Original Article

Therapeutic potential of Bulgarian Propolis against antibiotic-resistant *Salmonella* species

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Abstract

The emergence of multidrug-resistant (MDR) *Salmonella* spp. has increased the need for investigation of antimicrobial alternatives. Ethanolic extract of propolis (EEP) has been identified to have wide-ranging medical applications related to its antimicrobial activity. The current study aimed to identify the major chemical components of EEP with antibacterial effects, evaluate the antibacterial efficacy of EEP against MDR *Salmonella* spp. isolates, and further examine the therapeutic efficacy of EEP against MDR *S.* Enteritidis infected mouse model. Bulgarian propolis EEP was prepared by using ethanol based extraction method. The chemical composition of EEP was characterized using the gas chromatography-mass spectroscopy (GC-MS). The in-vitro antibacterial activity of EEP against 20 isolates of MDR *Salmonella* spp. was investigated. Furthermore, the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of EEP were determined along with in vivo therapeutic efficacy with reference to hematobiochemical and histological analyses. The main identified compounds belonged to flavonoids, aromatic acids, and esters which may be attributable to its antibacterial activity. Antimicrobial efficacy of EEP was detected against all isolates with variable bactericidal to bacteriostatic efficacy and with an MIC of ≤0.012-6.250 mg/mL (mean 1.294 ± 1.557) and an MBC of 1.563-12.50 mg/mL (mean 4.531 ± 2.678). A therapeutic efficacy against *S.* Enteritidis was also determined. Statistical analyses for hematological and serum biochemical tests have showed a significant increase due to infection effect in band neutrophil counts, eosinophil counts and serum alanine aminotransferase (ALT) levels in comparing two infected groups with two non-infected groups; simultaneously, there was no significant difference in between both infected groups and in between both non infected groups whereas the results were significant at (*p value*<0.05). As well as, there was no any evidence of significance regarding neither treatment nor infection*treatment interaction effects. In infected groups, histological examination of the liver revealed degenerative changes in hepatocytes; these changes had almost disappeared in the EEP-treated group. The current study demonstrated a potential therapeutic effect of Bulgarian propolis ethanolic extract on clinically recovered antibiotic-resistant *S.* Enteritidis from diarrheic goat, and the results herald a promising supplementary therapy of EEP during resistant *Salmonella* infection.

Keywords: antibacterial, anti-inflammatory, MDR, propolis, resistance, *Salmonella*

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Introduction

Salmonella has been identified as one of the most frequent causes of infectious diarrhea which takes a toll on both human and animal health worldwide (Singh *et al*., 2013). In recent years, multidrug-resistant (MDR) *Salmonella* has become a major public health concern because of the increased resistance to conventional antimicrobials (Alam *et al*., 2020). The current increase of drug resistance in many bacteria has been attributed to the inappropriate use of antimicrobials in agriculture and veterinary sectors (Ayukekbong *et al*., 2017), which are further transmitted to humans through the food chain and meat products (Bartlett *et al*., 2013). Human infection with antimicrobial resistant bacteria may lead to adverse health concerns (CDC, 2013). Rising levels of antimicrobial resistance (AMR) and the concurrent decline in new advances in antimicrobial agents both pose a significant threat to global health, resulting in an increased risk of treatment failure and the emergence of severe infections in animals and humans (Capita and Alonso-calleja, 2013). Therefore, antimicrobial alternatives may reduce the use of conventional antibiotics for the prevention and control of animal bacterial diseases; here, identification of effective and safe alternatives is very relevant for the improvement of livestock health and productivity (Ghosh *et al*., 2019).

