

EFFECT OF PROPOLIS EXTRACT ON HEMATOTOXICITY AND HISTOLOGICAL CHANGES INDUCED BY *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM IN BALB/C MICE

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Received: September 2, 2015; Revised: September 21, 2015; Accepted: September 22, 2015; Published online: March 30, 2016

Abstract: Typhoid fever, a life-threatening disease, causes several pathological changes due to the involvement of one or more organs. In the present study, the effect of typhoid on hematological indices and spleen histology in infected mice were investigated. The ameliorative efficacy of the ethanolic extract of propolis was tested and compared with treatment with the typhoid drug, cefixime. BALB/c mice were divided into six groups: group 1 was the normal control, group 2 was infected with *Salmonella enteric* serovar Typhimurium, group 3 was *Salmonella* infected and treated with cefixime, groups 4 and 5 were *Salmonella*-infected mice treated with 100 mg and 300 mg of propolis respectively. Group 6 was given only 300 mg propolis without infection. Mice were killed after completion of treatment and examination of spleen histology and hematological studies were performed. Significant differences were observed in the propolis-treated group of mice as compared to the infected group without antibiotic, further confirming the ameliorative effect of propolis on *Salmonella enterica* serovar Typhimurium-induced toxicity in mice.

Key words: Propolis; hematotoxicity; *Salmonella enteric* serovar Typhimurium; blood cells; typhoid; spleen; BALB/c mice

INTRODUCTION

Typhoid fever is an acute life-threatening disease caused by *Salmonella typhi* [1], a Gram-negative bacterium. It ranks second after malaria [2] with respect to incidence and severity. Around 21 million cases of typhoid are reported every year out of which 1-4% prove fatal [3]. Transmission may occur by ingestion of contaminated food, mainly meat, or by fecal oral route from an infected individual [4]. Poor sanitation facilities add to the cases of typhoid especially in developing countries.

Typhoid fever influences adversely the hematological profile of patients [5], causing serious hematological derangements [6]. During infection, *Salmonella typhi* escapes from macrophages and invades the organs of the mononuclear phagocyte system, such as the liver and spleen, damaging these organs by the bacterial toxins, as well as other tissues [7].

With increasing awareness of the functionality of different foods [8], attention has focused on honeybee-related products, such as honey [9], pollen [10-11], propolis [12], beeswax [13], bee venom [14] and royal jelly [8], with bee products also being examined and utilized in a branch of medicine known as Apitherapy. Propolis is a complex resinous material produced by honeybees [15], used as an emollient, hepatoprotective, immunomodulator, antioxidant and anti-inflammatory agent in traditional medicine [16]. We previously described the *in vitro* and *in vivo* antimicrobial activity of propolis against *Salmonella enterica* serovar Typhimurium [17], while its therapeutic effect was observed against *Salmonella* in liver of mice [18]. By measuring hematological indices and examining blood components and the spleen by scanning electron microscopy (SEM), in the present study we studied the effectiveness of propolis against *Salmonella enterica* serovar Typhimurium.

MATERIALS AND METHODS

Collection of propolis and preparation of extracts

Propolis was obtained from honey bee hives kept in an apiary maintained by Department of Zoology, Panjab University, Chandigarh. Ethanolic extract was prepared according to the standard protocol [17].

Microorganisms

The bacterial strain of *Salmonella enterica* serovar Typhimurium MTCC 98 was procured from IMTE CH, Sector-39, Chandigarh, and stored in the form of small aliquots at -20°C before subculturing. The strain was examined biochemically before storage and use.

Experimental animals

BALB/c mice of either sex, 4-6 weeks old, weighing 25-30 g were used in the experiments. The animals were obtained from the Central Animal House, Panjab University, Chandigarh, India. The mice were fed a standard pellet diet and water *ad libitum*. All the experiments were carried out strictly according to the guidelines and under the approval of the Animal Ethical Committee, Panjab University, Chandigarh. Animals were checked regularly for bacterial infection by streaking the tail vein blood directly on MacConkey agar.

Treatment regimens

Animals were divided into six groups with eight animals in each group: group 1: normal control mice; group 2: *Salmonella enterica* serovar Typhimurium infection at 2×10^4 CFU/ mL intraperitoneally; group 3: *Salmonella enterica* serovar Typhimurium infected and then treated with the antibiotic cefixime (4mg/kg body weight of mice) orally for 5 days; group 4: *Salmonella enterica* serovar Typhimurium infected and then treated with propolis (100mg/kg bw) orally for 30 days; group 5: *Salmonella enterica* serovar Typhimurium infected and then treated with propolis (300 mg/kg bw) orally for 30 days; group 6: treated only with

propolis (300 mg/kg bw) for 30 days. Each experiment was conducted in triplicate.

