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Antioxidant potential and protective effects of bee pollen extract against *Salmonella* induced hepatic and renal toxicity in BALB/c mice

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Apicultural products comprise honey, bee pollen, propolis, bee wax and royal jelly which are known for their medicinal and health promoting properties. Among these, bee collected pollen allure much attention for its high nutritional properties. Here, we have investigated the protective role of bee pollen against *Salmonella typhimurium* induced biochemical alteration in BALB/c mice. Experimental animals (BALB/c mice) were divided equally into 10 different groups including normal and treated. Oxidative stress was induced by injecting *Salmonella typhimurium* (0.2 mL of 2×10^4 CFU/mL) intraperitoneally in mice. Bacteria induced sufficient alterations in serum enzymes within 5 days. Aqueous extracts of bee pollen of different crops (250 mg/kg) were administrated orally to control and experimental mice for 21 days. Then, hepatic and renal enzymes were measured with the help of standardized kits. Results of this study have revealed that bacterial infection increases the levels of the hepatic and renal enzymes levels (P <0.001) but after treatment with bee pollen extracts, altered levels of enzymes were normalized up to the normal levels. This normalization was highest with bee pollen of *Helianthus annus*. Administration of bee pollen alone did not produce any negative effects in mice.

Keywords: *Apis* spp., Apitherapy, Honeybee products, Oxidative stress, Polyphenols

Pesticides are used globally in agriculture to increase the yield and profit. They control various pests of different crops. Most of the pests exposed to these pesticide chemicals are killed by absorption through the body surface. All the crops like vegetables, fruits, cereals also absorb these chemicals. Pesticide chemicals enter into the body of human through the air, water and food. The pesticide after reaching into the human body affects many bio-chemical activities. Most of the people involved in the farming practices are affected by different diseases caused by these pesticides. Pesticide alters the metabolic activity of the body and causes the most common deadly diseases in society by creating hormonal mediated disruption like the insecticide parathion, malathion, and estrogen, which are responsible to cause breast cancer in women[1].The pesticidal activity also disrupts the vital organs functioning inside the body.

Polyphenols are produced as secondary metabolites by the plants which provide protection against harmful radiations, herbivores, pests and pathogens and are also known for their health-enhancing and disease-preventing activities. The polyphenolic composition of bee pollen is responsible for its antioxidant properties act via reducing catalytic capacities of free radicals in the electron transport system and the ability to neutralize free radicals by H-atom transfer[9-11]. Various reports suggest that bee pollen has a radical scavenging effect with or without toxic agents on several tissues, which may account for its beneficial effect on oxidant-induced injury[12-15]. Bee collected pollen of Chinese wolfberry enhanced the level of GSH, CAT and SOD and reduced the activity of ALT and lipid peroxidation, thereby decrease the oxidative stress and improve hepatic functions. Administration of bee pollen increase thymus and spleen indices and also enhances the production of cytokines such as IgA, IL-10, TNF-α and TGF-β1 in serum of cyclophospamide-treated mice[16]. However, little research is carried out on the antioxidative properties of bee collected pollen against *Salmonella typhimurium*. Around 11.9 million cases and 1,29,000 deaths were reported in low-income and middle-income countries[17,18]. The identified risk factors include intake of contaminated food, ice creams, water and poor sanitary conditions at home as well as excessive use of synthetic antimicrobial drugs. Fluoroquinolones, cephalosporins and azalides are the common antimicrobial drugs used against typhoid. Increasing multidrug resistance (MDR) creates the worrisome possibility of recurrence of untreatable typhoid[19].

The plant-based medicines are in use since the ancient period for improvement of health as well as

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treatment of various serious ailments. Here, we investigated the beneficial effect of bee pollen extract against *Salmonella* induced hepatic and renal toxicity using biochemical approaches.

