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Antioxidative Potential of Pollen, Propolis and Bee Bread against Damage Caused by *Staphylococcus Aureus* in Liver and Kidney of BALB/c Mice: A Biochemical Study

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Microbial resistance towards antibiotic is menacing all over the world and therefore considered to be a global health issue. Their potentialities for adapting new resistance mechanisms threaten our ability to treat common infectious diseases. Hence it is important to explore alternative therapies to combat microbial diseases. Among them, honeybee products comprise a wide variety of dietary/nutritional supplements with wide range of pharmaceutical properties. Therefore the present studies were designed to evaluate the therapeutic potential of propolis, pollen and bee bread against *Staphylococcus aureus* mediated oxidative stress in BALB/c mice. For this, different experimental groups were taken and decapitated at the end of experimental regimen. Liver and kidney were excised/homogenized and used for estimation of oxidative stress caused to them by following standard protocols. Observations revealed severe biochemical alterations by 5th day of infection. Among them, activity of antioxidant enzymes and reduced glutathione level were found to be decreased after *S. aureus* infection while the lipids peroxidation increased significantly in both liver and kidney. Bee products (250 mg/kg/bw of mice) restore the values of antioxidant enzymes/molecules and liver/kidney function enzymes to near normal by 30th day and results obtained were comparable with the positive control i.e. amoxicillin treatment group.

Keywords: Antibiotic resistance, Antioxidative, Bee bread, Lipids peroxidation, Microbial resistance

Introduction

Staphylococcus, a natural inhabitant of our skin especially nostrils is found to be responsible for several clinical manifestations and can be life threatening. This tenacious human pathogen has the ability to outwit the immune system and in addition to this it also has remarkable multidrug resistance capacity which makes it most ungovernable pathogenic microbe in the history of antibiotic chemotherapy.[1] Multidrug resistance among microorganisms has become a challenging situation worldwide which necessitates research on complementary/alternative antimicrobial substances. This led discovery of antibiotics of the beehive.[2] The beehive antibiotics/products are naturally functional foods/ nutraceuticals responsible for pharmaceutics or medical benefits[3] and hence led the scope and objectives of this research. Among beehive products we have used propolis, pollen and bee bread for present study.

Among them propolis also called bee glue has notably demonstrated as antimicrobial substance and it could complement the effect of other antimicrobial substances such as; antibiotics.[4,5] This resinous material is gathered from plant exudates by worker honey bees, added its salivary secretions and beeswax which enhances its pharmaceutical properties including; antimicrobial, anti-inflammatory, antioxidant and anti-cancer.[6–10] Pollen, another bee product is basically "the male gametophyte of plants" gathered by worker honey bees, added its salivary secretions and nectar while collecting. It's a rich source of protein, lipids and several micro/macro nutrients for developing brood of the beehive. Beebread, in simple terms is the fermented/processed bee pollen which is ready for immediate consumption by developing brood or the nurse bees. The fermentation process promotes its nutritional values.[10,11] Bee bread and bee pollen demonstrates antimicrobial activity.[10–12]

A lot of research has been done on *in vitro* studies, while the comparative analysis for propolis, pollen and bee bread was required and the present study represent results of comparative analysis undertaken to evaluate their antioxidative potential against damage caused by *S. aureus* in liver and kidney of BALB/c mice.

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Material and Methods

Collection and Preparation of Propolis Extract

This was collected by scrapping with a clean hive tool from the top bars of the combs in a honey bee colony. The extract was prepared by the method of Rana and Kumar.[7] For this, crude propolis (30g) was added in different flasks containing 100 ml of 70% ethanol. The components were dissolved by occasional shaking at room temperature. It was kept in dark for 8 to 10 days. The solutions were filtered through Whatman filter paper and evaporated to dryness. Final concentrations were made on the basis of dry weight in water and kept at −5°C till use. The solutions were filtered through 0.22 μ Millipore membrane in a sterile tube and stored for further use at −5°C.

Collection and Preparation of Pollen/Beebread Extract

For this 'pollen trap' was installed at the entrance of the beehive and the extract was prepared by following standard protocols of Rana.[11]

Microorganism

The organisms (*S. aureus*-MTCC-1144) were procured from IMTech Chandigarh, India. The culture was grown and maintained in BHI broth/agar for further experiments

Colony Forming Units

The numbers of viable bacterial colonies were calculated by standard protocol of Rana[11]

$$\mathbf{CFU/ml} = \frac{\text{No. of colonies} \times \text{dilution factor}}{\text{Vol. of culture plated on agar plates}}$$

Animal Model

For experimental design, 5/6 weeks/25–30g weight and of either sex BALB/c mice were taken and kept under 12 hours light/dark period at (25 ± 2°C) temp in animal house of Panjab University, Chandigarh. Mice were fed with standard feed from Ashirwaad Industries, Kharar, Punjab and the guidelines for experiments on animals were followed under **(PU/IAEC/S/14/136)**.