Hematological analysis

Animals in group 2 were killed on the 5th day of infection, group 3 were killed 24 h after the last treatment, whereas animals in groups 4 and 5 were killed after 30 days of treatment by suffocating with diethyl ether. Blood was drawn from the jugular vein. Hematological investigations carried out within 24 h of sample collection. Complete blood analysis including red blood cell (RBC) and white blood cell (WBC) counts, differential leucocyte count (DLC), hemoglobin (Hb), packed cell volume (PCV) and red cell indices, including mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and mean cell hemoglobin concentration (MCHC) determination, as described [19].

Bacterial load

To determine the bacterial load in the spleen, mice were killed on different days post infection. Bacterial load was assayed by plating the 10-fold serially diluted tissue homogenate on deoxycholate citrate agar (DCA) and keeping it overnight at 37°C [20].

Histological examination

After blood collection, animals were killed and the spleen was removed aseptically. The spleen was weighed and a portion was kept for histological analysis. Histological processing included dehydrating, paraffin embedding, block making, section cutting, staining with hematoxylin and eosin (H&E) and finally mounting, according to the standard method [21].

SEM

Blood was aspirated out in citrate saline after jugular vein incision. Separation of WBCs was done by double density gradient centrifugation. Mononuclear (MN) and polymorphonuclear (PMN) cells were separated by double density gradient centrifugation using histopaque-1119 and 1077 [22]. A single drop of cell suspen-

sion was fixed in 2.5% (v/v) glutaraldehyde in PBS (pH 7.2) for 20 min at room temperature. After fixation and washing, the stubs were prepared. Stubs were sputtered for 30 min in a sputterer and viewed in SEM at the Central Instrumentation Laboratory, Panjab University, Chandigarh, under different magnifications [23].

Statistical analysis

All values were expressed as means±standard deviation. Statistical differences between the various groups were evaluated by Student's t-test. P values<0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Salmonella enterica serovar Typhimurium is a very important enteric pathogen causing typhoid. There are increasing incidences of mortality and morbidity due to this disease in developing countries [24]. Understanding the pathophysiology of typhoid infection is a very tedious process involving various stages [25]. In the present study, the recorded physical parameters of mice in all the groups were recorded, including activity level, feeding, appearance of fur, hunched back-are presented in Table 1. Infected mice had erect hair and hunched backs. In animals treated with propolis, after seven days of treatment with propolis, the above signs/symptoms disappeared and a return to normal appearance was observed.

The bacterial count in the spleen was high in the group of mice infected with *Salmonella enterica* serovar Typhimurium by the 5th day of infection (8.14±0.055 CFU/g), and no survival was observed by the 30th day post infection. The *Salmonella enterica* serovar Typhimurium-infected mice were killed on the 5th day. Treatment with cefixime reduced the bacterial load to negligible units after five days of treatment. Comparison of the bacterial load of the propolis-treated groups on the 5th day of treatment with the infected control (group2) revealed no significant difference. However, as the treatment continued, a significant reduction (p<0.050) in the bacterial load was observed in group 5 (5.29±0.29 CFU/g) on the 30th day (Fig.1).

Table1. Physical parameters of different groups of BALB/c mice.

clinical findings	group 1	group 2	group 3	group 4	group 5	group 6
activity level	++	-	++	+	++	++
Feeding	++	-	++	+	++	++
appearance of fur	++	-	++	-	++	++
hunched back	-	++	-	+	-	-
Vomiting	-	-	-	-	-	-

++ :normal, + : nearly normal, - :reduced

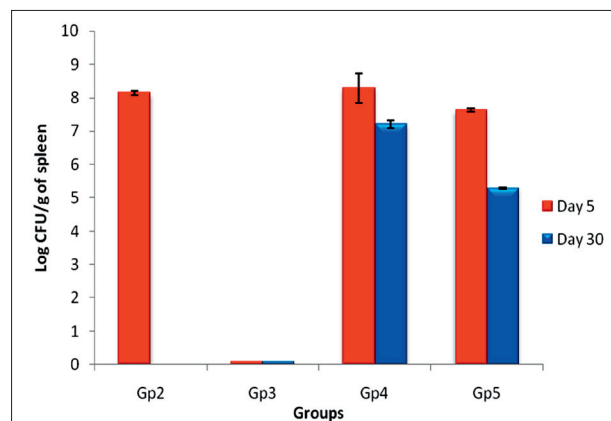


Fig. 1. Bacterial count in spleen of mice in infected and treated groups. Data is expressed as means±SD. * p< 0.050, infected vs P100, P300 (Student t-test).

