Journal of Applied and Natural Science 9 (1): 144 -149 (2017)

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Efficacy of different extracts of propolis against *Salmonella enterica* serovar Typhimurium: *In vitro* and *in vivo* study

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Received: May 9, 2016; Revised received: November 3, 2016; Accepted: January 14, 2017

Abstract: Present study focussed on the antibacterial and antioxidative effect of honey bee propolis on typhoid causing bacteria *i.e. Salmonella.* Water, ethanol, methanol were used as solvents for making of extracts. Both Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were calculated for all the three extracts. MIC of ethanolic extract of propolis was 160 mg/ml. It was 200 mg/ml for methanolic and 220mg/ml for water extracts respectively. Moreover, time kill analysis results confirmed that there was a significant reduction ($p<0.05$) in log count of bacteria when treated with ethanolic extraxt of propolis (3.98±0.15 log cfu/mL) and methanolic (4.66±0.05log cfu/mL) extract of propolis as compared to *Salmonella* control (7.72±0.03 log cfu/mL) in *in- vitro* experiments. For the *in vivo* studies, BALB/c mice was used as an murine model of typhoid. Levels of different liver marker enzymes and antioxidants like Lipid peroxidation (LPO) and Reduced Glutathione (GSH) were observed in infected and all the treated groups. By comparing the results, it was concluded that ethanolic extract of propolis showed maximum antimicrobial activity as compare to the rest two. So the results of present study encourages the potential of ethanolic extract of propolis as an alternative treatment for typhoid and its use in combination with standard antibiotics can also be explored.

Keywords: Antibacterial, Propolis, *Salmonella,* Typhoid

INTRODUCTION

Salmonella is a rod shaped gram negative bacterium and is responsible for causing different kinds of infection including enteric fever (typhoid and paratyphoid) gastroenteritis and septicaemia . *Salmonella enterica* serovar Typhimurium causes an invasive disease in mice that has similarity with human typhoid (;Santos, 2001; Ozkaya *et al.,* 2012). It also causes salmonellosis in humans. Transmission may occur by ingestion of contaminated food, mainly meat, or by faecal oral route from infected individual. In case of typhoid, the problem of Multi Drug Resistance (MDR) is very common. MDR typhoid is more severe with high toxicity and complications (Colledge *et al*., 2010). WHO rated antibiotic resistance as "one of the three greatest threats to human health". Because of the alarming incidence of multi drug resistance in bacteria (Monroe and Polk, 2000) the need of the hour is identification and development of new and effective therapeutic agents (Bhavnani and Ballow, 2000).

Propolis is a glue-like substance that honey bees collect from plant bark and buds. It is obtained as a result of the biochemical alteration of the resinous materials and plant secretions by the enzymes secreted from the glands of the bees. Some of its physical properties include its colour that range from dirty yellow to dark brown, a strong and nice odour, water insolublility and semi-solid nature at room temperature (Hepsen *et al*., 1996; Sahinler, 2000). The chemical composition of propolis depends on the vegetation, climate, season and environmental conditions of the area from where it was collected (Santos *et al*., 2003; Virda-Martos *et al*., 2008). It is mainly composed of resin and vegetable balsam (50%), wax (30%), essential and aromatic oils (10%), pollen (5%) and various other substances including organic compounds and minerals (5%) (Tylowski *et al*., 2006; Kaur *et al*., 2013). Propolis has been used in folk medicines in many regions of the world and has been reported to have various biological activities, such as antibacterial (Grenho *et al*., 2015), antiinflammatory (Chen *et al*., 2004) antitumor effects (Watanabe *et al*., 2011 and Hasan *et al*., 2014)) and immunomodulatory effects (Sforcin, 2007). There are a lot of studies favoring the use of different biologically active natural products for the treatment of serious ailments are being emphasized. Various clinical studies are in progress to verify the preventive and therapeutic potential of propolis as an antibiotic alone as well as synergistically. The present study aimed to investigate the antibacterial property of different extracts of ropolis against *Salmonella enterica* serovar Typhimurium .

ISSN : 0974-9411 (Print), 2231-5209 (Online)

MATERIALS AND METHODS

Collection of propolis and preparation of different extracts: Propolis was obtained from honey bee hives kept in an apiary maintained by Department of Zoology, Panjab University, Chandigarh. Hand collected propolis was kept in a dry place and stored at 4°C until processed. The sample (10 g) was cut into small pieces ground and subsequent solvent extraction was done using different solvents (ethanol, methanol, water). The volume was made to 40ml and it was kept for 5 days with occasional shaking. It was filtered through a Whatman # 41 filter paper and then dried (Kumar *et al*., 2008). The three extracts obtained were ethanolic extract of propolis (EEP), methanolic extract of propolis (MEP) and water extract of propolis (WEP).

