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"*In vivo*" free radical scavenging efficacy of beebread against biochemical alterations induced by *Salmonella enterica* serovar *Typhimurium*

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Salmonella typhimurium induced hepatic and renal toxicity was investigated using standard laboratory techniques in mice. BALB/c mice were divided into ten groups (8 mice per group). Group (Gp) 1 served as normal, and were administrated with normal saline orally, and Gp2 served as infected, and were injected bacteria (2×10^4 CFU/mL) only, while Gp3, 5, 7 and, 9 were administrated beebread (250 mg/kg body weight) of *Helianthus annus*, *Brassica campestris, Zea mays* and vitamin C respectively for 21 days without *Salmonella* infection, and Gp 4, 6, 8 and, 10 were administrated beebread (250 mg/kg bw) of *Helianthus annus*, *Brassica campestris, Zea mays* and vitamin C respectively for 21 days with a bacterial infection. Serum activities of hepatic and renal enzymes were analysed. A significant increase was observed in biochemical enzymes in *Salmonella* infected group on the 5th day but after the administration of beebread of different crops, relief was observed to be near normal. This alleviation was more with beebread of *Helianthus annus.* Furthermore, administration of beebread without bacteria did not show any negative effects in mice. Thus, the results indicate that aqueous extract of beebread of different crops is safe and can be exploited in healthcare delivery systems.

Keywords: Antioxidant, Honey bee products, Multidrug resistance, Oxidative stress, Protective effect, Typhoid

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Typhoid fever is caused by gram negative rod-shaped bacteria, *Salmonella typhi*. Transmission may occur through the ingestion of food or drink contaminated by the urine and faeces of an infected person. Symptoms include headache, high fever, constipation or diarrhoea, enlargement of liver and spleen, rose-colored spots on the chest, develops after 1-3 weeks after the exposure. *Salmonella typhimurium* causes a similar disease in mice as *Salmonella typhi* causes in human[1-5]. The problem of multidrug resistance (MDR) in the case of typhoid is very common. MDR increases the treatment cost, reduces the effective treatment options and causes deleterious effects on the various organs of the body and finally death. Bacterial invasion produces genetic changes in the body that can create oxidative stress in the body. Active oxygen (ROS) types such as hydroxyl radical, singlet oxygen, superoxide anion, peroxyl radicals and hydrogen peroxide are produced in the cell by endogenous (metabolic processes), exogenous (smoking, microbial infections) and pathogenic (inflammation, immune function etc.) sources[6]. Pro-oxidants are balanced by endogenous antioxidants but under pathogenic conditions, the balance shifts in favour of ROS, which causes oxidative stress. Hence, the higher concentration of pro-oxidants lead to deleterious effects on the cells lipids, proteins and nucleic acids[7] thereby resulting in loss of integrity and function of cell membranes[8]. Oxidative stress causes destructive conditions including cancer, diabetes, pulmonary disorders, cardiovascular and neurological disorders[9].

The use of natural medicine in the traditional system has been replaced by chemically synthesized drugs (allopathic medicines) modern scientific era. Once their use was considered as a blessing for human health, although technological advancements soon showed that prolonged use of these chemical drugs adversely influence the body organs. The aforesaid problem was reversed by the use of natural products that provides an efficient solution with the no or minimum adverse effects. Ancient Assyrians, Chinese, Egyptians, Greeks and Romans used

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honeybee products to treat intestine associated diseases and in wound healing. However, their use in modern medicine is limited because of the availability of insufficient scientific support[10]. Honey bees are wonder alchemists since times immemorial that contribute a lot towards human health-related issues. Honey consumption for the cure of cough, colds and several types of allergies was recorded in our ancient epics. Lofty, 2006 also recorded the use of propolis in many Georgian medicines to cure diseases and its application on wounds during World War II and Anglo-Boer war[11]. Bee venom also acts as a powerful tool in Chinese medicine for the treatment of joint pain and rheumatoid arthritis. The Greeks thought that beebread is the food of kings, provide youth and life. The earliest Egyptians narrated it as "a life-giving dust". Hippocrates, Pliny the Elder and Pythagoras are the "Fathers of Western Medicine" advised it to their patients for its healing properties. Consumption of beebread on large scale started after the Second World War with the advancement of pollen traps[12]. Its positive effects in public health were documented in Bible and ancient Egyptian texts. They considered it as a gold mine of nutrition due to its composition that has notable health promoting and medicinal properties. Its application to the skin helpful to make it soft, hydrates and, rejuvenates[13]. The therapeutic use of honey bee products generates a new branch of medicine named apitherapy.