Material and Methods

Collection and extraction of bee pollen

Bee pollen of different crops (*Helianthus annus*, *Brassica campestris* and *Zea mays*) was collected by installing a pollen trap (Fig 1) at the entrance of the beehive. In order to collect bee pollen from different crops, honeybee colonies were placed in different fields. Water was used to extract different pollen samples by using the protocol of Nagai *et al*.[20]

Phytochemical studies

Qualitative tests to identify the bioactive richness were performed on water extracts of different pollen by following the methods as described earlier[21]

Preparation of bacterial strain

Salmonella enterica serovar *Typhimurium* (MTCC 98) was used in the study. This strain was purchased from the Institute of Microbial Technology (IMTECH), Chandigarh, further identified biochemically according to Bergey's Manual of systemic bacteriology. The strain was maintained in nutrient agar slants at 4ºC. For further use, fresh agar slant was taken out each time, transferred into the nutrient broth and kept overnight. The next day, isolated colonies were procured by streaking on the nutrient agar plate, kept at 37ºC overnight. This was used for further studies as follows.

Animals and Housing

BALB/c mice (weighing 25-30 g) of either sex aged between 5 to 6 weeks were used for the experiments. Animals were kept in a temperature-controlled room under a 12 h light 12 h dark cycle. Animals had free access to commercial solid food (Ashirwaad Industries, Kharar, Punjab) and water *ad libitum*. All mice experiments in this study were approved by the Animal Ethical Committee (vide letter no. IAEC/411, dated September 11, 2013), Panjab University, Chandigarh in accordance with the guidelines of Animal Care.

Oxidative stress induction and Experimental groups

To induce oxidative stress, groups of animals were intraperitoneally injected with *Salmonella typhimurium* (0.2 mL of 2×10^4 CFU/mL). Bacteria-injected mice exhibited alterations in serum parameters unlike the control mice within 5 days. Animals were divided into ten groups (8 mice/group) as follows: group (N), normal, administered with normal saline orally; Group (I), infected, injected bacteria only; group (HP), normal mice received only bee collected pollen from *H. annus* orally without bacteria; group (I+HP), treated with bee collected pollen from *H. annus* (orally) in *S. typhimurium* infected mice, group (BP), normal mice receiving only bee collected pollen from *B. campestris* orally without bacteria; group (I+BP), treated with bee collected pollen from *B. campestris* (orally) in *S. typhimurium* infected mice; group (ZP), normal mice receiving only bee collected pollen from *Z. mays* orally without bacteria; group (I+ZP), treated with bee collected pollen from *Z. mays* (orally) in *S. typhimurium* infected mice; group (Vit C), normal mice receiving only Vitamin C orally without bacteria; group (I+Vit C), treated with Vitamin C (orally) in *S. typhimurium* infected mice.

Biochemical assays

Under diethyl ether, the animals were lightly anesthetized. Infected (Gp I) was sacrificed on the 5th day, as this was the peak day of infection while other groups were sacrificed immediately after the 21st day of treatment. Mice from all groups were sacrificed and blood was collected from the jugular vein in the Eppendrof tubes. The blood samples were left to clot for 20 min at room temperature. Clear serum was collected and then centrifuged at 3000 rpm for 30 min. Alanine aminotransferase (ALT)/ serum glutamate pyruvate transaminase (SGPT), Aspartate aminotransferase (AST)/ serum glutamate oxaloacetate trans-aminase (SGOT), Alkaline phosphatase (ALP), Bilirubin, Lactate dehydrogenase (LDH), Blood urea nitrogen (BUN), Urea, Uric acid and Creatinine levels in serum of normal, infected and treated groups were evaluated using standardized assay kits (Enzopak, Reckon diagnostic pvt Limited, Baroda).

Statistical analysis

The results obtained in the research were expressed as Mean±Standard deviation. The difference between mean values was analyzed by Two way ANOVA. p values of ≤0.05, ≤0.001 and ≤0.0001 were considered to be significant, very significant and extremely significant, respectively.