Microorganism: 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.

Determination of minimum inhibitory concentration (MIC): MIC was determined as the lowest concentration of the propolis extract which inhibited the growth of the tested microorganisms. The minimum inhibitory concentration (MIC) of propolis was determined using the broth dilution method. For this a series of tubes (three replicates of each tube) were prepared with broth to which various concentrations of propolis extracts were added *viz*., 0mg/ml (negative control), 100mg/ml, 120mg/ml, 140mg/ml, 160mg/ml, 180mg/ml, 200mg/ml, 220mg/ml, 240mg/ml, 260mg/ml, 280mg/ml and 300mg/ml. The antibiotic cefixime was taken as positive control. The tubes were then inoculated with standardized suspension 2X 10^4 cfu of test organisms. After incubating overnight at 37°C the tests tubes were examined and MIC was determined. All sets were read visually and MIC values were recorded as the lowest concentration of propolis that had no visible turbidity.

Determination of minimum bactericidal concentration (MBC): MBC was determined by transferring 0.1ml from MIC test tubes and spreading on Agar plates. The culture was incubated at 37°C for 24 h. The lowest concentration of the extract that did not yield any colony growth on the solid medium after the incubation period was regarded as MBC (Kalia *et al*., 2013).

Time kill assay: A series of nutrient broth tubes containing different concentrations (MIC) of all the three extracts of propolis and cefixime were taken. Around 10^4 cfu of *Salmonella* in log phase (6 hours) was added to each tube. The tube containing *Salmonella* but no propolis acted as Control. All tubes were incubated at 37ºC overnight. Samples from each tube was taken out at different time intervals *viz*. 0, 2,4,6,8,10,12 and 24 hours, O.D. was noted down at 600nm and then plated on nutrient agar plate. The plates were incubated at 37ºC overnight. Viable cells were counted and expressed as $\log_{10}$cfu/ml. Whole experiment was performed in triplicate.

Experimental model for *in vivo* studies: BALB/c mice of either sex, 4-6 weeks old, weighing 20-25 g were used in the experiments. The mice were obtained from The Central Animal House, Panjab University, Chandigarh, India. They were fed 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 Mac Conkey agar.

Treatment regimens: BALB/c mice were divided into nine groups with six animals in each group.

Group 1: Normal control (Normal mice given saline orally).

Group 2: *Salmonella enterica* serovar Typhimurium infection at 2×10^4 CFU/ ml intraperitoneally.

Group 3: *Salmonella* infected + Antibiotic (Cefixime) [4mg/kg body weight (bw) of mice] orally for 5 days.

Group 4: *Salmonella* infected + EEP (300mg/kg bw) given orally for 30 days.

Group 5: *Salmonella* infected + MEP(300mg/kg bw) given orally for 30 days.

Group 6: *Salmonella* infected + WEP(300mg/kg bw) given orally for 30 days.

Group 7: Only EEP.

Group 8: Only MEP.

Group 9: Only WEP.

Each experiment was conducted in triplicate.

Mice in group 2 were sacrificed on day 5 post infection (Group 2: 5^{th} day as peak day of infection). Animals of the rest of the groups were sacrificed after the respective days of treatment.

Collection of blood and tissue: The animals were lightly anaesthetized with di-ethyl ether. Blood was drawn from jugular vein for biochemical investigations. After blood collection animal was sacrificed and liver was removed aseptically. Weight of liver was taken and it was homogenised in saline in a glass homogeniser for quantitative bacterial culture and measuring the antioxidant levels.

Assay of liver marker enzymes: The serum was collected from the blood and was used for analysis of various liver function tests like Serum glutamate oxaloacetate transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), Alkaline phosphatase and Bilirubin by using standard kits (Avecon).

Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration of Propolis.

Extract	MIC	MBC
EEP	160 mg/ml	250mg/ml
MEP	200 mg/ml	260mg/ml
WEP	220 mg/ml	310mg/ml

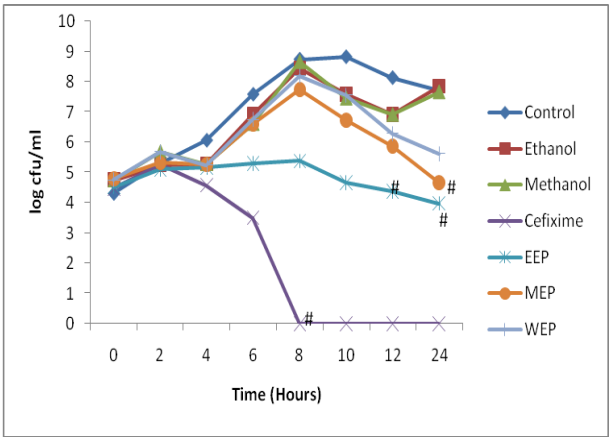

Fig. 1. *Time kill curve of different extracts of propolis. p-value #: IControl vs Cefixime, EEP, MEP(#: p<0.05: statistically significant).*

Antioxidants: LPO and GSH assay were determined from liver homogenate by the following standard protocol (Kaur *et al*., 2014).