Recently, beebread has received much attention because of its therapeutic applications[14]. Beebread is a modified mixture of bee collected pollen and nectar from different flowers. Bees add their own secretions containing enzymes with this mixture and, store it for the lactic fermentation within the hive. Medicinal values of beebread lie in its chemical composition that produces a definite physiological action on the human body. It can be used as a food supplement and source of nutraceuticals depends on its chemical richness, geographical origin, nutritional components (moisture, ash, fats, proteins and sugars), the presence of vitamin E, the polyphenolic content, antioxidant activity, which varies upon the flora diversity[15]. Several studies indicate its versatile pharmacological properties: antioxidative, antibacterial, hepatoprotective, antiviral and food preservative[12,16-18]. Thus, the present study aimed to investigate the protective effect of beebread in *Salmonella*-induced hepato-renal toxicity in mice by biochemical methods.

Material and Methods

Collection and extraction of beebread

Beebread of different crops (*Helianthus annus, Brassica campestris* and *Zea mays*) were collected from the cells of the hive from January 2013 to December 2015 with the help of forceps and spatula. For the collection of beebread of different crops, honeybee colonies were placed in different fields. Samples were extracted with water by using the protocol of Nagai *et al*. (2004)[19] and Kaur *et al*. (2013a)[20]. 3 g of fresh bee bread was suspended and extracted by shaking with 10 volumes of distilled water at 20ºC for 1 day and the extracts were centrifuged at 5000 rpm for 1 h. The supernatants were collected, filled up to 30 mL with water for biochemical analysis.

Phytochemical studies

Qualitative tests were performed according to the protocols of Misra *et al*. (2011)[21]; Vijaylakshmi and Ravindhran (2012)[22]; Kaur *et al*. (2013b)[23] to identify the bioactive richness of different samples of beebread.

Preparation of bacterial strain

Salmonella enterica serovar *Typhimurium* (MTCC 98) was used as a standard strain. This strain was obtained 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, a fresh agar slant was transferred each time in the sterilized nutrient broth and kept overnight. The bacterial culture was maintained on a nutrient agar medium.

Animals and housing

5 to 6 weeks old BALB/c mice of either sex weighing between 25 g-30 g were used for the studies. Animals were kept in plastic cages with an open-top (sealed with wire gauze) and temperature-controlled room under a 12 h light 12 h dark cycle. Animals were fed with commercial solid food (Ashirwaad Industries, Kharar, Punjab) and clean drinking water *ad libitum*. All mice experimentations were approved by Animal Ethical Committee, Panjab University, Chandigarh (vide letter no. IAEC/411, dated September 11, 2013) under the guidelines of Animal Care.

Oxidative stress induction and experimental groups

The dose of *Salmonella enterica* serovar *Typhimurium* used to induce oxidative stress was

2×10^4 CFU/mL. On the 5th day sufficient alterations were observed in the biochemical parameters in mice. The dose of bee bread of *H. annus* was adjusted to 250 mg/kg body weight of mice (bw) in distilled water and given orally for 21 days.