Results and Discussion

Several studies have shown that bee pollen of different crops is rich in considerable amounts of important compounds which may serve as antioxidants. These compounds can be classified

as Primary and Secondary metabolites. Sugars, amino acids, proteins and nucleic acids are included under primary metabolites. Secondary metabolites comprise flavonoids, terpenoids, alkaloids, saponins, tannins, resins, coumarins, *etc*. The latter is present in small amounts, complex structure and has restricted distribution as compared to the primary metabolites. They show remedial properties and are used traditionally to treat many ailments in humans. In the present study, polyphenolic composition of bee pollen from different crops was assessed. It showed the presence of tannin, flavonoids, carbohydrates, steroids, terpenoids, alkaloids, coumarins and quinones (Table 1). The concentration of tannins, steroids and terpenoids was found to be higher in *H. annus* as compared to *B. campestris* and *Z. mays.* Further, flavonoids, carbohydrates, alkaloids, coumarins and quinones were present in the same concentration in *H. annus* and *B. campestris* but less in *Z. mays* (except coumarins and quinones, which were found to be absent in *Z. mays*). However, the order of their overall concentration in bee pollen of different crops was *H. annus*>*B. campestris*>*Z. mays*. Freire *et al.*[22] found that monofloral pollens from Brazil contained flavonoids which are responsible for the antioxidant potential of the pollen. Ulusoy & Kolayli[23] reported the phenolic compounds comprising p-OH benzoic acid, caffeic acid, p-coumaric acid, ferulic acid, syringic acid, trans-cinnamic acid, cis and trans-abscisic acid, vanillic acid and rutin in all Anzer pollens but rutin, p-coumaric acid and ferulic acid were present in higher concentration function as natural antioxidants. Honey bee products or their bioactive chemical constituents like polyphenolic compounds showed antioxidant, antibacterial, anti-inflammatory, immunomodulatory and neuroprotective properties[24]. Bee pollen of *Camellia japonica* has abundant polyphenolic composition which cut down the release of serum uric acid and helps in the improvement of renal function. It also decreases the level of liver xanthine oxidase (XOD) and positively regulated the expression of urate transporter 1, organic anion transporter 1, organic cation transporter 1, glucose transporter 9, and ATP-binding cassette in the kidney[25].

Presented in Table 2 is the serum biochemistry of the mice in infected and treated groups. The level of ALT (138.5 ± 0.58), AST (96.45 ± 0.61), ALP (26.3 ± 0.58), Bilirubin (2.3±0.1), LDH (264.6±1.61), Urea (89.0±0.59), Uric acid (7.2±0.42), Creatinine

Table 1 — Phytochemical analysis of bee collected pollen of *H. annus*, *B.campestris* and *Zea mays*

Tests	HP	BP	ZP
Tannins	++	+	+
Flavonoids	++	++	+
Carbohydrates	++	++	+
Steroids	++	+	+
Terpenoids	++	+	+
Alkaloids	++	++	+
Coumarins	+	+	-
Quinones	+	+	-

[+++ = high concentration, ++ = moderate concentration, + = low concentration. HP, BP and ZP = bee collected pollen of *H. annus*, *B. campestris* and *Z. mays*, respectively]

