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Synergistic effect of propolis with cefixime against *Salmonella enterica* serovar Typhimurium: An *in vitro* study

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Antibiotic resistance is one of the world's most pressing health problems in 21st century. Multi Drug Resistance (MDR) is a phenomenon where the microorganism develops resistance against more than one drug. MDR typhoid is one such emerging problem. This calls for some alternative drugs or use of some compounds alone or in combination with drugs against typhoid. Apitherapy provide us the cure for this. In apitherapy, the honey bee and its hive products are used for the treatment for various ailments. In the present study, a honey bee product i.e. propolis which act as a strong antibacterial is combined with standard antibiotic i.e. cefixime against *Salmonella*. The time kill analysis and results of checkerboard method confirmed when propolis and cefixime were used in combination, synergy was observed.

Keywords: Antibiotics, Cefixime, *in vitro,* Propolis, *Salmonella*, Synergy, Typhoid.

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Introduction

Apitherapy is an emerging branch of medicine which focuses on the use of honey bee and its products in the treatment of various ailments. Earlier, apitherapy was only limited to the use of bee venom, but nowadays apitherapy is a much broader term covering the medicinal use of all the products of honey bees or bee hive. Bee products include honey, bee pollen, wax, propolis, royal jelly, and bee venom. Because of their natural occurrence and quality, bee products are also added as adjuvant in the formulation of different drugs and medicines.

Propolis is a generic name for the complex resinous material produced by honey bees from plant exudates, bees wax, and bee secretions[1]. The major botanical sources of propolis include *Acacia* spp*, Azadiracta indica*, *Mangifera indica, Populuseur americana, Populus italica*, *Populus nigra*, *Populus suaveolens*, and *Populus tremula*[2-4]. The composition of propolis depends upon various factors like geographical origin, plant source, and the season of collection. In general, it is composed of 50 % resin and vegetable balsam, 30 % wax, 10 % essential and aromatic oils, 5 % pollen, and 5 % various other substances including organic debris as studied by various researchers[5,6].

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Since the composition of propolis varies from area to area, so is the case with its different activities. The biological activity of propolis depends upon the concentration of the active compounds. Propolis is used as an emollient, immunomodulator, antioxidant, anti tumor growth agent, a dental antiplaque agent and has anti inflammatory, antibacterial, and pain-killing (analgesic) properties. Certain bacteria have built resistance to antibacterial dressings. So, several groups of researchers have focused their attention on the biological activity of propolis and its active principles[7]. Propolis is often named as "Russian Penicillin".

According to the World Health Organization (WHO), foodborne illnesses are becoming a major global threat. Around 200 diseases are caused due to unsafe food and millions of deaths occur every year due to consumption of contaminated or unsafe food. The most important contaminants are microbes, adulterating substances, and improper cooking. Amongst the microbes, the genus *Salmonella* is very important as the causative agent of several foodborne diseases the world over[8].

Typhoid is a major public health problem caused by *Salmonella* serotypes including Typhi, Paratyphi A, Paratyphi B, and Paratyphi C. Susceptible conditions prevail because of poor sanitation and

improper hygiene conditions, contaminated food and water[9]. Travelers visiting Indian subcontinent are highly prone to the disease and 3-30 cases per 10,000 persons annually have been reported for typhoid infection[8]. Typhoid is characterized by persistent high fever (40 °C/104 ºF), inflammation, profuse sweating, rose- colored spots, malaise, chills, myalgia, and colic pain.

The aim of an antibacterial treatment should be proper availability of drug for oral as well as intravenous use by adults and children, early recovery of patient, low side effects, and low cost of drug. Continuous use of antibiotics, however, leads to development of antibiotic resistance. *S. typhimurium* has been reported to exhibit resistance to several commonly used antibiotics[10]. The increase in the multi drug resistant strains of *Salmonella* is a matter of grave concern and calls for exploration of some alternative drugs or for the use of some natural compounds alone or in combination with drugs against typhoid[10]. The problem of antimicrobial resistance is particularly pressing in the developing countries, where due to cost constraints the application of newer, expensive drugs is discouraged. Usually, microorganisms develop resistance by developing mutations in the chromosome or plasmids which affect the mode of action of drug[11]. The antimicrobial resistance has reached unacceptable levels. Hence, some alternative approaches must be tested to interrupt the unwarranted trends of *Salmonella* resistance. There is, therefore, renewed interest in the use of propolis, a bee product, whose natural antibiotic action has been known to be very effective[8]. Previous studies by the authors showed the hepatoprotective role of propolis against typhoid causing *S. typhimurium* in mice. The results showed significant recovery in mice. Biochemical parameters and histology of liver were restored to normal in propolis treated mice[12,13].

The present study focused on the *in vitro* effect of propolis in combination with standard antibiotic cefixime against *Salmonella enterica* serovar Typhimurium to investigate if propolis could help in reducing the effective dose of antibiotic and could possibly effect in increasing MDR cases.

Materials and Methods

Collection of propolis and preparation of extracts

Propolis was obtained from honeybee hives kept in an apiary maintained by Department of Zoology, Panjab University, Chandigarh. Ethanolic extract of propolis (EEP) was prepared by following standard protocol[12].