Propolis (bee glue) has been defined as a resinous product derived from plants and collected by honeybees (Lotfy, 2006). Propolis is still used in traditional and alternative medicine because of its broad spectrum of biological activities and potential therapeutic properties (Lotfy, 2006). The therapeutic and antibacterial properties of propolis have contributed to a growing interest in its chemical composition and origin (Przybyłek and Karpinski, 2019). Propolis exhibited variable antimicrobial activity against a wide range of microorganisms (Pobiega *et al*., 2017, Przybyłek and Karpinski, 2019). The antibacterial activity of ethanolic extract of propolis (EEP) alone or in synergy with antibiotics against *Salmonella* has been examined (Ayhan TEMİZ *et al*., 2011). Propolis has been observed to exhibit anti-inflammatory effects in acute and chronic models of inflammation (Castaldo and Capasso, 2002).

The emergence of MDR *Salmonella* spp. is of great concern and calls for investigation into antimicrobial alternatives in order to control salmonellosis. The previous studies investigated the antibacterial efficacy of Bulgarian propolis in vitro only and on standard *Salmonella* spp. strains including *S*. Typhimurium and *S*. Typhi without any referral to their antibiotic-resistance pattern or effect on pathogenicity (Orsi *et al*., 2005, Orsi *et al*., 2012). Moreover, the antibacterial components of EEP are yet to be fully identified.

The current study aimed to 1) Identify the chemical composition of the ethanol extract of the Bulgarian propolis using gas chromatography-mass spectroscopy (GC-MS). 2) Investigate the antibacterial activity of EEP *in vitro* against MDR *Salmonella* isolates recovered from sheep and goats through determination of MIC, MBC and MBC/MIC ratio. 3) Determination of *in vivo* antibacterial activity of EEP against *Salmonella* Enteritids experimentally infected mice.

Materials and Methods

Ethical approval for animal experimentation: The current study was approved by Cairo University-Institutional Animal Care and Use Committee (CU-IACUC) with an approval number: CU/II/F/97/18. The study was started in September, 2019 and launched till mid-November, 2019. All experiments of the study were implemented at the Central Laboratory of Molecular Epidemiology and Infectious Diseases which is located in the Department of Internal Medicine and Infectious Diseases, Faculty of Veterinary Medicine-Cairo University.

Salmonella spp. Isolates: A total of 20 isolates of *Salmonella* spp., were recovered from sheep and goats suffering from diarrhea were used for the in vitro investigation in the current study. The phenotypic multiple antimicrobial resistance (MAR) indices of the used strains were previously characterized where all strains were identified as MDR as mentioned in the previous study (Farouk *et al*., 2020; Supplementary table 1).

Preparation of ethanol extracts of propolis (EEP): A macerated Bulgarian propolis sample was donated from the Desert Research Institute, Egypt. Ethanol-based extraction of Bulgarian propolis was performed according to Sawaya *et al*. (2004). Following the extraction, a rotary evaporator set at 50°C was then used to remove the excess ethanol. The remaining dried ethanolic extract of the Bulgarian propolis (EEP) was stored in dark sterile glass bottles at 4°C until use.

Determination of the chemical composition of EEP: Chemical composition analysis of EEP was performed using the Trace GC1310-ISQ mass spectrometer (Thermo Scientific®, Austin, TX, USA), with a direct capillary column TG– 5MS (30 m ×0.25 mm × 0.25 µm film thickness), in the Faculty of Medicine, Zagazig University, Egypt. The components were identified by comparison of their retention times and mass spectra with those of WILEY 09 and NIST 11 mass spectral database according to Kalia *et al*. (2015). The propolis yield was calculated by the following equation:

percentage yield (%) = Amount of the recovered pure product / amount of crude material used x100

In vitro antibacterial efficacy of EEP against Salmonella spp. Isolates: First, the antibacterial activity of EEP against all 20 *Salmonella spp.* isolates was examined. The minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of EEP were determined for each isolate separately upon confirmation of an antibacterial effect of EEP on the isolates tested.