Hematological examination showed that the *Salmonella*-infected mice (group 2) developed anemia, monocytosis and lymphocytosis. The data presented in Tables 2 and 3 show the effect of *Salmonella enterica* serovar Typhimurium infection and treatment with different doses of ethanolic extracts of propolis on hematological parameters. Hematological parameters of an organism are affected by a variety of different factors [7,14,26,27], with typhoid fever causing severe hematological changes, include anemia, leucopenia and eosinophilia [5]. *Salmonella enterica* serovar Typhimurium infection significantly decreased the mean levels of RBC, Hb, PCV, MCV, MCH and MCHC. Suppression of hemophagocytosis responsible for hematological changes [6,28], and the observed low levels of RBC, Hb and PCV were probably caused by inhibition of hematopoiesis which led to anemia. Studies by Dangana and Kayode [29,30] reported decreased levels of PCV in typhoid and paratyphoid patients. The destruction of RBC and decrease in Hb

Table 2. Hematological indices measured in the blood of infected and treated groups.

hematological indices	group 1	group 2	group 3	group 4	group 5	group 6
RBC ($10^6/\text{mm}^3$)	8.36±1.20	4.37±0.95	7.76± 0.58*	5.8±0.47	8.06±0.66*	7.86±0.75
Hb (g/dL)	12.48±0.66	9.06±0.11	11.56±0.4*	10.9±0.43	12.6±0.55*	12.1±0.1
Hematocrit (%)	42±1	28±1.0	40.33± 0.57*	39.33±1.15	44±1.73	40.33±0.57
MCV (μm^3)	48.33±3.85	64.17±0.14	52.12±3.84*	67.77±6.69	53.49±3.79*	53.9±4.3
MCHC (%)	29.77±0.82	34.13±3.89	28.68±1.35*	27.73±1.62	28.69±2.3*	30.03±3.25
MCH (pg)	13.68±1.88	21.02±0.75	15.89±3.26*	18.15±0.15	14.82±1.23*	12.86±1.02

Data is expressed as means±SD.*- $p < 0.050$, Group 2 vs Group 3, 4 and 5 (Student t-test)

Table 3. Total and differential leucocyte counts (TLC and DLC) measured in the blood of infected and treated groups.

groups hematological indices	group 1	group 2	group 3	group 4	group 5	group 6
WBCcount/ mm^3	7730±170.8	5766.6±28.86	7563±32.14*	6980±97.59	7348.33±47.52*	7348.33±47.52
lymphocytes (%)	69.16±1.44	83.33± 1.4	72±2.64*	77.83± 0.57	74.16±1.14*	71.66±1.44
neutrophils(%)	20.83± 1.4	14.33±0.57	20.3±0.3*	16.66±0.57	19.33±0.6*	19.33±0.57
monocytes(%)	1.66±0.28	3.16±0.28	1.83±0.28*	2.66±0.3	1.88±0.28*	1.15±0.5
eosinophils(%)	1± 0	2.16±0.30	0.6±0.28*	1.16±0.2	0.83±0.3*	0.66±0.28

Data is expressed as means±SD.*- $p < 0.050$, Group 2 vs Group 3, 4 and 5 (Student t-test). (n=6)

were responsible for the decrease in MCH, MCV and MCHC. Treatment with the ethanolic extract of propolis produced a significant revival of different hematological parameters, such as RBC, Hb, PCV, WBC and neutrophils ($p < 0.05$). Our investigations revealed a significant decrease in the WBC count in group 2 mice as compared to control mice (group 1), suggesting that typhoid leucopenia was caused by invasion of hemopoietic organs, such as the spleen and bone marrow with *Salmonella* which further slowed down leucopoiesis [31,32]. The DLC findings showed a significant increase in lymphocytes, eosinophils and monocytes in the infected group most likely as a result of the allergic reaction to the infection [33]. An increase in lymphocytes has also been suggested to be due to the increased release of cells from lymphoid/myeloid tissues [34]. Monocytosis and decreased hematocrit in *Salmonella*-infected mice has been reported [35]. Treatment with propolis, particularly at the higher dose (group 5), showed ameliorative effects and significantly increased the RBC count, Hb and PCV.

SEM studies of blood cells in typhoid mice have not been reported so far. The present SEM studies revealed that the normal red blood cells (group 1) were biconcave and elliptical in shape (Fig. 2A), whereas in *Salmonella*-infected mice (group 2), the RBCs were

distorted in shape. Folds and depressions of RBCs and activated WBCs with finger-like projections were a commonly observed in infected mice (Fig. 2B, C, D). In groups 3, 4, 5 and 6, the cells exhibited morphology comparable to normal (Fig. 2E, F). Morphological distortions were observed in blood cells in the *Salmonella*-infected group (group 2), because *Salmonella* resides in phagocytes, escaping killing. These distortions receded with propolis treatment (group 5). Reports also confirmed that infections like *Plasmodium* in mice showed distortions in both red and white blood cells [23].