BALB/c mice were divided into ten groups (8 mice/group): group (Gp)1, normal control group, the animals were given distilled water orally, Gp2, infected, animals were intraperitoneally injected with *Salmonella typhimurium* (0.2 mL of 2×10^4 CFU/mL); Gp3, normal mice receiving only beebread from *H. annus* orally without bacteria; Gp4, mice treated with beebread from *H. annus* (orally) in *S. typhimurium* infected mice, Gp5, normal mice receiving only beebread from *B. campestris* orally without bacteria; Gp6, with beebread from *B. campestris* (orally) in *S. typhimurium* infected mice; Gp7, normal mice receiving only beebread from *Z. Mays* orally without bacteria; Gp8, treated with beebread from *Z. mays* (orally) in *S. typhimurium* infected mice; Gp9, normal mice receiving only vitamin C orally without bacteria; and Gp10, treated with vitamin C (orally) in *S. typhimurium* infected mice.

Biochemical assays

Under diethyl ether, the animals were lightly anesthetized. Mice from all groups were sacrificed and immediately all blood samples were collected from the jugular vein in the Eppendorf tubes. The blood samples were left to clot for 20 min at room temperature before centrifugation at 3000 rpm for 30 min.

Assessment of liver function tests

Alanine aminotransferase (ALT)/ serum glutamate pyruvate transaminase (SGPT), Aspartate aminotransferase (AST)/ serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), bilirubin, lactate dehydrogenase (LDH), were measured according to standardized assay kits (Enzopak, Reckon diagnostic Pvt. Limited, Baroda).

Assessment of renal function tests

Blood Urea Nitrogen **(**BUN), urea, uric acid and creatinine were measured with the help of standardized diagnostic kits (Enzopak, Reckon diagnostic pvt. Limited, Baroda).

Statistical analysis

Statistical analysis was done by using ANOVA and Student's t-test. The data were presented in the form of mean± standard deviation. p-values of ≤0.05, ≤0.001 and ≤0.0001 were considered to be significant, very significant and extremely significant respectively.

Results

Phytochemical investigations (Table 1) showed the presence of tannin, flavonoids, carbohydrates, steroids, terpenoids, alkaloids, coumarins and quinones in the bee bread of different crops. However, the order of phytochemical concentration of beebread of different crops was *H. annus>B. campestris>Z. Mays*.

The alterations were observed in the behaviour of infected mice as compared to the normal mice. Externally, *Salmonella* administered mice showed reduced water and feed intake, unstable movements, tangled fur, partially closed eyes and hunched posture, which are the signs of illness. Biochemically, there was an extremely statistically significant (p≤0.0001) elevated effect was observed in the level of liver and kidney serum enzymes in the infected group (Gp2) as compared to normal group (Gp1) (Table 2), which indicates the production of oxidative stress in mice. Conversely, upon treatment with beebread of different crops (Gp4,6,8) and vitamin C (Gp10), stress effect was restored to near normal as compared to the infected group. These normalizations were at a significant level in the beebread of *H. annus*, *B. Campestris, Z. mays* (except bilirubin and uric acid) and vitamin C. The mean values of serum enzymes in beebread and vitamin C alone (without infection) treated groups (Gp3,5,7 & 9) were measured to be up to statistically significant (p≤0.0001).

Discussion

Elevated concentration of free radicals has the power to damage a tissue or cell damage by reacting

Table 1 — Phytochemical analysis of bee bread of *H. annus*, *B. campestris* and *Z. Mays*

S.No.	Tests	HB	BB	ZB
1	Tannins	+++	+	+
2	Flavonoids	+++	+++	++
3	Carbohydrates	+++	+++	++
4	Steroids	++	++	++
5	Terpenoids	++	++	+
6	Alkaloids	+++	+++	+
7	Coumarins	++	+	-
8	Quinones	++	++	-

+++ = high concentration, ++ = moderate concentration, + = low concentration (HB = bee bread of *Helianthus annus*, BB = bee bread of *Brassica campestris*, ZB = bee bread of *Zea mays*)