Statistical analysis: All the values were expressed as Mean ±Standard deviation. Statistical differences between the various groups were evaluated by Student- t- test. p-values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Honey bees have been called Master alchemists since times immemorial because of the beneficial effects of the majority of bee products. Apitherapy is the art and science of treatment and holistic healing through honey bee products for the benefit of mankind. Today, however, the novel system of healing "Apitherapy" has been extended to the use of all bee products for the treatment of a variety of problems. It was in view of this background information that the present study originated.

MIC and MBC: The present studies tested on the antibacterial efficacy of popolis against *S. enterica* serovar Typhimurium using different solvent extracts. It was observed that all three extracts showed antimicrobial activity against *S. enterica* serovar Typhimurium at different concentrations ranging from 160 mg/ml to 220mg/ml of the extracts. The MICs and MBCs of different extracts are given in Tables 1. On the basis of the calculated MIC of the three extracts it was concluded that best results were shown by ethanolic extract of propolis rather than methanolic and water extracts.

Time kill curve: *In-vitro* growth culture of *S. enterica* serovar Typhimurium was used to perform the growth kinetics of *Salmonella* alone and along with propolis to analyse its antibacterial effect. The O.D. (600nm) was noted at 2 hours intervals for 24 hours using U.V. spectrophotometer (Fig. 1.).

MEP and WEP showed significant reduced log count at 24 and 12 hour respectively. After plotting, the results supported greater efficacy of EEP as compared to that of MEP and WEP. Moreover previous studies have (Silici and Kutluca, 2005; Wagh, 2013) also reported the effectiveness of EEP. Earlier studies

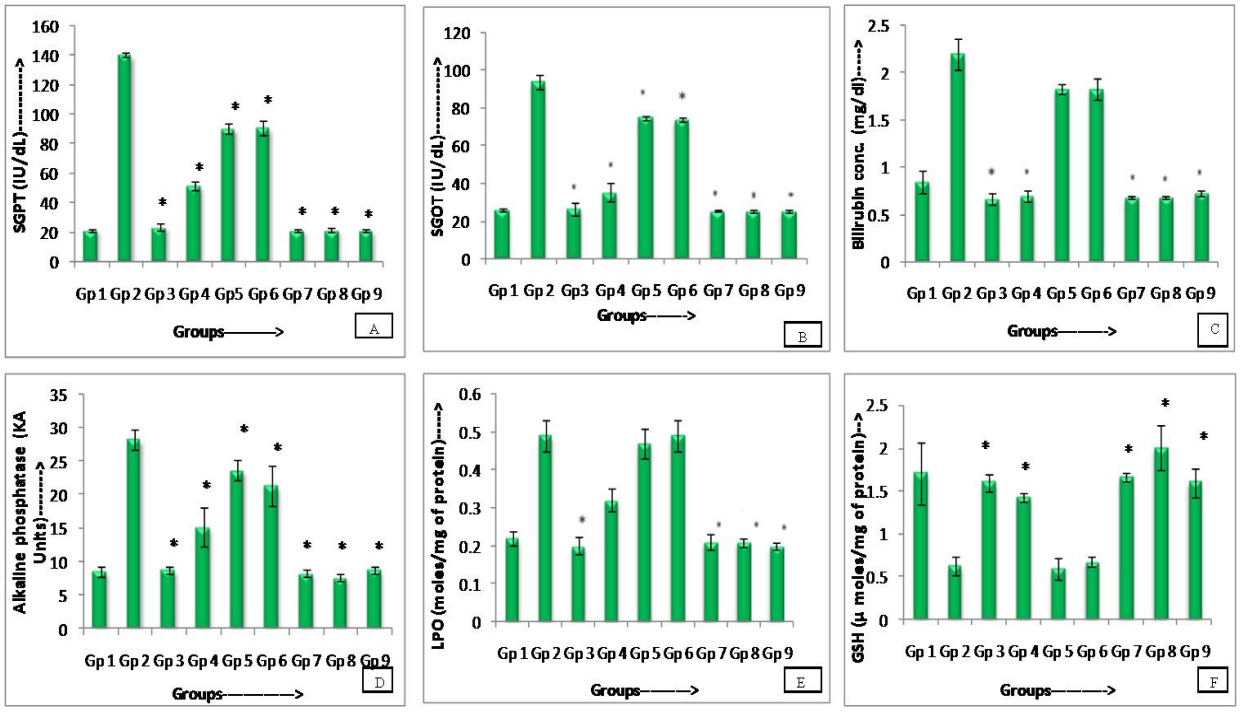

Fig. 2. *(A-F) Showing concentrations of different enzymes and antioxidants in infected and treated groups of mice (Data is expressed as mean±SD. p-value*:Infected vs all cefixime and propolis treated groups (*:p<0.05:statistically significant).*