Experimental Regimen

For experimental purpose the animals were divided in to nine groups. Group-1 comprises control mice administered with normal saline only. Group-2 is *S. aureus* infected mice (0.2 mL/ip/0.5 × 10^6 CFU/mL). Group-3 is *S. aureus* infected + propolis extract. Group-4 is *S. aureus* infected + pollen extract. Group-5 is *S. aureus* infected+ bee bread extract. Group-7 is *S. aureus* infected+ amoxicillin/positive control. Group-7 is *S. aureus* infected+ propolis+ amoxicillin. Group-8 is *S. aureus* infected+ Pollen+ amoxicillin. Group-9 is *S. aureus* infected+ bee bread + amoxicillin at dose of 250 mg/kg/bw.

Selection of Bacterial Dose

The selection of the dose was determined by inoculating different infection doses in mice such as; $10^{3,}$ 10^4, 10^5 and $10^{6.}$ After inoculating different doses, mice were sacrificed on 3rd, 4th, 5th and 6th day. Enough biochemical alterations were observed with a dose of 0.5 × 10^6 CFU/ml, so this was selected as the final dose for experimental regimen.

Bacterial Load

The bacterial burden/bacterial load was calculated directly by taking blood from the jugular vein and homogenates of liver and kidneys, which was after serial dilutions plated on nutrient agar. These nutrient agar plates containing *S. aureus* were kept for incubation overnight at 37°C. The bacterial colonies were counted after 24 hrs of incubation and the number of CFU per gram of liver/kidney and CFU per milliliter of blood were calculated.

Selection of Bee products Dose and Day of Sacrifice

Different doses i.e. (50,150, 250 and 350) mg/kg bw/day were tested for propolis, pollen and bee bread. Similarly mice were sacrificed on 5th day, 15th day and 30th day. After seeing enough biochemical alterations the dose decided was 250 mg/kg bw/day and the experimental regimen was up to 30th day.

Biochemical Studies on Liver and Kidney

The untreated group of animals (*S. aureus* infected) was sacrificed on 5th day, while treated groups were sacrificed after 30th day by decapitation. Liver/kidney were excised/washed and homogenized at pH 7.4 in the ice-cold buffer containing 1mM EDTA, 1mM Tris-HCl and 0.25 M sucrose solution. Lipid per-oxidation and glutathione levels were estimated directly from the homogenate and biochemical estimation of all antioxidant enzymes was done from supernatant.

Assay of Liver and Kidney Function Tests

Biochemical assays of SGPT/ SGOT, urea, uric acid, bilirubin, creatinine and alkaline phosphatases was done by using kits from Reckon Diagnostics Pvt Limited, India.

Results

In the present study antioxidative potential of propolis, pollen and bee bread was tested along with a

positive control (amoxicillin) against infection caused by *S. aureus.* The concentration of bee products and positive control used were corroborated from our earlier studies[6–8] i.e. 250 mg/kg bw.

Survival Percentage

Survival percentage of mice was calculated by comparing number of mice taken at the start of experiment and the number of mice survived at the end of the experimental regimen. Results obtained revealed that, at end of the experiment zero percentage survival was observed in *S. aureus* infected group, while propolis, pollen and bee bread showed survival rate 50–62% when used alone for treatment. Pollen and bee bread when used along with amoxicillin showed 100% survival rate at the 30th day of experiment (Table 1). From this, therapeutic potential of bee products was confirmed and was further authenticated/ validated by biochemical tests performed on liver and kidney of BALB/c mice.

Bacterial Load

Bacterial load was calculated up to peak day of infection i.e. for a period of 5 days in blood, liver and kidney. The bacterial load after *S. aureus* infection in blood was found to be 5.62 ± 0.13 log CFU/ml on 5th day. Significant decline in *S. aureus* load was observed in the combinational treatment of pollen and bee bread along with amoxicillin. The bacterial load in liver and kidney was also found to be decreased after bee products treatment especially in combinational treatment of pollen/bee bread along with amoxicillin (Table 2).