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

S. No.	Biochemical tests	Gp1	Gp2	Gp3	Gp4	Gp5	Gp6	Gp7	Gp8	Gp9	Gp10
1	ALT (IU/L)	20.6±1	138.5±0.58$^{@}$	19.07±0.94$^{\wedge}$	28.65±0.92$^{\wedge}$	21.83±0.07$^{\wedge}$	52.36±1.28$^{\wedge}$	21.09±1.27$^{\wedge}$	132.04±0.05$^{\wedge}$	20.04±0.68$^{\wedge}$	20.48±0.50$^{\wedge}$
2	AST (IU/L)	26.82±0.87	96.45±0.61$^{@}$	27.54±0.53$^{\wedge}$	31.43±0.93$^{\wedge}$	28.56±0.33$^{\wedge}$	38.72±0.81$^{\wedge}$	28.08±0.52$^{\wedge}$	79.04±0.48$^{\wedge}$	25.7±0.27$^{\wedge}$	26.77±0.10$^{\wedge}$
3	Alkaline phosphatase (KA units)	9.3±0.6	26.3±0.58$^{@}$	9.27±0.35^	10.45±0.78^	10.3±0.46^	19.1±0.35^	10.03±0.47^	23.33±1.83^	9.03±0.42^	9.67±0.5^
4	Bilirubin (mg/ml)	1.05±0.13	2.3±0.1$^{@}$	1.03±0.06^	1.37±0.12^	1.09±0.11^	1.8±0.21*	1.12±0.18^	2.07±0.15	0.97±0.02^	1.16±0.06^
5	Lactate Dehyrdrogenase (IU/L)	197.39±2.12	264.6±1.61$^{@}$	198.05±1.97^	202.91±0.96^	199.86±1.17^	209.77±1.65^	202.53±1.33^	234.01±1.2^	197.56±0.81^	201.79±3.08^
6	Urea (mg/dL)	47.77±1.22	89.0±0.59$^{@}$	47.24±1.22^	64.88±1.33^	44.69±1.09^	75.32±0.86^	48.74±0.59^	83.67±0.67*	45.54±1.59^	54.89±2.55^
7	Uric acid (mg/dL)	3.2±0.26	7.2±0.42$^{@}$	3.2±0.2^	3.53±0.15^	3.43±0.21^	5.9±0.26^	3.6±0.36^	6.6±0.2	3.03±0.15^	3.43±0.47^
8	Creatinine (mg/dL)	0.43±0.04	1.19±0.22$^{@}$	0.40±0.03^	0.52±0.01^	0.41±0.02^	0.65±0.05^	0.41±0.03^	0.83±0.04^	0.39±0.02^	0.47±0.02^
9	BUN (mg/dL)	18.14±0.69	40.84±0.94$^{@}$	19.14±0.36$^{\wedge}$	21.07±0.67$^{\wedge}$	19.69±0.22$^{\wedge}$	26.59±0.34$^{\wedge}$	19.70±1.27$^{\wedge}$	36.67±0.40$^{\#}$	18.37±0.66$^{\wedge}$	19.66±0.10$^{\wedge}$

Gp N v/s Gp I; $ p<0.05 (statistically significant); %: p≤0.001 (very statistically significant); @: p≤0.0001 (extremely statistically significant)
Gp I v/s Treated groups *: p<0.05 (statistically significant); #: p≤0.001 (very statistically significant); ^: p≤0.0001 (extremely statistically significant)

with the biochemical components of the cell such as lipids, carbohydrates, proteins, and DNA. In general, an antioxidant is a substance that inhibits or delays the peroxidation by scavenging or neutralizing the free radicals thereby preventing the cell from damage. Currently, the antioxidants are receiving more attention as they are a crucial factor in the biochemical management of oxidative stress[24]. The antioxidant property of any substance may be due to its polyphenolic composition. They relieve oxidative stress by various mechanisms, including shut down of chain initiation, decomposition of peroxides, binding of transition metal ion catalysts, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging[25]. Vast numbers of natural products have been detected to contain such antioxidants in the form of bioactive compounds[26]. Among these, apicultural products receive great attention due to their therapeutic potential. Beebread is one of the apicultural hive products and, has proved to be a good source of nutrients, with high carbohydrate and protein content. It is also rich in fatty acids such as palmitic, stearic, arachidonic, oelic, eicosenoic, erucic and linolenic acid. Its high nutritional value, together with the richness in phenolic and its high bioactivity, makes beebread an excellent choice to be consumed as a nutraceutical in food supplementation[27]. The results of the present study demonstrated that the *Salmonella*-infected mice exhibited significantly higher levels of hepatic and renal enzymes as compared to normal mice (Table 2). Our results are in agreement with several studies such as by Shamim *et al*. (2012)[28]; Kaur and Jain, (2013)[29]; Yamaguchi *et al*. (2007)[30] and, Salim *et al*. (2011)[31]. Alteration of the level of these enzymes may be due to many factors, including hepatic lysis by cell necrosis, cell leakage, and loss of functional integrity of the cell membrane of liver and kidney cells[32]. Moreover, these disturbances are due to lipid peroxidation induced by the generation of ROS under the effect of bacterial toxicity and alterations of the cell membrane causing the seepage of enzymes in the blood.