supported the fact that the organic solvents for plant extraction is better option as compared to water extract, as many components are extracted through organic solvents only (Gajera *et al.*, 2005; Negi and Dave, 2010).The reason could be that the solubility of phytochemicals like flavonoids, terpenes (Harborne, 1973; Cunha *et al.,* 2004) responsible for the biological properties of propolis (Cowan, 1999) was greater in ethanol as compared to other solvents because this extract gave the best results for parameters tested and recommended for further use. Earlier *in vitro* studies were supported the effectiveness of EEP as compared to other extract (Kalia *et al*., 2013) and this was due to the phytochemicals which are extracted well with ethanol as a solvent. Several studies elucidated use of ethanolic extract of propolis for studying its biological activities (Bankova *et al*., 1999). Recent research observed that phenolic compounds like caffeic acid, naringenin and quercetin considered to be most effective and active components against studied microorganism (Ristivojevic *et al*. 2016).

***In vivo* experiments:** During the *in vivo* studies, the biochemical analysis involves the liver function tests. The levels of liver markers i.e. SGOT, SGPT, alkaline phosphatase and bilirubin were significantly high in case of infected group as compared to that of normal control group ($p<0.05$). The mice which were treated with 300mg/kg bw of EEP showed significant difference from infected control (Fig.2. A, B, C, D). Whereas with the rest two extracts that were MEP and WEP, the results were significantly different when compared with infected values but the EEP treated mice showed results that were more towards normal range. The increase in the concentrations of liver marker enzymes was due to the fact that the *Salmonella* infection caused hepatic granulomas that led to the release of liver enzymes into serum thus increasing or that the extent of hepatic dysfunction in typhoid fever depended upon various contributory factors like endotoxins produced by *Salmonella*, damage to hepatocytes and invasion of hepatocytes by microorganisms (Hasbun *et al.,* 2006; Kalia *et al*., 2015, 2016).). Reports suggested that the high serum concentration of liver markers indicated cellular leakage due to the disintegration of liver cell membranes (Yanpallewar *et al*., 2003). In the only propolis treated group (Gp 7, 8 and 9) all the parameters are within control values. Earlier studies also confirmed that EEP showed no toxicological manifestations in different organs of BALB/c mice at different concentration (Kalia *et al*., 2014). Studies by Kolankaya *et al*. (2002) and Al-Amoudi (2015) supported that the treatment with propolis significantly prevented the release of liver marker enzymes like transaminases suggesting its hepatoprotective potential. Propolis helped in reducing the increased activity of ALP and AST in rats treated with $AlCl_3$ (Newairy *et al*., 2009).

The antioxidant analysis also showed increased lipid peroxidation and decrease levels of GSH in case of infected control. But the treatment with EEP reduced the levels of LPO towards normal range significantly as compared to infected group. Some honey bee products like propolis, pollen act as strong antioxidants and as a free radical scavengers. Both detoxifies a variety of free radicals and reactive oxygen intermediates. The strong antioxidant activity is due to the polyphenolic compounds which chelate the metal ions and helped in scavenge singlet oxygen, proxy radicals and also the peroxynitrite (Kumazawa *et al.,* 2004; Cottica *et al*., 2011; Saleh, 2012; Daleprane and Abdalla, 2013; Kaur *et al*., 2014). The present results showed that propolis decreased lipid peroxidation possibly by its antioxidant activity. Studies supported that the propolis improve lipid profile,MDA and SOD activity in mice (Shinohara *et al*. 2002 and Laun *et al*., 2000).

Conclusion

The mice treated with EEP showed considerable therapeutic efficacy against *Salmonella enterica* serovar Typhimuirium. This was revealed by the restoration of normal values in various biochemical parameters used for testing. The results of both *in vitro* and *in vivo* experimentation concluded that ethanolic extract of propolis performed best with respect to antibacterial activity against *Salmonella enterica* serovar Typhimurium. With these results, the effectiveness of ethanolic extract of propolis as a prospective candidate for treating infections cannot be ignored rather it opens new avenues for research to consider propolis as an alternative treatment for developing MDR diseases.

ACKNOWLEDGEMENTS

The authors would like to thank department of science and technology for their assistance at various stages of this research work through INSPIRE fellowship and the DST-FIST grant.

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