Table 1 — Survival percentage after *S. aureus* infection and treatment with propolis, pollen, bee bread, amoxicillin alone and their combinations.

Experimental regimen	Number of mice in each group (each group repeated thrice)	Mice survived at the end of experiment (30th day)	Survival percentage (%)
Group 1.	8	8 ± 0	100 ± 0
Group 2.	8	0 ± 0	0 ± 0
Group 3.	8	4.0 ± 0.67	50 ± 0.23
Group 4.	8	4.5 ± 0.71	56.25 ± 0.41
Group 5.	8	5.0 ± 0.34	62.50 ± 0.59
Group 6.	8	6.5 ± 0.51	81.25 ± 0.62
Group 7.	8	7.0 ± 0.57	87.50 ± 0.57
Group 8.	8	8.0 ± 0.78	100 ± 0.78
Group 9.	8	8.0 ± 0.99	100 ± 0.81

Each value in the table is expressed as Mean ± S.D

Biochemical Studies

The biochemical studies were performed by sacrificing *S. aureus* infected group (Gp. 2) on 5th day of infection, and other treatment groups (Gp. 3–9) at the end of the experimental regimen by decapitation. Liver/kidney was excised from all the groups and lipid Peroxidation and reduced glutathione levels (Fig. 1a & b) were studied directly from the homogenate while the antioxidant enzymes such as; SOD (Fig. 1c), CAT (Fig. 1d), GST (Fig. 1e), GR (Fig. 1f) and GPx (Fig. 1g) were estimated from the supernatant obtained after centrifugation of the homogenate.

Lipid Peroxidation (LPO): in Liver and Kidney of BALB/c Mice

The end products of lipids oxidation such as; malondialdehyde (MDA) and 4 hydroxynonenal (HNE) are reactive aldehydes and act as second messenger of free radicals mediated oxidative stress. Hence lipid peroxidation is an important parameter for determining oxidative stress caused by *Staphylococcus aureus.*[13,14] The malondialdehyde levels were measured in *S. aureus* infected liver and kidney in comparison with control group and the levels were found to be increased significantly. Treatment with pollen, propolis and bee bread leads a significant reduction in the MDA levels. Pollen and bee bread on combination with amoxicillin leads restoration of LPO levels in *S. aureus* infected mice (Fig. 1a).

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

	Liver			Kidney		
Groups	5th Day	15th Day	30th Day	5th Day	15th Day	30th Day
Gp.1	0 ± 00	0 ± 00	0 ± 00	0 ± 00	0 ± 00	0 ± 00
Gp.2	8.96 ± 0.59	0 ± 00	0 ± 00	6.62 ± 0.23	4.23 ± 00	0 ± 00
Gp.3	8.09 ± 0.63	7.39 ± 0.22	6.01 ± 0.33	5.63 ± 0.11	3.99 ± 0.25	3.00 ± 0.46
Gp.4	7.37 ± 0.32	6.89 ± 0.49	5.37 ± 0.45	4.92 ± 0.67	3.82 ± 0.51	2.89 ± 0.39
Gp.5	7.09 ± 0.76	6.02 ± 0.62	4.89 ± 0.61	4.00 ± 0.71	3.62 ± 0.43	2.62 ± 0.51
Gp.6	6.54 ± 0.50	5.49 ± 0.98	4.02 ± 0.63	3.89 ± 0.28	2.89 ± 0.67	2.01 ± 0.57
Gp.7	6.00 ± 0.58	5.01 ± 0.62	3.86 ± 0.74	3.04 ± 0.78	2.09 ± 0.49	1.69 ± 0.61
Gp.8	5.77 ± 0.71	4.00 ± 0.77	2.99 ± 0.81	2.09 ± 0.39	1.89 ± 0.61	1.50 ± 0.74
Gp.9	5.32 ± 0.69	3.22 ± 0.92	2.02 ± 0.79	1.89 ± 0.61	1.68 ± 0.74	1.23 ± 0.81

Data was expressed as Mean ± S.D; At the end of experiment, significant reduction in bacterial load was observed in all treated groups