Microorganism

The bacterial strain of *Salmonella enterica* serovar Typhimurium (MTCC 98) was procured from CSIR-IMTECH, Chandigarh (Letter no: MTCC/11/5/6869) and stored in the form of small aliquots at -20 °C before subculturing. The strain was examined biochemically before storage and use.

Minimum inhibitory concentration (MIC)

MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC values were calculated according to the Clinical and Laboratory Standards Institute (CLSI) guidelines[14]. Broth dilution method was used for testing *in vitro* the inhibitory concentration of the antimicrobial agent against specific bacteria.

In vitro synergistic nature of propolis

Synergistic effect of propolis with standard antibiotic cefixime (Checkerboard method: *CB method)*

The combination interactions of propolis extract with standard antibiotic cefixime (Sigma-Aldrich) were determined in 96-well microtitre plates by checkerboard micro dilution method[15]. Propolis extract was diluted horizontally and cefixime was diluted vertically to get a matrix of different combinations of the two. Plates were incubated at 37 °C for 24 h after the addition of 2×10^4 CFU/mL of *S. typhimurium*. After 24 h, with the help of MIC of the drug alone and in combination, the fractional inhibitory concentration (FIC) and the FIC index (FICI) were calculated.

FIC was calculated for cefixime as well as for propolis according to the following formulae:

$$\text{FIC of drug cefixime} = \frac{\text{MIC of drug cefixime in combination}}{\text{MIC of drug cefixime alone}}$$

$$\text{FIC of EEP} = \frac{\text{MIC of EEP in combination}}{\text{MIC of EEP alone}}$$

FIC index (FICI) = FIC of drug cefixime + FIC of EEP

Synergy is defined as an FIC index of ≤0.5. Additivity is defined as an FIC index of >0.5to ≤ 1. Indifference is considered when FIC >1 but ≤4.0. When the FIC index is >4.0 then it is antagonism.

Time kill assay

The time kill analysis was performed using only combinations which showed synergistic or additive results in CB method. Mueller Hinton broth (HiMedia) tubes containing combination of propolis and cefixime (from the CB results) and concentrations of propolis and cefixime alone were taken. Around 10^4 CFU/mL of *S. typhimurium* in log phase (6 h) were added to each tube[16]. The tube containing *S. typhimurium*, but no propolis acted as infected control (Inf Control). All tubes were incubated at 37 ºC overnight. Samples from each tube were taken out at different time intervals (0, 2, 4, 6, 8, 10, 12, and 24 h), O.D. was noted down at 600 nm and were inoculated on Trypticase Soy agar plates. The plates were incubated at 37 ºC overnight. Viable cells were counted and expressed as $\log_{10}$CFU/mL. Whole experiment was performed in triplicate. Synergy was defined as 2 $\log_{10}$ decrease in colony count at 24 h as compared to the most active single agent or 2 $\log_{10}$ decrease in colony count as compared to starting inoculums. If <10 fold change in colony count was observed in combination as compared to most active agent alone at 24 h, then it was indifference. Antagonism was defined as 2 $\log_{10}$ increase in colony count at 6 or 24 h with the combination compared with that by the most active drug alone[17].

Statistical analysis

Data were expressed as mean±S.D. All experiments were repeated thrice. The statistical significance of inter group difference of biochemical parameters and microbial counts was determined by Student '*t*' test and Analysis of Variance (ANOVA) using Holm Sidak test. Differences were considered statistically significant at p <0.05 and highly significant at p <0.001.

Results

Combination of antimicrobial agents and natural products has become a need of the hour in view of drug resistance and multi drug resistance reported for many pathogenic species. Some research is documented to prove the efficacy of this new trend. Much more is however, needed to put it on a firm footing before its safe application. The present study is an attempt to test propolis, a natural product of the bee hive, for its antimicrobial effect in combination with cefixime against *S. typhimurium*.The results of biochemical tests for strain confirmation are tabulated in Table 1.

MIC and FIC indices

The MIC of cefixime was found to be 0.08 µg/mL and MIC of EEP was 160 mg/mL. Combination of propolis extract and cefixime exhibited a synergistic response at combination of ¼ MIC of propolis (P) (40 mg/mL) and ¼ MIC of cefixime (C) (0.02 µg/mL). Based on this, the value of FIC index was found to be 0.5 indicating synergy.