Screening for EEP antimicrobial activity by growth inhibition test: Acetone has been reported to have a neglected antimicrobial effect, so a known amount of

dry crude EEP was dissolved in acetone at a concentration of 25 mg/mL (VanVuuren *et al.*, 2010). Briefly, to examine the antibacterial activity of the EEP against *Salmonella* spp. isolates, the bacterial culture of each isolate in brain heart infusion was adjusted to a concentration 10^6 colony-forming units (CFU)/mL and confirmed using plate counting. Equal volumes of the prepared EEP and each *Salmonella spp.* isolate broth were mixed in order to reach a concentration 5×10^5 CFU/mL, and, in parallel, a negative control tube was prepared for each strain by mixing equal volumes of broth containing bacteria and sterile deionized water. Next, all tubes were incubated overnight in a shaking incubator at 225 rpm at 37°C. After that, 0.1 mL from each tube was streaked onto a Müller-Hinton agar plate (Oxoid®) and incubated overnight at 37°C, followed by CFU counting and comparison between the initial bacterial counts (5×10^5 CFU/mL) and the bacterial plate counts.

Supplementary Table 1 Details of antimicrobial susceptibility profile of the used *Salmonella* strains in the study

Salmonella strain	Susceptibility profile to 10 antimicrobials		MAR index
	Pattern (No)	Antimicrobials	
S. Paratyphi A	S (3)	Amikacincin, Enrofloxacin, Gentamicin	0.6
	I (1)	Florfenicol	
	R (6)	Amoxicillin, Ciprofloxacin, Colistins, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Ferruch	S (4)	Amikacincin, Enrofloxacin, Florfenicol, Gentamicin	0.4
	I (2)	Ciprofloxacin, Amoxicillin	
	R (4)	Colistin, Doxycycline, Oxytetracycline, Sulfa-Trimethoprim	
S. Durham (strain No. 1)	S (2)	Amikacin, Enrofloxacin	0.7
	I (1)	Gentamicin	
	R (7)	Amoxicillin, Ciprofloxacin, Colistin, Doxycycline, Florfenicol, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Durham (Strain No. 2)	S (4)	Amikacin, Enrofloxacin, Florfenicol, Gentamicin	0.6
	I (0)	-	
	R (6)	Amoxicillin, Ciprofloxacin, Colistin, Doxycycline, Oxytetracycline , SULFA-TRIMETHOPRIM	
S. Mississippi (Strain No. 1)	S (5)	Amikacin, Amoxicillin, Enrofloxacin, Florfenicol, Gentamicin	0.5
	I (0)	-	
	R (5)	Ciprofloxacin, Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Mississippi (Strain No. 2)	S (4)	Amikacin, Enrofloxacin, Florfenicol, Gentamicin	0.6
	I (1)	Ciprofloxacin	
	R (5)	Amoxicillin, Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Mississippi (Strain No. 3)	S (4)	Amikacin, Enrofloxacin, Florfenicol, Gentamicin	0.6
	I (0)	-	
	R (6)	Amoxicillin, Ciprofloxacin, Colistin, Doxycycline, Oxytetracycline , SULFA-TRIMETHOPRIM	
S. Mississippi (Strain No. 4)	S (5)	Amikacin, Amoxicillin, Enrofloxacin, Florfenicol, Gentamicin	0.5
	I (0)	-	
	R (5)	Ciprofloxacin, Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Mississippi (Strain No. 5)	S (5)	Amikacin, Ciprofloxacin, Enrofloxacin, Florfenicol, Gentamicin	0.5
	I (0)	-	
	R (5)	Amoxicillin, Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Mississippi (Strain No. 6)	S (5)	Amikacin, Amoxicillin, Enrofloxacin, Florfenicol, Gentamicin	0.5
	I (0)	-	
	R (5)	Ciprofloxacin, Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Mississippi (Strain No. 7)	S (6)	Amikacin, Amoxicillin, Ciprofloxacin, Enrofloxacin, Florfenicol, Gentamicin	0.7
	I (0)	-	
	R (4)	Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Mississippi (Strain No. 8)	S (6)	Amikacin, Amoxicillin, Enrofloxacin, Florfenicol, Gentamicin, SULFA-TRIMETHOPRIM	0.8
	I (0)	-	
	R (4)	Ciprofloxacin, Colistin, Doxycycline, Oxytetracycline	
S. Mississippi (Strain No. 9)	S (4)	Amikacin, Enrofloxacin, Florfenicol, Gentamicin	0.9
	I (1)	Ciprofloxacin	
	R (5)	Amoxicillin, Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Stanleyville	S(4)	Amikacin, Enrofloxacin, Florfenicol, Gentamicin	0.6
	I (0)	-	
	R (6)	Amoxicillin, Ciprofloxacin, Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Mississippi (Strain No. 10)	S (4)	Amikacin, Enrofloxacin, Florfenicol, Gentamicin	0.6
	I (0)	-	
	R (6)	Amoxicillin, Ciprofloxacin, Colistin, Oxytetracycline , SULFA-TRIMETHOPRIM, Doxycycline	
S. Kottbus	S (4)	Amikacin, Enrofloxacin, Florfenicol, Gentamicin	0.5
	I (1)	Ciprofloxacin	
	R (5)	Amoxicillin, Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Bonariensis	S (3)	Amikacin, Enrofloxacin, Gentamicin	0.7
	I (0)	-	
	R (7)	Amoxicillin, Ciprofloxacin, Colistin, Doxycycline, Florfenicol, Oxytetracycline, SULFA-TRIMETHOPRIM	