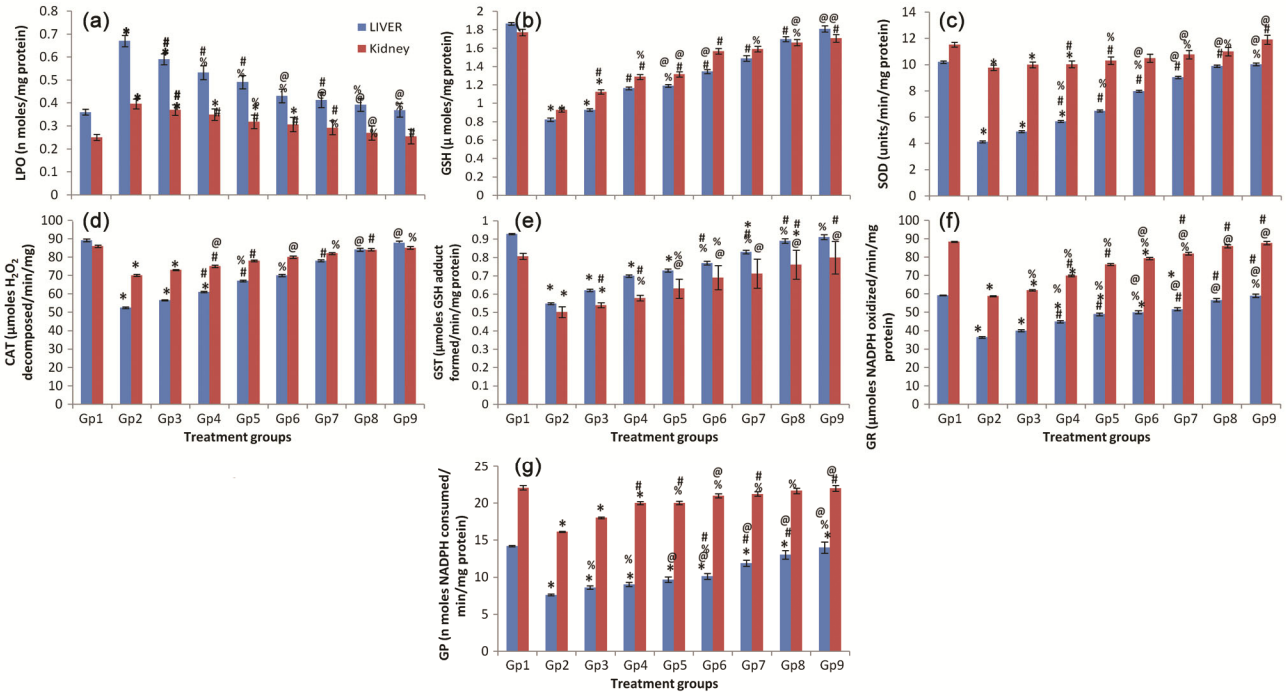

Fig. 1— Histogram showing effect on lipid peroxidation, antioxidant molecules and antioxidant enzymes in the liver and kidney of S. aureus infected mice and on treatment with bee products (propolis, pollen, bee bread) alone as well as in combination with amoxicillin as positive control: (a) decrease in lipid peroxidation level, (b) increase in GSH level, (c) increase in SOD activity, (d) increase in CAT activity, (e) increase in GST levels, (f) increase in GR activity, and (g) increase in GP activity, after treatment

Glutathione (GSH): in Liver and Kidney of BALB/c Mice

To understand the microbial infection, both reduced and oxidized forms of glutathione are important parameter/antioxidant molecule to be studied in the cellular system. The reduced form of glutathione is more stable and active while the oxidized one is unstable, so during bacterial infection oxidized glutathione levels got increased which were then further converted back to its stable form i.e. reduced glutathione by glutathione reductase (GR); an antioxidant enzyme. In the present studies reduced glutathione levels were found decreased significantly ($p \leq 0.01$) in the liver/kidney of *S. aureus* infected mice (Fig. 1b) which indicated oxidative stress. Propolis (Gp. 3), pollen (Gp. 4), bee bread (Gp. 5) and amoxicillin (Gp. 6) treatment leads significant increase in the GSH levels. The use of bee products (Gp. 7–9) in combination with amoxicillin restores the values to normal level which also authenticated synergistic behavior of bee products.

