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Efficacy of bee pollen and beebread against *Salmonella typhimurium* in BALB/c mice

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ABSTRACT: The present work evaluate the antibacterial and antioxidant potential of bee pollen and beebread against the toxic changes induced by *Salmonella typhimurium* in BALB/c mice. The experiment was divided into six groups as; Gp1 was normal, Gp2 was infected with *Salmonella enterica* serovar Typhimurium at 2×10^4 CFU, Gp3 and Gp5 were administrated with bee pollen and beebread of *Helianthus annus* crop alone at 250mg kg^{-1} bw respectively; Gp4 and Gp6 were with *S. typhimurium* and bee pollen and beebread *H. annus* at 250mg kg^{-1} bw respectively. Different hematological and oxidative stress parameters were assessed in animals. It has been observed that Gp2 showed alteration in the level of all tested parameters as compared to the Gp1, which indicated the toxicity induced by bacteria, but after the treatment with bee pollen and beebread as in Gp4 and 6, their level ameliorated to near normal, which showed the effectiveness of the tested bee products against bacteria.

KEY WORDS: Apitherapy, antibacterial, antioxidant, typhoid, *Helianthus annus* crop

INTRODUCTION

Typhoid is caused by rod shaped bacteria, *Salmonella typhi* which is transmitted through contaminated food or water. Symptoms include continued fever, rose like spots on chest, abdomen and back, spleenomegaly and inflammation of intestine. Once bacteria entered in the body through contaminants, it evades the acidic barrier and attaches itself to the epithelium of intestine and then penetrates, divides inside the mesenteric lymph nodes and finally, reaches the blood stream. Here, immune system gets activated and action of antibodies and complement system gets started against the bacteriaEverest *et al.* (2001). As a result, few of them get lysed and release endotoxins, which further cause typhoidal fever. While some cleared from the blood and come in contact with macrophages of liver, spleen, lymph nodes, bone marrow where they survive and again multiply. If untreated, some of the typhoid patients develop complications that include intestinal ulcers and perforation, further leads to bleeding, sudden rise in pulse rate, abdominal discomfort and rigidity (Butler, 2011). A number of synthetic drugs used to treat the bacterial infection. But with time, bacteria develop resistance against these drugs and this phenomenon, by which pathogenic microorganism became resistant to a group of related or unrelated drugscalled multiple drug resistance MDR (Parry*et al.*, 2002). Continue use of these drugs develop a number of undesirable effects in the human body

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that effects the normal functioning of body organs.

Nowadays, research shift towards the development of safe and cost effective natural remedies against MDR. Natural products including bee products have gained greater popularity over widely used synthetic pharmaceuticals. Honey bee products such as honey, beeswax, bee venom, propolis, bee pollen and royal jelly, out of these three (beewax, venom and royal jelly) are chemically synthesized by the bees themselves and other three (pollen, honey and propolis) are derived from plants and are modified by the bees for their own use. All these products have amazing beneficial effects.Bee collected pollen recently drawn more attention as sources for new drug discovery as well as an additive agent to reduce negative effects of popularly used drugs. Honey bees collect pollen from different flowers of the plant in order to feed their larvae. Pollen is a good source of protein and bee required it for the proper growth and maintence of their larvae. Bees collect pollen, modify it with their own abdominal secretions and honey, further they store it in their comb cells for lactic acid fermentation. This ripened product is known as beebread. Chemically, the composition of bee pollen varies according to the age, type and nutritional status of plant species and environmental conditions of the area visited by bees (Almaraz-Abarca *et al.*, 2004). Analysis of chemical composition in previous studies revealed that it contains different types of polphenolic compounds such as flavonoids, phenolic acids, tannins and other compounds (Bonvehi *et al.*, 2001), these compounds are responsible forantibacterial (Morais *et al.*, 2011; Fatrcová-Šramková *et al.*, 2013) and antioxidant properties (Kroyer and Hegedus, 2001; Silva *et al.*, 2006; Moita *et al.*, 2013).The antibacterial and antioxidant effect of polyphenols are mainly due to their redox properties, which are helpful in scavenging free radicals, neutralizing ROS and decomposing peroxides (Nijveldt *et al.*, 2001). Besides, bee pollen also act as dietary supplement that prove beneficial to cope up with the side effects produced by drug therapies and to boost up the immunity at the time of pathological conditions (Radimer *et al.*, 2004). The present work evaluate the antibacterial and antioxidant potential of bee pollen and beebread against the toxic changes induced by *Salmonella typhimurium* in BALB/c mice.

