only 12.5%. On combining propolis with amoxicillin, 96.25% survival was recorded at the end of the treatment regimen which showed additive effect of the combination (Table 1). In earlier studies it was observed that *S. aureus* caused heavy mortality from 5th day of infection, which could be due to severe infection in vital organs causing disruption of physiological parameters in *S. aureus* infected mice. Treatment with propolis in combination with antibiotics showed ameliorative effect on tissue damage caused by *S. aureus* infection as confirmed by histological studies on liver, kidney and spleen. In the present studies, heavy bacterial load (Table 2) was observed in *S. aureus* infected mice which showed least survival rate at end of experimental regimen. However, on treatment with propolis and antibiotics alone and in combination, a significant reduction in bacterial load and increased survival percentage of the infected mice was observed which proved therapeutic potential of propolis. Pharmaceutical industries have always used natural products, as an encouraging alternative source of drugs that are used in health system[29].

Liver and kidney function tests

The damage caused by *S. aureus* to liver and kidney was assessed by estimating the levels and activities of enzymes and macromolecules. For liver, estimation of SGPT, SGOT, ALP and bilirubin was done in serum samples of all experimental groups (Table 3). These enzyme molecules are present in liver cells in normal healthy individuals and their raised levels in blood indicate some kind of infection or injury to the hepatocytes. The injury caused by *S. aureus* infection might be attributed to its cytokines production and

inflammation of the Kupffer cells[30]. In earlier studies, propolis was observed to be hepato-protective[31,32]. It showed inhibitory activity against puffiness, leakage and clustering in inflamed hepatic cells caused due to microbial infiltration[33]. The hepatic damage was reduced when treated with propolis, antibiotics alone and in their combination. Propolis along with antibiotics showed synergistic activity and restored the values to near normal (Table 3). It acts as a strong antioxidant and its curative effect on liver cells was thought to be due to its suppression of leakage of enzymes through the plasma membrane and repair to the damaged liver cells[34]. Similarly, in case of kidney the levels of urea, uric acid and creatinine were raised in *S. aureus* infected group, which were restored to near normal values after treatment with propolis and amoxicillin when used in combination (Table 4).

Biochemical assays

Oxidative stress is imbalance between production and accumulation of reactive oxygen species (ROS) in cells and tissues as a result of microbial infection or as metabolic byproducts. Overproduction of reactive oxygen species can cause damage to lipids, proteins, nucleic acids and other macromolecules. This increase in ROS is simultaneously accompanied by an immediate compensatory increase in the activities of antioxidant molecules like GSH and enzymes[35].Glutathione is an important antioxidant molecule involved in protection against reactive oxygen species generated during oxidative stress. In present studies, it is evident that *S. aureus* infection in mice caused increased lipid peroxidation, decreased GSH levels and decreased activities of antioxidant enzymes (GST, SOD, CAT GPx and GR), in liver, kidney and spleen of *S. aureus* infected mice.