Antioxidant Enzymes in Liver and Kidney of BALB/c Mice

In a natural defense system, cells are able to defend themselves against oxidative stress mediated by microbial infection through the use of antioxidant molecules and antioxidant enzymes. The increased DNA/protein/lipid oxidations caused by microbial infections were decreased to near normal levels by using our antioxidant defense system which mainly includes the antioxidant molecules and antioxidant enzymes such as; GST, SOD, CAT GPx and GR. The present research revealed decreased levels of all antioxidant enzymes in both liver and kidney of *S. aureus* infected mice (Fig. 1c–g), which demonstrated that antioxidant enzymes led elimination of reactive oxygen species or mitigating the oxidative stress caused by *S. aureus* infection. So to increase levels of antioxidant enzymes in the body regular exercise and dietary supplements which are rich in antioxidants should be added. Pollen, propolis and bee bread are some of the natural bee products which are responsible for increasing the levels of antioxidant enzyme. On treatment with propolis, pollen and bee bread significant increase in enzymatic activities was observed (Gp. 3–5). Pollen/bee bread along with amoxicillin (Gp. 8–9) restored the enzymatic activities (Figs 1 (c–g) which authenticated/validated the synergistic behavior of bee products when used in combination with the antibiotic amoxicillin.

Liver and Kidney Functions Tests

The levels and activities of different enzymes were measured for determining the liver and kidney

Table 3 — Liver function tests

Groups	SGPT (IU/L)	SGOT (IU/L.)	ALP (KA units)	Bilirubin (mg/ml.)
Group 1	26.068 ± 0.67	23.206 ± 0.424	6.97 ± 0.171	0.590 ± 0.013
Group 2	127.67 ± 1.05*	89.153 ± 0.651*	23.314 ± 0.217*	1.735 ± 0.018*
Group 3	37.106 ± 0.703$^{*\#}$	37.580 ± 0.535$^{*\#}$	15.306 ± 0.198$^{*\#}$	0.994 ± 0.093$^{*\#}$
Group 4	36.306 ± 0.521$^{*\#}$	35.610 ± 0.293$^{*\#}$	13.292 ± 0.274$^{*\#}$	0.890 ± 0.110$^{*\#}$
Group 5	34.474 ± 0.518$^{*\#}$	33.407 ± 0.243$^{\%\#}$	11.953 ± 0.312$^{*\#}$	0.730 ± 0.107$^{*\#}$
Group 6	33.908 ± 0.204$^{*\#}$	29.809 ± 0.203$^{*\#\%}$\$	10.716 ± 0.212$^{*\#}$\$	0.69 ± 0.899$^{*\#}$\$
Group 7	31.208 ± 0.310$^{*\#\%}$	27.045 ± 0.301$^{*\#\%@}$	9.89 ± 0.278$^{*\#@}$	0.663 ± 0.098$^{*\#}$\$
Group 8	29.345 ± 0.218$^{*\#\%@}$	25.234 ± 0.343$^{*\#\%@}$	8.67 ± 0.314$^{*\#@}$	0.602 ± 0.678$^{*\#@}$
Group 9	27.042 ± 0.138$^{*\#\%@}$	23.806 ± 0.546$^{*\#\%@}$	7.023 ± 0.512$^{*\#@}$	0.520 ± 0.879$^{*\#@}$

All the values are expressed as mean ± S.D (n = 5), N v/s I (*:p ≤ 0.0001, &: p ≤ 0.001), I v/s Treated groups (#: p ≤ 0.0001, %: p ≤ 0.001), I+ propolis v/s other treated groups (@:p ≤ 0.0001, $: p ≤ 0.001).

Table 4 — Kidney function tests

Groups	Urea (mg/dl)	Uric Acid (mg/dl)	Creatinine (mg/dl)
Group 1	49.128 ± 0.503	5.29 ± 0.0615	0.409 ± 0.0236
Group 2	92.314 ± 1.308*	9.99 ± 0.433*	0.908 ± 0.026*
Group 3	61.210 ± 0.629$^{*\#}$	7.44 ± 0.267$^{\#\%}$	0.699 ± 0.023$^{\#\%}$
Group 4	57.155 ± 0.391$^{*\#}$	6.832 ± 0.183$^{\#}$	0.606 ± 0.107$^{\#}$
Group 5	56.603 ± 0.610$^{*\%}$	6.432 ± 0.120$^{\#}$	0.590 ± 0.134$^{\#\%}$
Group 6	55.203 ± 0.438$^{*\%}$	6.091 ± 0.231$^{\#\%}$	0.5380 ± 0.123$^{\#\%}$
Group 7	53.368 ± 0.396$^{\#@}$	5.56 ± 0.187$^{\#@}$	0.520 ± 0.211$^{\#\%}$
Group 8	52.879 ± 0.721$^{\#@}$	5.031 ± 0.234$^{\#@}$	0.499 ± 0.301$^{\#@}$
Group 9	51.032 ± 0.568$^{\#@}$	4.998 ± 0.327$^{\#@}$	0.423 ± 0.412$^{\#@}$