MATERIALS AND METHODS

Bee pollen was collected by installing pollen trap at the hive entrance of the bee colonies, placed in the *Helianthus annus* crop. After that, for the collection of beebread from the comb cells, forceps and spatula were used. Collected samples were stored at -20^{o}C for further experimentations. Aqueous extracts of bee pollen and bee bread were prepared by following the protocols of Nagai *et al*. (2004) and Kaur *et al.* (2013b).

Bacterial strain of *Salmonella typhimurium* (MTCC 98) was obtained from IMTECH (Institute of Microbial Technology), Sector -39, Chandigarh and tested biochemically prior to use according to Bergey's Manual of systemic bacteriology. Further, strain was maintained in the nutrient agar and stored in the form of small aliquots at -20°C before sub culturing.

Determination of minimum inhibitory concentration (MIC): Broth dilution method was performed to test the value of MIC. A series of broth containing test tubes were prepared, to which different concentrations of extracts were added viz., 0mg ml^{-1} (negative control), 50, 100, 150, 200, 220, 240, 260, 280, 300, 320, 340, 360, 380 and 400mg ml^{-1}. After that, inoculate the tubes with standardized suspension 2X 10^{4} CFU of test pathogen. Then incubate the tests tubes at 37°C for 24hours and next day, MIC was determined. Turbidity in the test tubes indicates the presence of bacteria and clear broth or absence of turbidity indicates as absence of pathogen. It is defined as the lowest concentration of the pollen extracts, where no visible growth of bacteria is seen in the test tubes. Determination of minimum bactericidal concentration (MBC): MBC of tested bee products analyzed by transferring aliquots of 0.1ml from MIC test tubes (means test tubes, which show no visible bacterial growth), spreading on Agar plates and incubate at 37°C for 24h. When 99.9 per cent of bacterial population is killed at the lowest concentration of the extract, is regarded as MBC.

BALB/c mice were obtained from the Central Animal House, Panjab University, Chandigarh, India. All the experimental protocols using mice were carried out strictly under the approval of the Animal Ethical Committee, Panjab University, Chandigarh. Mice weighing 20-25g and aged between 4 to 6 weeks were used in all biochemical studies. These were fed on standard pellet diet (Ashirwaad Industries, Kharar, Punjab) and water ad libitum. Animals were acclimatized for one week before the beginning of the research experiments.

BALB/c mice were divided into six groups with 6-8 animals in each. Group 1: Control (Normal mice given saline orally). Group 2: Mice were challenged intraperitoneally with *Salmonella enterica* serovar Typhimurium at 2×10^4 CFU ml^{-1}. Group 3: Animals given bee pollen (250mg kg^{-1} bw) collected from *H. annus* orally for 21 days. Group 4: *Salmonella* infected + water extract of bee pollen collected from *H. annus* (250mg kg^{-1}bw) given orally for 21 days. Group 5: Animals given beebread collected from *H. annus* (250mg kg^{-1}bw) orally for 21 days. Group 6: *Salmonella* infected + water extract of beebread collected from *H. annus* (250mg kg^{-1} bw) given orally for 21 days. Mice in group 2 were sacrificed on day 5 post infection as it was the peak day of infection. Experiments were conducted in triplicate.

Blood was aspirated from jugular vein of animals of different experimental groups in sodium salt of ethylenediaminetetraacetic acid (EDTA). After that, hemoglobin (Hb), red blood cell count (RBC), total leucocyte count (TLC), Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were also calculated by following standard protocols.

Organs (Liver, Spleen and Kidney) were homogenized in an ice-cold 100mM potassium phosphate buffer (pH 7.4) containing 150 mM KCl to obtain 10 per cent homogenate (w/v). This homogenates of different tissues was divided into two portions. One portion of homogenate was used for the estimation of LPO (lipid peroxidation; Beuge and Aust, 1978) and GSH (glutathione; Moron *et al*., 1979). Other portion was subjected to cold centrifugation at 10,000g for 30 min. The pellets were discarded and supernatants regarded as post-mitochondrial supernatant, were used for further estimation of other enzymes such as SOD (superoxide dismutase; Kono, 1978) and CAT (catalase; Luck, 1971), GST (glutathione-S-transferase; Habig *et al*., 1974), GP (glutathione peroxidase; Pagila and Velentine, 1967) and GR (glutathione reductase; Carlberg and Mannervik, 1985). The quantity of protein in homogenate and supernatant of different samples was determined by the method of Lowry *et al*. (1951). The data was presented as a mean ± standard error and analyzed by ANOVA and Student t-test. p values of 0.05, 0.001 and 0.0001 were considered to be significant, very significant and extremely significant, respectively.