Table 2 — Effect of treatments on serum parameters of BALB/c mice

Biochemical tests	N	I	HP	I+HP	BP	I+BP	ZP	I+ZP	Vit.C	I+Vit.C
ALT	20.6	138.5	19.31	30.1	21.59	58.56	22.34	136.59	20.04	20.48
(IU/L)	±1	±0.58@	±1.25^	±0.72^	±0.49^	±1.02^	±0.46^	±0.95	±0.68^	±0.50^
AST	26.82	96.45	27.98	35.39	28.20	42.41	28.68	83.98	25.7	26.77
(IU/L)	±0.87	±0.61@	±0.72^	±0.44^	±0.39^	±0.51^	±0.56^	±0.74^	±0.27^	±0.10^
Alkaline phosphatase	9.3	26.3	9.37	13.87	10.2	20.3	10.17	24.4	9.03	9.67
(KA units)	±0.6	±0.58@	±0.61^	±0.65^	±0.3^	±0.4^	±0.35^	±0.36	±0.42^	±0.5^
Bilirubin	1.05	2.3	1.07	1.5	1.13	1.9	1.06	2.13	0.97	1.16
(mg/mL)	±0.13	±0.1@	±0.12^	±0.1^	±0.16^	±0.2	±0.12^	±0.15	±0.02^	±0.06^
Lactate dehyrdrogenase	197.39	264.6	199.2	210. 04	201.58	221.95	204.39	249.61	197.56	201.79
(IU/L)	±2.12	±1.61@	±1.32^	±1.07^	±3.19^	±0.24^	±0.33^	±0.52^	±0.81^	±3.08^
Urea	47.77	89.0	48.6	68.49	48.24	77.75	49.12	85.19	45.54	54.89
(mg/dL)	±1.22	±0.59@	±1.07^	±0.79^	±0.35^	±1.56^	±0.78^	± 0.97	±1.59^	±2.55^
Uric acid	3.2	7.2	3.43	3.9	3.47	6.37	3.63	6.97	3.03	3.43
(mg/dL)	±0.26	±0.42@	±0.16^	±0.2^	±0.12^	±0.45*	±0.15^	±0.59	±0.15^	±0.47^
Creatinine	0.43	1.19	0.42	0.59	0.43	0.73	0.44	0.89	0.39	0.47
(mg/dL)	±0.04	±0.22@	±0.04^	±0.04^	±0.02^	±0.04^	±0.04^	±0.03^	±0.02^	±0.02^
BUN	18.14	40.84	19.14	25.07	19.69	29.59	19.70	39.83	18.37	19.66
(mg/dL)	±0.69	±0.94@	±0.36^	±0.67^	±0.22^	±0.34 ^	±1.27^	± 0.42	±0.66 ^	±0.10^

[Gp N *vs*. Gp I; $ P <0.05 (statistically significant); %:P ≤0.001 (very statistically significant); @:P ≤0.0001 (extremely statistically significant). Gp I *vs*. Treated groups, * : P <0.05 (statistically significant); # : P ≤0.001 (very statistically significant); ^ : P ≤0.0001 (extremely statistically significant)]

(1.19±0.22), BUN (40.84±0.94) in the infected group was increased up to extremely statistically significant level ($P \leq 0.0001$) as compared to normal group as ALT was (20.6 ± 1), AST was (26.82 ± 0.87), ALP (9.3 ± 0.6), Bilirubin (1.05±0.13), LDH was (197.39±2.21), Urea (847.77±1.22), Uric acid (3.2±0.26), Creatinine (0.43±0.04) and BUN was (18.14±0.69). While in treated groups, serum enzymes levels become significantly normalized near to normal in mice treated with bee pollen collected from *H. annus*, *B. campestris* (except bilirubin) and vitamin C. However, in the case of bee pollen collected from *Z. mays*, the serum enzyme values of infected groups were near to normal although this normalization was not significant (except LDH, creatinine, AST ($P \leq 0.0001$). The mean level serum enzymes of the mice assessed in this study, were extremely statistically significant ($P \leq 0.0001$) influenced by dietary (administration of bee pollen and vitamin C without infection) treatments.

The liver and kidney are important organs, responsible for the metabolism, storage, detoxification and excretion of xenobiotics and their metabolites. Damage to the hepatic cell membrane results in the release of cytosolic enzymes into the blood, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), which act as indicators of cellular damage. Therefore, their determination in serum could be used to assess any type of organ damage. In the present study, liver damage is manifested by increases in serum AST, ALT, ALP, bilirubin and LDH levels in the infected group as compared to the normal group (Table 2). It is well known that the kidney performs three main functions including the production of hormones which could be used to assess the renal status, elimination of toxic substances that are produced during metabolism and regulation of internal liquid medium homeostasis[26]. Al-Saleem *et al*.[27] experimented that administration of bee pollen showed protective effects in liver, kidney and brain against oxidative stress and free radical species produced by propionic acid in rat.