All the values are expressed as mean ± S.D (n = 5), N v/s I (*:p ≤ 0.0001, &: p ≤ 0.001), I v/s Treated groups (#: p ≤ 0.0001, %: p ≤ 0.001), I + propolis v/s other treated groups (@:p ≤ 0.0001, $: p ≤ 0.001).

functions tests. Liver functioning was evaluated in serum samples of all experimental groups by measuring liver marker such as; alkaline phosphatase (ALP), bilirubin, serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT). *S. aureus* infection caused increased levels of these parameters in comparison with normal control group (Table 3). Kidney functioning was assayed by measuring markers such as; creatinine, urea and uric acid levels in different experimental groups. Here also we observed increased levels of kidney function markers in bacterial infected group (Table 4). The levels and activities of liver/kidney function markers were brought down to normal level on treatment with bee products alone as well as in combination with antibiotic amoxicillin. The combinational treatment restored their values to normal level which authenticated the synergistic potential of bee products along with amoxicillin.

Discussion

The World Health Organization has reported microbial resistance towards drugs as the most severe threat to mankind. It not only makes use of antibiotics ineffective but also minimizes their use in medical practices.[15,16] Here in the present study we have taken *Staphylococcus aureus* which is found to be resistant to several antibiotics and hence multidrug resistance microbe. It is most commonly found drug resistant bacterium responsible for serious infectious diseases to mankind which necessitates search for the alternative natural antimicrobial substances which can eliminate the microbial infection through different action mechanisms and can improve the existing ones.[17,18] Over the time research on antimicrobial activity of bee products has been increased. In present studies propolis, pollen and bee bread are tested for their natural therapeutic potentiality against *Staphylococcus* infection in liver and kidney of BALB/c mice.

The bacterial load was found to be very high on 5th day of infection in liver (8.96 ± 0.59) and kidney (6.62 ± 0.23) of *S. aureus* infected mice (Gp. 2), which caused zero percent survival by 30th day of infection. Hence the mice were killed on 5th day and other groups were treated with pollen, propolis and bee bread alone as well as along with amoxicillin. Treatment with propolis (Gp. 3) was not found significant in comparison with pollen (Gp. 4) and bee bread (Gp. 5), while pollen and bee bread caused significant reduction in bacterial load and enhanced survival of experimental mice. The present studies showed 50–62% survival when observed for propolis, pollen and bee bread (Gp 3–5) as compared to the

infected group (Gp. 2). While the combinational treatment of pollen (Gp. 8) and bee bread (Gp. 9) along with amoxicillin showed 100% survival at the end of the experimental regimen. This can be due to reduction in bacterial load/cell lyses mediated by bacterial toxins or exposure to cell wall active agents might get reduced with this combinational therapy. The antimicrobial activity against *Staphylococcus* have been studied since long but the use of natural bee products along with antibiotics is a new approach towards multi drug resistance bacteria following a new delivery mechanism to maximize the antimicrobial agents concentrations. In the present study, we have observed *Staphylococcus aureus* mediated oxidative stress in liver and kidney of BALB/c mice which authenticated the phenomena that increased oxidative stress/free radicals are simultaneously accompanied by an immediate compensatory increase in the activities of antioxidant molecules and enzymes as evident from present observation i.e. *S. aureus* infection caused increased lipid peroxidation and decreased GSH levels and activities of antioxidant enzymes: (GST, SOD, CAT GPx and GR), in liver/kidney of bacterial infected mice.