RESULTS AND DISCUSSION

MIC and MBC activities of bee pollen and beebread investigated to evaluate their antibacterial activity against gram negative bacteria i.e. *S. typhimurium,* revealed that both extracts were potentially effective in suppressing the bacterial growth with different potency, but beebread was the effective to retard the growth of the tested pathogenic bacteria as compared to bee pollen. Antimicrobial activities of bee pollen and beebread were 320 and 260mg ml^{-1} respectively for MIC; it was 380 and 320mg ml^{-1} respectively for MBC.

In mice, alterations in the blood parameters and antioxidant enzymes were observed in G2 as compared to the G1 but after the treatment, these alterations were ameliorated to the near normal in G4 and G6 (Table 1, 2).

The problem of multi drug resistance is growing by time therefore; actions must be taken to face this problem by introducing the safe and cost effective, natural products (Nostro *et al.*, 2000; Osman *et al.*, 2013). Apitherapy is the alternative branch of medicine that deals with the use of the honey bees and their different products. They are also known as Master alchemists as their products have many beneficial effects. The use of bee venom for the treatment of rheumatoid arthritis and joint problems and honey for the cure of common cold, cough and

Table 1: Hematological alteration with different groups due to bee products

Parameters	Gp1	Gp2	Gp3	Gp4	Gp5	Gp6
Hb	13.4 ± 0.34	9.69 ± 0.39@	13.5 ± 0.31^	12.5 ± 0.76^	13.3 ± 0.43^	13.3 ± 0.52^
RBC	8.64 ± 0.24	5.7 ± 0.54@	8.7 ± 0.43^	7.8 ± 0.73^	8.2 ± 0.97^	7.5 ± 1.35*
MCH	19.30 ± 1.22	32.91 ± 2.6@	19.59 ± 2.92^	26.94 ± 1.59#	18.70 ± 3.28^	21.94 ± 3.52^
MCV	52.46 ± 2.29	71.97 ± 3.97@	53.38 ± 2.47^	65.04 ± 2.46*	50.14 ± 4.30^	56.63 ± 2.21^
MCHC	29.73 ± 3.11	35.89 ± 1.19@	29.12 ± 2.23^	30.10 ± 3.43^	29.00 ± 4.25^	31.82 ± 2.46^
Hematocrit	39.52 ± 1.71	26.87 ± 1.03@	40.08 ± 2.59^	32.84 ± 2.09#	38.62 ± 1.39^	36.40 ± 0.80^
WBC	7767.81 ± 66.79	5620.97± 51.25@	7861.11± 48.45^	6569.57± 50.05^	7711.43±2.62^	6681.71±7.20#
Lymphocytes	67.23 ± 1.24	87.21 ± 0.70@	68.44 ± 0.71^	79.35 ± 2.69 ^	69.34 ± 1.98^	74.27 ± 1.87^
Neutrophils	25.42 ± 1.30	16.24 ± 0.58@	24.82 ± 0.63^	23.01 ± 2.74^	25.78 ± 0.75^	22.63 ± 0.60^
Eosinophils	1.00 ± 0.82	2.29 ± 0.76	0.86 ± 0.69*	1.14 ± 0.90	1.29 ± 0.95	1.14 ± 0.69

allergies are well documented in our ancient epics (Haleem *et al.*, 2015; Kaur *et al.*, 2015). With time, the therapeutic affects other bee products, also came to light (Kalia *et al.*, 2017).

In the present study, a number of blood parameters tested to investigate the effect of bee pollen and beebread against the *Salmonella*. There was extremely statistically significant (except in case of eosinophils) alterationsin the level of blood parameters were recorded in the Gp2as compared to the Gp1but after the administration of bee pollen and beebread (Gp4 and 6), these alterations were restored to the near normal, which showed the effectiveness of the bee products. This restoration was up to extremely statistically significant except in case of MCH and hematocrit, which were very statistically significant and in case of MCV, which was statistically significant in case of Gp4 and also in case of Gp6, the level of blood parameters were up to extremely statistically significant except in case of WBC, which was very statistically significant and in case of RBC, which was statistically significant. The inhibitory effects of bee pollen against bacterial infection were also reported by Aboude *et al.* (2011) and Sramkova *et al.*(2013).