The GSH level was significantly decreased in *S. aureus* infected mice. On treatment with propolis and antibiotics, GSH levels were increased significantly. It was observed that propolis in combination with antibiotics restored the values to near normal (Fig. 2B).The decreased GSH levels represent its increased utilization to counter bacterial infection. Moreover, the reduction in reduced glutathione (GSH) levels might be due to increased lipid peroxidation which may be associated with less availability of NADPH, which is required for GR activity to transform the lesser active form of glutathione (GSSG) to more stable and active form of glutathione (GSH)[36]. Antioxidant enzymes play important role in primary defense of biological macromolecules against oxidative damage caused by microbial infections. Among them superoxide dismutase (SOD), is an important antioxidant enzyme present in nearly all living cells exposed to oxygen. It rapidly catalyzes dismutation of superoxide anions to ordinary oxygen molecules and less dangerous hydrogen peroxide, which is further degraded to water and oxygen by another antioxidant enzyme catalase (CAT) and glutathione peroxidase (GPx)[37].Superoxide is produced as metabolic byproduct of oxygen utilization during bacterial infection, which when not regulated, causes damage to biological macromolecules[38]. This was observed during present studies as well which showed a significant reduction in SOD and CAT activities in liver, kidney and spleen of *S. aureus* infected mice (Figs 2C & D). On treatment with propolis and antibiotics (Gp.3, 4 & 5) significant increase in SOD and CAT activities was observed. Their values were restored to near normal when propolis was used in combination with ampicillin and amoxicillin (Gp.6 & 7), which showed synergistic behavior of propolis along with antibiotics. This reduction in SOD activity may be due to its utilization during disposing off free radicals produced during oxidative stress caused by *S. aureus*. Similarly CAT (Fig. 2D) and GPx (Fig. 2G) activities were reduced while converting hydrogen peroxide generated by superoxide dismutation to molecular oxygen and water[39]. This depletion in antioxidant enzymes activity might be due to protein/enzymatic degradation or inactivation caused by *S. aureus* infection and also due to down-regulation of transcription and translation processes. In present studies, there was fall in glutathione-s-transferase activity in *S. aureus* infected group (Gp.2). Propolis and antibiotics, when used alone (Gp.3-5), gave significant increase in GST activity and the values were restored to normal when propolis was used in combination (Gp.6&7) with ampicillin and amoxicillin respectively (Fig. 2E). This reduction in GST activity during *S. aureus* infection is due to its utilization for catalyzing the conjugation of reduced glutathione (GSH) to xenobiotic substrates and electrophilic compounds for their detoxification[40.] GST along with GSH plays important role in defending cells from mutagens and carcinogens as a free radical scavenger. Thus in present study, the significant decrease in GSH (Fig. 2B) level and GSH-dependent enzymes, that is, GST (Fig. 2E), GR (Fig. 2F), and GPx (Fig. 2G) in liver kidney and spleen of *S. aureus* infected mice may be due to increased utilization to scavenge free-radicals generated. Treatment with

propolis and antibiotics in combination significantly increased the GPx, GR and GST activity.

Efficacy of propolis and its mechanism of action

In present studies, the observed activities of propolis were due to presence of several pharmacologically active compounds, which act by two different ways, first stimulating and enhancing the immune system and thus activation of natural defense mechanism of the organism, 2nd by killing or attenuating the microorganism's directly[41]. This mechanism of action is attributed to increase in cell membrane permeability, reduction in adenosine triphosphate (ATP) production, decrease in bacterial mobility, disturbances in membrane potential and also inducing the activity of body's immune system[41]. It was further observed that, the biological activities were not only due to single component present in propolis, rather it is due to total extract which further supported synergism between various components of propolis responsible for therapeutic activities[42].

Efficacy of antibiotics and synergism between propolis and antibiotics

The antibiotics acted through penetration in to monocytes, thus killing or attenuating microbial growth[43]. Synergistic behavior was observed for propolis with antibiotics. Synergistic effect of propolis ethanolic extract was also observed in our previous studies, where antibiotics like ampicillin, amoxicillin, acted synergistically with propolis on growth inhibition of *S. aureus*[10,11]. The observed synergistic behavior can be effective in preventing microbial resistance, increasing antimicrobial efficacy and can provide broader spectrum for antibacterial activity than antibiotic monotherapy[44]. However, the reason and mechanism of action, behind synergistic behavior is not yet fully known. This might be due to some complex formation which inhibits bacterial growth by damaging cell membrane, inhibiting ATPases, cell division and biofilm formation. Moreover, cinnamic acid and its derivatives in propolis have been reported to exhibit anti-quorum sensing activity and hence causing death of microorganisms[45].

Conclusion

The study described here showed that liver, kidney and spleen were susceptible to *S. aureus* infection through increased production of reactive oxygen species which led to increased lipid peroxidation and reduced glutathione as well as decreased antioxidant status. The findings of liver and kidney function assays also confirm the negative situation. Treatment with propolis and antibiotics showed ameliorative effect and protected liver, kidney and spleen from such infections by decreasing free radical generation, lipid and protein damage and also by increasing the antioxidant status. Hence, propolis along with antibiotics can be used as a potent free radical scavenger antioxidative product and can be used as a potential therapeutic agent against staphylococcal infection.

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Conflict of interest

Authors declare no conflict of interest.

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