Spleen architecture in control mice (group 1) exhibited normal morphology, with white and red pulp regions separated by a marginal zone (Fig. 3A). In group 2, the spleen showed enlargement of the marginal zone, reactive enlargement and an increase in the number of follicles (Fig. 3B). Transverse section of a cefixime-treated spleen (group 3) showed normal structural organization at low magnification. At higher magnification, red and white pulp regions and the marginal zone were clearly evident (Fig. 3C). Signs of recovery were noteworthy in the propolis-treated group 5 (Fig. 3D). Splens of animals treated with the lower dose of propolis showed similar architecture to that of infected splens. Enlargement of the white

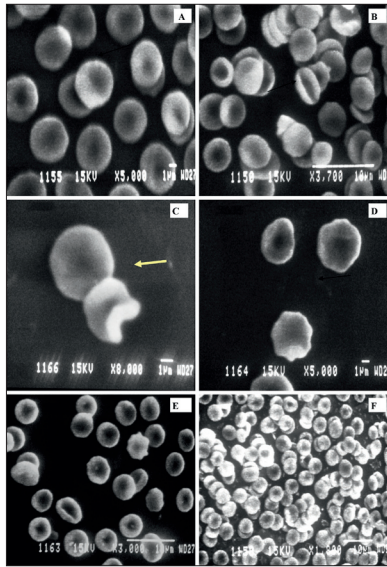


Fig. 2. SEMs showing red and white blood cells. **A.** Normal RBC: group 1 showing biconcave and elliptical shaped RBCs. **B.** *Salmonella* infected RBC: group 2 showing the RBCs with distorted shape. **C.** and **D.** Folds and depressions in RBCs and activated WBCs with finger-like projections were a commonly observed feature of infected mice. **E.** and **F.** Almost normal morphology of RBCs: groups 3, 4, 5 and 6.

pulp and marginal zone was observed. Infiltration of WBCs was evident in the red pulp region. In contrast, treatment with the higher dose of propolis resulted in normal spleen architecture with a red pulp, white pulp and marginal zone. Histology of an infected spleen (group 2) showed an expansion of the red pulp and depletion of the white pulp regions. Microscopical examination revealed multifocal histiocytic infiltration, lymphoid follicular disruption and thrombosis in the spleen, resembled pathology in hemophagocytic lymphohistiocytosis [34, 35]. Due to the expansion of the red pulp and inflammation, splenomegaly was observed as previously reported [36-39]. The effectiveness of the standard antibiotic cefixime was due to its inhibition of bacterial growth [40]. In the group treated with propolis alone (group 6), no changes in biochemistry and histology were observed, and this supports our previous work [41]. Treatment with propolis has been suggested to increase membrane permeability, inhibiting bacterial motility; thus, its bactericidal activity reduces the toxic effect of bacteria

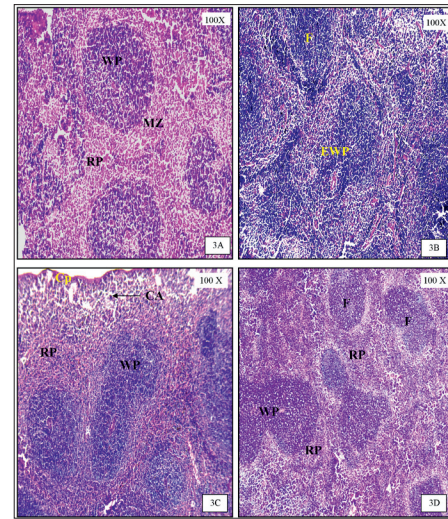


Fig. 3. Light micrographs of the spleen. **A.** Normal control: group 1 spleen showing red, white pulp and marginal zone. **B.** Infected spleen: group 2 showing expanded white pulp region. **C.** Cefixime-treated spleen: group 3 with almost normal morphology. **D.** Propolis-treated: group 5 with similar architecture as that of control group 1. Cp – capsule, CA – central artery, EWP – expanded white pulp, F – follicles, MZ – marginal zone, RP – red pulp, WP – white pulp.

on blood cells as reported previously [42]. Propolis acts as an activator of macrophages, which further increases phagocytic activity [43-45] and stimulates natural killer cells [46-49], contributing to its fungicidal and bactericidal properties [50-51].

To conclude, these results describe the effectiveness of propolis in treating infections due to *Salmonella*, and open new avenues for research in to its active principles and the mechanisms of their actions.

Acknowledgments: The authors would like to thank the Department of Science and Technology (DST) for their assistance at various stages of this research work through the INSPIRE fellowship.

Authors' contributions: The first author performed the practical research work and wrote the manuscript. The second and third authors supervised and helped in difficult situations during the experimental work and in writing the manuscript.

Conflict of interest disclosure: The authors declare that there are no conflicts of interest.

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