Phytochemical screening of bee pollen and beebread revealed several types of secondary metabolites such as alkaloids, polyphenols, flavonoids, coumarins, saponins, tannins and steroids (Kaur *et al.*, 2013a and b). These molecules are act actively against pathogenic microorganisms, as antibacterial and antioxidant activities (Tsopmo *et al.*, 2013; Erfan and Marouf, 2019). The steroids exerts its action by forming complex with membrane lipids and thus causing leakage of enzymes and other components from cell, which in turn effect the stability of the bacterial cell (Marjorie, 1999), similarly Saponins have detergent like properties that cause leakage of proteins and important enzymes from cell (Shimada, 2006). Further, tannins present in these apicultural products have ability to react with the protein components of the cell wall of the bacteria to form a stable insoluble component, which in turn effects the functioning of the cell (Dangoggo *et al.*, 2012) while, the antibacterial effects of alkaloids are due to its ability to form interchelate with nucleic acid of both Gram positive and negative bacteria and interfere with cell division (Bukar *et al.*, 2015).

Antioxidant activity of bee pollen and beebread also assessed in liver, spleen and kidney of the mice (Table 2). In Gp2, oxidative stress was increased as compared to Gp1 but restoration was observed after the treatment with bee pollen and beebread in Gp 4 and Gp 6.Peroxidation of lipids induced due to generation of ROS and results in oxidative stress. In present study, there is an increase in the level of LPO in the infected group as compared to the normal group, which indicates the production of

Table 2. Activity of antioxidant enzymes in different groups of Liver, Spleen and Kidney

Organs	Biochemical	G1	G2	G3	G4	G5	G6
Liver	LPO	0.21 ± 0.02	0.49 ± 0.03@	0.18 ± 0.02^	0.29 ± 0.01^	0.17 ± 0.01^	0.24 ± 0.04^
	GSH	1.6 ± 0.35	0.62 ± 0.11@	1.8 ± 0.38^	1.43 ± 0.06*	1.88 ± 0.31^	1.57 ± 0.12#
	SOD	9.6 ± 2.18	3.67 ± 2.4@	12.77 ± 1.29^	7.62 ± 1.41^	13.03± 1.65^	9.47 ± 1.3^
	CAT	74.81± 2.09	46.03 ± 1.99@	75.77± 1.42^	61.37± 4.03^	76 ± 3.0^	67.48± 2.14^
	GST	0.86 ± 0.04	0.43±0.03@	0.97 ± 0.03^	0.8 ± 0.02^	1.03 ± 0.06^	0.83 ± 0.07^
	GR	49.35± 1.32	32.8 ± 1.86@	52.5 ± 1.22^	43.99± 1.33^	52.97± 1.13^	48.27± 1.56^,á
	GP	13.28± 0.89	6.43 ± 0.54@	14.92± 0.82^	10.83± 0.87^	15.21± 1.1^	12.38± 0.42^,á
Spleen	LPO	0.30 ± 0.02	0.48 ± 0.03@	0.28 ± 0.01^	0.35 ± 0.04^	0.27± 0.02^	0.33 ± 0.02^
	GSH	1.33 ± 0.15	0.67 ± 0.12@	1.5 ± 0.1^	1.08 ± 0.08*	1.56 ± 0.15^	1.26 ± 0.06^,á
	SOD	10.7± 3.27	5.5 ± 1.85@	12.67 ± 2.67^	7.99 ± 2.01#	12.97 ± 1.5^	9.76 ± 3.7^
	CAT	73.17± 1.75	27.2±1.78@	75.1 ± 5.63^	64.2± 2.69^	75.3± 4.03^	67.0± 3.83^
	GST	0.74 ± 0.04	0.23 ± 0.04@	0.90 ± 0.05^	0.69 ± 0.03^	0.92 ± 0.03^	0.73 ± 0.03^
	GR	52. 45±1.21	36.16 ±1.59@	54.44± 1.23^	45.04± 1.59^	54.94± 1.76^	48.33 ± 1.40^
	GP	9.46 ± 0.6	5.27 ± 0.32@	11.12± 0.8^	7.87 ± 0.23^	11.36± 0.32^	9.31 ± 0.56^,á
Kidney	LPO	0.11 ±0.006	0.18 ± 0.006$	0.09 ± 0.02#	0.17 ± 0.006	0.08 ± 0.06^	0.12 ± 0.001á
	GSH	1.23 ± 0.23	0.75 ± 0.05%	1.39 ± 0.01^	1.14 ± 0.05*	1.43 ± 0.13^	1.26 ± 0.09^
	SOD	10.82 ±4.25	8.2 ± 1.7@	13.0 ± 1.57^	8.17 ± 1.20	13.53 ±2.07^	9.93 ± 1.90^
	CAT	79.8 ± 2.88	60.21 ±1.05@	80.8 ± 1.19^	74.28 ± 1.11^	80.94 ±3.60^	77.9 ± 2.19^
	GST	0.71 ± 0.02	0.52 ± 0.03%	0.91 ± 0.03^	0.67 ± 0.03*	0.96 ± 0.03^	0.71 ± 0.04#
	GR	78.8 ± 1.55	75.48 ± 1.84$	79.9 ± 1.98^	71.19 ± 3.71^	80.3 ± 4.17^	75.95 ± 1.3
	GP	11.16 ±0.66	8.13 ± 0.74@	12.1 ± 0.37^	9.3 ± 0.78	12.35 ±1.02^	10.79 ± 0.23#,á