S. Allerton	S (4)	Amikacin, Enrofloxacin, Florfenicol, Gentamicin	0.6
	I (0)	-	
	R (6)	Amoxicillin, Ciprofloxacin, Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Enteritidis(Strain No. 1)	S (4)	Amikacin, Enrofloxacin, Florfenicol, Gentamicin	0.5
	I (1)	Ciprofloxacin	
	R (5)	Amoxicillin, Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	
S. Enteritidis(Strain No. 2)	S (4)	Amikacin, Enrofloxacin, Florfenicol, Gentamicin	0.6
	I (0)	-	
	R (6)	Amoxicillin, Ciprofloxacin, Colistin, Doxycycline, Oxytetracycline, SULFA-TRIMETHOPRIM	

S: Sensitive; ***I:*** Intermediate; ***R:*** Resistant; ***(No):*** Number; ***MAR index:*** Multiple Antimicrobial Resistance index.

Minimum inhibitory concentration (MIC) of EEP and bacterial viability assay: To quantify the MIC values of EEP for each *Salmonella* isolate, serial dilution assays were performed in microtiter plates. A total of 100 microliters of sterile Muller-Hinton broth was introduced into each well of a 96-well microtiter plate, except for one well that was left as blank according to the microplate reader software instructions. Furthermore, three rows were used as controls, culture control row was included to verify that the broth was capable of supporting microbial growth, an EEP negative control row at a starting concentration of 25 mg/mL was used as blanks, each well's value was deduced from the corresponding test well's value, and a positive control row of a standard ciprofloxacin antibiotic at a starting concentration 0.1 mg/mL was included. Later on, 100 µL of EEP at a starting concentration of 25 mg/mL was individually transferred into the top row of the microtiter plate, and double-fold serial dilutions were then performed. All isolates were tested in triplicates in order to ensure accurate and reproducible results. Hereafter 100 µL of sub-culture broth (with adjusted turbidity to the 0.5 McFarland standard to ensure an approximate concentration of 1.5 ×10^8 CFU/mL) was added to all 96 wells, except for the EEP negative control row, where 100 µL of sterile Muller-Hinton broth was added to each well in this row. Each plate was subsequently sealed using a sterile adhesive sealing film to prevent evaporation of the test sample; then, it was incubated at 37°C for 24 hours. After incubation, 40 µL of tetrazolium salt AR (Research Lab) at a concentration of 5 mg/mL was added to each well, followed by incubation in a rotary incubator (150 rpm) at 37°C for 15 min. When the tetrazolium salt indicator was added, the color change (from colorless to pink) was monitored in the culture control row. Once an observable color change was noted within the culture control column after completion of the incubation period, the optical density of each plate was measured at 570 nm using a microplate plate reader (BioTek ELX-800). The percentage of bacterial inhibition by EEP concentration was computed using the following equation after blank correction:

= (OD of Culture Control-OD of Test)/(OD of Culture Control)x100

where MIC was defined according to Shekar *et al*. (2016) as the minimum concentration of EEP inhibiting 20% of the bacterial growth.