Time kill assay

In vitro growth culture of *S. typhimurium* was used to study the growth kinetics of *S. typhimurium* alone and with propolis to analyse its antibacterial effect. The O.D. (600 nm) was noted at 2 h intervals for 24 h using UV spectrophotometer. The results of time kill analysis showed a non significant reduction in the log count of ¼ MIC propolis (1/4 MIC P= 7.72±0.03 log CFU/mL) alone group and ¼ MIC cefixime (1/4 MIC C = 7.22±0.05 log CFU/mL) alone group as compared to infected control treatment (p >0.05) at all intervals. On the other hand quite significant decrease was observed in combination group (2.42±0.005 log CFU/mL) (p <0.001) as compared to infected control (7.72±0.03 log CFU/mL) at 24 h (Fig. 1). While comparing the log counts of ¼ MIC P,

Table 1 — Biochemical tests for identification of bacteria

S. No.	Biochemical tests	Results
1	Catalase	+ve
2	Oxidase	-ve
3	Indole	-ve
4	MR	+ve
5	VP	-ve
6	Citrate	+ve
7	TSI	K/AG
8	Motility	+ve

K-Alkaline (yellow), A-Acidic (pink), G-Gas

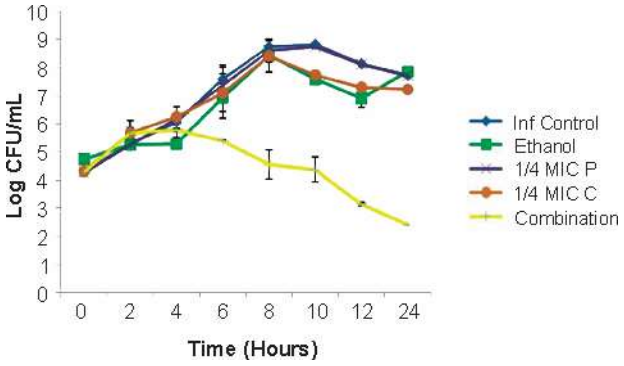

Fig.1 — Synergistic activity of cefixime and propolis. (# : p <0.001)

¼ MIC C, and combination group, among themselves, significant difference was observed in combination treatment (p <0.001) indicating that cefixime and propolis when used in combination, not only reduced the doses of each other but also significantly increased each other's effectiveness.

Discussion

Chemical or drug resistance is a consequence of evolution and is a response due to pressures imposed on any living organism. Increasing antibiotic resistance among microbes urgently necessitates the development of novel antimicrobial agents. The alternative therapeutics incorporating natural products with standard medication is a promising approach in disease remediation. Plant and animal sources offer good potential for exploitation and bee products because of their documented application in home remedies are of great interest for such researches. The present study was planned to focus on antibacterial role of propolis against *S. typhimurium* so as to analyse if it could help in reducing the antibiotic clinical doses.

The effectiveness of propolis was checked in combination with the antibiotic cefixime during *in vitro* experiments. It was observed that the sub MIC of EEP (40 mg/mL) in combination displayed synergistic effect with sub MIC of cefixime (0.02 µg/mL). The bacterial count was reduced from 7.72±0.03 log CFU/mL in control (only *S. typhimurium*) to 2.72±0.005 log CFU/mL in combination treated group. A previous study[18] discussed the *in vitro* synergistic effect of combinations of different plants like *Mumefructus*, *Coptidisrhizoma*, and *Schizandraefructus* against *Salmonella* and MIC varied from 0.49 to 7.8 mg/mL. The present study was the first of its kind where propolis was used synergistically with cefixime against typhoid. Earlier studies reported the synergistic effect of Brazilian propolis and some antibiotics against *S. typhi*[19,20]. Synergistic combinations to fight MDR have been experimented earlier also and were quite successful like propolis with clarithromycin against *H. pylori* had synergistic or additive activity[21,22]. The results of present study further supported the outcome of previous experiments. The possible reason for the effectiveness of propolis could be the active components, which were confirmed by phytochemical analysis as well as GC-MS studies performed by the authors earlier[12,13]. The results showed the presence of flavonoids like 4, 5, 7 trihydroxyflavone (galangin), 4 H-1-benzopyran-4-one (pinocembrin), cinnamic acid, tannins, alkaloids, terpenoids, fructofuranose, fructopyranose, tagatofuranose . The phytochemicals detected in the present study have previously been shown to exhibit biological activities, such as antibacterial, antitumor, and antihelminthic[13,23-25].

Studies have proved the effectiveness of propolis against both Gram positive and Gram negative bacteria[26]. This might be due to components of propolis like flavonoids, benzoic acid, cinnamic acid, caffeic acid that may have acted on membrane or cell wall of microorganism and caused structural as well as functional deformities[4,27-31]. Moreover, the effectiveness of cefixime was due to its reasonable penetration into the monocytes, thus, inhibiting the growth of bacteria[32]. Its effectiveness has been proven by several studies[33-39]. The possible mode of action for synergistic effect could be some complex formation that might have inhibited the microorganisms by inhibiting its cell wall synthesis or by lysing the cell and thus, causing death[40]. Both cefixime and propolis complemented each other's activity. The data obtained from the present study revealed a significant reduction in the *S. typhimurium* bacterial count in combination groups. These results point to the synergistic effect of propolis and cefixime.

Conclusion

The overall results of the present work provide baseline information for the possible use of the ethanolic extract of propolis (EEP) in combination with antibiotic in reducing its effective dosage for the treatment of salmonellosis, especially typhoid fever. The effectiveness of propolis as a prospective candidate in combination with antibiotic against *Salmonella* cannot be ignored. 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 is a step forward to support the use of propolis as "global remedy".

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