Gp 1 v/s Gp 2 $ p<0.05 (statistically significant); %:p < 0.001 (very statistically significant); @:p < 0.0001 (extremely statistically significant)

Gp 2 v/s Treated groups * : p < 0.05 (statistically significant); # : p < 0.001 (very statistically significant); ^ : p < 0.0001 (extremely statistically significant)

G4 v/s G6 (á : p < 0.05; â: p < 0.001; &: p < 0.0001)

(Gp 1: Normal mice - administered with normal saline orally. Gp 2: Infected mice - administered intraperitonelly 0.1 ml of 2×10^4 CFU/ml of *Salmonella typhimurium*. Gp 3: Normal mice administrated bee collected pollen from *Helianthus annus* orally. Gp 4: Treatment with bee collected pollen from *H. annus* (orally) in *S. typhimurium* infected mice. Gp 5: Normal mice administrated bee bread from *H. annus* orally. Gp 6:Treatment with bee bread from *H. annus* (orally) in *S. typhimurium* infected mice)

oxidative stress. Administration of bee pollen in Gp 4 and 6 significantly reduced the adverse effect induced by the bacteria. Glutathione is the most abundant cellular antioxidant, prevents damage to important cellular components by neutralizing the free radicals (Pompella *et al.*, 2003). Oxidative stress deplete the GSH level in Gp2 as compared to the Gp1, which adversely affects the cellular thiol redox balance and in turn makes the cells more susceptible to a number of internal stresses. But in treatment groups, i.e. Gp 3 and 4, the level of GSH raised to near normal, indicating the effectiveness of bee pollen and beebread. Similarly, in case of Gp2 the other level of other antioxidants such as SOD, CAT, GST, GR and GP decreased as compared to Gp1 but after the administration of

bee pollen and beebread, the level of theses enzymes ameliorate up to the normal level (Kaur *et al.*, 2018, 2020). Superoxide radicals generation occurs in the body, when oxygen gains an extra electron produced during bacterial infection and other metabolic processes by different enzymatic reactions. Superoxide dismutase helps to detoxifying the highly reactive superoxide radicals into H_2O_2 and oxygen. Here, the H_2O_2 acts as a pro-oxidant for the cells and is thus, converted into simple water and oxygen with the help of enzyme CAT and GPx using either an manganese or iron as cofactor (Chelikani *et al.*, 2004). Alone administration of bee pollen and beebread did not harm the mice (Kaur *et al.*, 2014; Kaur *et al.*, 2022, 2023). The therapeutic effects of apicultural products are a new hope to combat the dangerous threats caused by increasing evidence of antimicrobial resistance. It was investigated that bee pollen and beebread of *H. annus* showed therapeutic potential such as antibacterial and antioxidant activities against typhoid bacteria but these activities were in case of beebread as compared to bee pollen. Besides this, alone administration of these two apicultural products did not harm the animal model showing the protective effects.

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