Oxidative stress and lipid peroxidation are interlinked with each other. More the oxidative stress more will be the oxidation of lipids, resulting in end products which further act as second messenger of free radicals mediated oxidative stress. In the present study it was observed that *Staphylococcus aureus* caused increased lipid peroxidation in liver and kidney of all experimental groups which can be assessed by measuring the end product malondialdehyde. Treatment with bee products such as; propolis (Gp. 3), pollen (Gp. 4), bee bread (Gp. 5) and amoxicillin (Gp. 6) alone (250 mg/kg.bw/day; for 30 days) showed significant reduction in Lipid peroxidation as compared to infected group (Gp. 2). Bee products when used along with amoxicillin (Gp. 7–9) showed significant reduction in lipid oxidation, this authenticated the combinational therapy of bee products along with antibiotics (Fig. 1a). Glutathione is an important antioxidant molecule capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides and heavy metals. *S. aureus* infection in mice caused significant reduction in the reduced glutathione level (Gp. 2). Treatment with bee products alone as well along with amoxicillin led significant restoration in the glutathione levels in liver as well as kidney of *S. aureus* infected mice. It is also evident from the results that liver; the main metabolic center of the body was more infected as compared to kidneys. Propolis (Gp. 3), pollen (Gp. 4), bee bread (Gp. 5) and amoxicillin (Gp. 6) when used in monotherapy increased glutathione levels to some extent, but when used in combination, restores the values near normal in group (Gp. 7 & 8), bee bread along with amoxicillin showed the best results in restoring the values to normal level (Fig. 1b). The reason behind reduction in glutathione activity might be due to its increased utilization to counter bacterial infection as well due to increased lipids peroxidation. Lipids peroxidation is linked with glutathione activity as it utilize the cofactor NADPH which is required to change the lesser stable oxidized form of glutathione to more stable reduced form by glutathione reductase. This is how on *S. aureus* infection GR activity also gets reduced along with glutathione (GSH).[19]

Similarly antioxidant enzymes maintain their balance between their production and degradation on encountering a bacterial infection. Among them superoxide dismutase (SOD), catalase (CAT), glutathione system including (GST, GPx, GR) represent enzymatic antioxidants used to scavenge excess of internal as well as external reactive oxygen and nitrogen species mediated through oxidative stress.[20]

Among them the most studied antioxidant enzymatic scavenger is superoxide dismutase (SOD). It is evident from present studies that the activity of superoxide dismutase (SOD); a metabolic byproduct of oxygen utilization during bacterial infection got reduced after *S. aureus* infection (Gp. 2). Treatment with pollen, propolis and bee bread alone leds significant increase in the SOD activity. Pollen and bee bread along with amoxicillin (Gp. 8 & 9) restores the values to normal level in liver and kidney of *S. aureus* infected mice (Fig. 3). The decrease in SOD activity might be contributed to dismutation of superoxide anions ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2), which is further converted to water and oxygen by other antioxidant enzymes such as; catalase (CAT) and glutathione peroxidase (GPx).[21,22] This is how, in dismutation reaction CAT and GPx activities also got reduced. Bee products (propolis, pollen, bee bread) and antibiotic (amoxicillin) alone treatment led significant increase in the CAT and GPx activity (Gp. 3–6) in liver and kidney of *S. aureus* infected mice. Bee pollen and bee bread along with

amoxicillin restores the values to normal level authenticated the combinational therapy/ synergistic behavior of bee product along with antibiotics (Fig. 1d & g). The results observed confirmed combined/interlinked action of SOD, CAT and GPx[23] on encountering microorganisms. Previous studies elucidate the role of superoxide dismutase and catalase as virulence factor of many intracellular pathogens.[24–27]

It is also evident from the present studies that, there is significant reduction in glutathione-s-transferase activity in liver and kidney of *S. aureus* infected mice (Gp. 2). Propolis (Gp. 3), pollen (Gp. 4), bee bread (Gp. 5) and amoxicillin (Gp. 6) treatment alone led's significant increase in GST activity and the values were restored to normal when propolis, pollen and bee bread were given along with amoxicillin i.e. combinational therapy (Gp. 7–9). Best results were observed for pollen/bee bread along with amoxicillin in liver and kidney of *S. aureus* infected mice (Fig. 1e). The reduction in GST activity might be due to its contribution in eliminating the *S. aureus* mediated free radicals and its utilization for catalyzing the conjugation of reduced glutathione (GSH) to xenobiotic substrates and electrophilic compounds for their detoxification.

It is evident prom the present studies that glutathione and glutathione dependent enzymes (GST, GPx and GR) along with SOD and catalase all work together in restoring the normal metabolic rate of a cell. The depletion in their activity on encountering *S. aureus* infection might be due to protein/enzymatic degradation or inactivation caused by bacteria.