The emergence of multidrug resistance (MDR) in *Salmonella* species creates a major problem for the treatment of typhoid fever. In addition to MDR, antibiotics are sometimes also associated with deleterious side effects such as hypersensitivity, immune suppression and allergic reactions[33]. Therefore, there is a need to find safe alternative medicine towards antimicrobial agents for the treatment of infectious diseases such as typhoid. Beebread has been identified a source of bioactive constituents that are responsible for antioxidant activities. Thus, the experiment found that after the administration of beebread of different crops after infection (Gp4, 6 & 8) revive the activities of hepatic and renal enzymes to near normal (Gp1) (Table 2). On comparing the activity of beebread of different crops and vitamin C (Table 2), it was found that *H. annus* (Gp4) possess the highest activity as compared to *B. campestris* (Gp6) and *Z. mays* (Gp8) but not more than vitamin C (Gp10). This is maybe due to the rich concentration of bioactive compounds in the beebread of *H. annus.* No side effects were observed after only administration of beebread of different

crops (Gp3, 5 & 7). Several studies showed that beebread has antioxidant potential and exhibited a protective effect against aluminium-induced blood and hepato-renal toxicity[34]. Bee bread, honey and propolis produced by stingless bees displayed antioxidant, antimicrobial anti-proliferation effects on cancer cells[35,36]. In the future, beebread can be used as medicine and health food due to its scavenging activities against active oxygen species[18]. Further, the alone administration of bee pollen and beebread did not cause any oxidative stress in *in vivo* studies in animal models[37-40]. It also possesses inhibitory effects against bacteria. It had been found that this inhibition was more against Gram-positive bacteria as compared to Gram-negative bacteria[41]. On comparing honey and beebread, beebread was shown to possess higher anti radical potential than honey[42]. Beebread holds enormous quantities of unsaturated fatty acids and sometimes has very favorable n-6/n-3 fatty acid ratios which indicate its high nutritive value[43].

Conclusion

Anciently in China, Egypt and Greece, beebread was used as a medicine. They use it to maintain their good health, improve skin texture and delay the aging. The present research delivers basic information about the antioxidant activity of the aqueous extract of bee bread. Therefore, the study concluded that administration of beebread from different crops has the potential of anti-stress activity-induced due to *Salmonella typhimurium*. But when we examine carefully, the activity of bee bread of *Helianthus campestris* is stronger as compared to bee bread of *Brassica campestris* and *Zea mays.* These effects might be mediated due to their polyphenolic composition related to their antioxidant potential and amelioration of hepatic and renal enzymes alteration.

Fluctuation in biochemical indices from normal levels will lead to deterioration of the normal functioning of the organs. Since no such major changes were found in the biochemical parameters on the alone administration (without bacteria) of beebread of different crops, it shows the safe intake of studied apicultural honey bee product at a concentration of 250 mg/Kg and that it has no adverse effect on the functioning of the liver and kidney in the mice. Further research has to be conducted to measure the amount of active components of beebread and their biological activity to support its potential use as an alternative therapy against MDR with the help of higher animal models.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

RK did the practical work and manuscript writing. NRK and KH were guided during experimental work.

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