Renal function in the blood can be assessed through the measurement of BUN, urea, uric acid and creatinine. Creatinine, a catabolic end product of phosphocreatine, is produced by irreversible reactions during metabolism in skeletal muscle. It is eliminated by the kidneys from the body with very little tubular reabsorption. When the glomerular filtration rate diminished, it starts to deposit in the blood, indicating the impairment in kidney functions[28]. When there is Chronic Kidney damage (CKD), the end product of nitrogen metabolism builds up, increasing nonprotein nitrogen levels which can be expressed in the form of elevation of blood urea nitrogen and serum creatinine. Urea is formed in the liver from ammonia and later eliminated by the kidney as the end product of protein metabolism[29]. The increase in serum creatinine levels was observed after the *Salmonella* infection in the guinea pig, which indicated renal damage[30]. Intraperitoneal injection of Cisplatin increase the levels of ALP, Creatinine, BUN and also altered the other antioxidant enzymes but after treatment with bee pollen significant recovery was observed, which showed that bee pollen helped to improve the antioxidant, anti-apoptotic and anti-inflammatory of the body[31]. Accordingly, an increase in the levels of uric acid, creatinine[32], plasma urea[33], ALP and bilirubin[34] were observed after infection with *S. typhimurium* or its components as LPS. Increased BUN level is associated with CKD, blockage of the urinary tract by a kidney stone, increase the risk of diabetes mellitus and bleeding in the gastrointestinal tract[35].

Treatment with bee pollen of different crops after infection restored the levels of hepatic and renal enzymes to near normal (Table 2). On comparing the mean values of activity of bee pollen collected from different crops (Table: 2), it was found that *H. annus* (Gp: I+HP) performed well followed by *B. campestris* (I+BP) and *Z. mays* (I+ZP) but this comparison was not statistically significant. This may be due to the highest concentration of polyphenolic compounds in bee pollen of *H. annus.* Bee pollen of *H. annus* possesses the highest activity but not more than vitamin C (Gp: I+Vit C). Vitamin C normalized the levels of serum LDH, ALP, gamma glutamine, aspartate aminotransferase, alanine aminotransferase and bilirubin in intoxicated animals[36]. The overall hepatorenal protective activity of bee pollen might be due to the presence of high amount of polyphenols, flavonoids and cholinergic acids, which further possess high antioxidant potential owing to their ability to neutralise free radicals[37]. Honey bees collect bee pollen and knead it with the honey and their own secretions which contain different enzymes, the final product which is formed after these modifications is called as beebread. Administration of aluminium in rats

elevate the level of C-reactive protein, monocyte and leukocyte, decrease the level of Hb as well as it also increase the liver enzymes and BUN. These alterations were significantly ameliorated in beebread treated rats[38].

Administration of bee pollen of different crops without bacterial infection did not show any negative effects on hepatic and renal enzymes. These results agree well with other previous experimental investigations[39-42,12,13]. Bee pollen and propolis alone or in combination attenuated the deleterious effects of D-glucose induced type 2 diabetes and restored the biochemical enzymes which played an important role in the proper functioning of the liver and kidney[43]. Treatment with thymoquinone and/or bee pollen reversed the hepatic alterations induced by fluvastatin towards the normal level in rat. But their co-treatment gave better results than each alone[44].

Conclusion

In this study, we investigated the protective effects of bee pollen against oxidative stress produced by *Salmonella typhimurium*. Bacterial infection produces oxidative stress that resulted in an increase in the level of hepatic and renal enzymes, showing impairment in their function. After treatment with bee pollen, restoration was observed in the level of enzymes towards the normal level, indicating the antioxidant activity of bee pollen. So, the experimentation concluded that the administration of honey bee collected pollen plays an important in the protection of different body organs, especially the liver and kidney against oxidative stress or bacterial infections. These protective effects of bee collected pollen may be due to their rich bioactive composition. However, more research is required to test the effects of bee pollen in higher animal models before it is applied to typhoid patients. The need is to quantitatively investigate active constituents from different bee pollens and their usage to improve the efficacy without developing Multidrug resistance or any side effects.

Conflicts of interest

Authors declare no competing interests.

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