For assessing damage caused by *S. aureus* to liver and kidney, level and activities of various enzymes and macromolecules was measured. Liver comprises one third part of the reticulo-endothelial system and imparts important role in host defense against invasive microorganism as it contain variety of immune cells; such as macrophages, natural killer cells, natural killer T cells, and gamma-delta T cells which contributes to immunologic elimination of microorganisms along with kupffer cells of the liver.[28] The effects of microbial infection on hepatocytes vary from asymptomatic elevation in aminotransaminases, acute liver failure, hepatic fibrosis, cirrhosis[29] and the elevated liver enzymes might be associated with mortality due to heavy infection in the adult population.[30–32]

In the present studies, liver functioning was estimated by measuring enzymes such as; SGPT/SGOT also called transferase and ALP in serum samples of all experimental groups (Table 3). These enzymes are present in the hepatocytes and to some extent in the blood stream of healthy individuals. If some microbial infection or any injury is caused to liver, these enzymes get leaked in an abnormal level by damaged liver cells, which results in their elevated level in bloodstream indicating disease. Liver functioning is also evaluated by measuring bilirubin (hemoglobin byproduct). Normal levels of bilirubin are maintained by healthy liver by continuous removal of it from bloodstream for further processing. If liver is injured or infected/inflamed then bilirubin is not removed and its level rises in bloodstream. In the present studies it was observed that *S. aureus* infection (Gp. 2) caused significant rise in the level and activities of liver function markers in the blood stream. *S. aureus* might be causing injury to hepatocytes especially kupffer cells either by direct invasion or indirectly through toxins and cytokines[33] and there must be some immune reaction against this microbe infection which caused rise in the activities of liver markers.

Since liver is infected, it is not capable of processing bilirubin as well which is why its levels also get increased in the blood stream indicating disease. Treatment with propolis (Gp. 3), pollen (Gp. 4), bee bread (Gp. 5) and amoxicillin (Gp. 6) alone caused a significant reduction in the levels and activities of these markers. Pollen and bee bread along with amoxicillin (Gp. 8 & 9) caused a significant restoration in the values and activities of these liver function markers to normal level indicating synergistic behavior of bee products with amoxicillin. Bee products are strong antioxidant and their therapeutic effect on hepatocytes might be due to its suppression on microbial infection and leakage of enzymes through the plasma membrane, it also caused repair to the damaged liver cells.[34] Microbial infection impact on liver (*Streptococcus milleri, Escherichia coli, Streptococcus fecalis, Klebsiella pneumoniae* and *Proteus vulgari, Salmonella typhimurium)* and hepato-protective activities of bee products is also elucidated in previous studies. It showed inhibitory activity against puffiness, leakage and clustering in inflamed hepatic cells caused due to microbial infiltration.[33–35]

The kidney functioning is not measured through enzymes; rather it is assessed by measuring the breakdown product of proteins like urea, uric acid and

creatinine. Among them, urea/uric acid are formed in liver and carried via blood to kidneys for elimination. Creatinine; a breakdown product of specific kind of muscle protein is formed in the muscles and carried to kidneys for elimination. If some microbial infection is caused to kidneys then their elimination gets hampered and their level rises in bloodstream which indicates disease. As evidenced in the present studies; the levels of urea, uric acid and creatinine were raised in *S. aureus* infected mice (Gp. 2). Propolis (Gp. 3), Pollen (Gp. 4), bee bread (Gp. 5) and amoxicillin (Gp. 6) treatment caused significant reduction in their levels to some extent. Bee products when used in combination with amoxicillin (Gp. 7–9) caused significant reduction in the raised levels of kidney function markers to normal level. This authenticated the combinational effect of bee products along with antibiotic (Table 4).

Conclusions

In the present studies, it was observed that, among bee products studied here, pollen and bee bread were found to be most effective in ameliorating damage caused by *S. aureus* infection to liver and kidney of BALB/c mice. Moreover the combinational treatment with amoxicillin showed significant results by 5th day of infection which was further supported by follow up studies. However, more studies are required to test the combinational treatment in higher animal models which further necessitates extraction of bioactive constituents from bee products and then their use in combination with standard antibiotics so that their efficacy can be improved without fear of developing drug resistance or any other harmful effects caused to body due to antibiotics treatment. Liver; a firewall for filtering out microorganisms has compromised this function in liver disease and was found to be more affected as compared to kidney. Collectively, the findings showed that, the treatment of microbial infections, especially those caused by drug resistant microorganisms, should be considered for apitherapy, potentially in combination with antibiotics. Furthermore, apitherapy could be an important strategy to combat infections and should be comprehensively trialed for use within a medical setting.

Declarations

Ethics approval and consent to participate

It was approved by Institutional Animal Ethics Committee **(PU/IAEC/S/14/136)** of Panjab University, Chandigarh, India.

Competing interests

The authors declare that they have no competing interests

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