Minimum bactericidal concentration (MBC): A total of 0.1 mL from each well was streaked on Muller-Hinton agar after optical density (OD) measurements; then, it was incubated at 37°C to determine the lowest concentration resulting in no obvious growth (MBC) after 20 hours of incubation.

In vivo antibacterial efficacy of EEP against MDR Salmonella Enteritidis.

Challenge Salmonella spp. Strain: *Salmonella* Enteritidis strain was selected from the 20 isolates that were under *in vitro* investigation of the current study; the strain infects a broad range of host animals, including mice (Suar *et al*., 2006). The partial codon nucleotide sequence of *stn* gene of the used strain was deposited in the National Center for Biotechnology Information GenBank database under the accession number (MN750322). Also, the sensitivity pattern of the used challenge strain to cefotaxime (30µg) antibiotic was identified by Kirby-Bauer disc diffusion method and the exhibited zone of inhibition diameter (30mm) was interpreted according to CLSI, 2014 guidelines, whereas the strain was sensitive to cefotaxime.

Experimental study: A mouse animal model used for the study. The mice were acclimated for 1 week before the start of the experiment; at the end of the acclimatization period, fecal samples were collected and subjected to routine bacteriological isolation of *Salmonella* spp., according to international organization for standardization (ISO-6579) standards (ISO, 1998) in order to ascertain that mice were free from *Salmonella* spp.

Animal housing and feeding: A total of 35 male mice of 25-28 g body weight were equally divided and randomized into 5 groups and housed in plastic cages (7 mice/group/cage); all cages were isolated from each other, and there was no reported contact between them. The mice were housed with free access to pellet feed (21% protein) and water.

Experimental design: The mice were randomly allocated into the following five groups: Group 1 (Neutral control), non-infected and non-treated mice; Group 2 (EEP control), non-infected and EEP–treated mice; Group 3 (Negative control), *S.* Enteritidis–infected and non-treated mice; Group 4 (Test), *S.* Enteritidis–infected and EEP–treated mice; and Group 5 (Positive control), *S.* Enteritidis–infected and cefotaxime–treated mice. This design was employed in

order to determine if there were significant effects on the tested parameters due to infection and/or EEP treatment as well as to detect if there was a significant difference between cefotaxime (standard antibiotic) and EEP treatment efficacies.

Experimental infection and treatment regime: The mice were injected intraperitoneally with cyclophosphamide (Endoxan®, Baxter Oncology GmbH) using a dose of 30 mg/kg/24 h for 3 successive days (Tala *et al.,* 2015) for induction of immune suppression, and, on the third day all mice were fasted overnight followed by oral administration of 0.1 mL of 5% sodium bicarbonate (Srinivasan *et al.,* 2004), followed by oral administration of 1mL normal saline solution containing 1.5×10^8 CFU of the *S.* Enteritidis strain to infected groups and 1 mL sterile normal saline to the non-infected groups by oral gavage. Fecal bacterial loads reflecting *S.* Enteritidis shedding were monitored daily to ascertain whether infection has occurred. The establishment of infection was confirmed by an observed steady increase in bacterial load within 2 days following oral experimental infection. After confirmation of infection, the treatment regime was initiated, which lasted for 1 month according to Kalia *et al.,* 2015. Positive control mice were given 4 mg/kg body weight daily of cefotaxime orally, while the test and EEP control groups were treated orally with 300 mg EEP/Kg body weight daily. In parallel, 1 mL of sterile saline was given orally to the neutral and negative control groups in order to exclude any effect of the experiment itself, including stress (Kalia *et al.,* 2015). The researcher was responsible for experimental infection, treatment regime and daily fecal sampling of animals.

Bacteriological analysis: The number of viable *Salmonella* spp. per gram of feces in the three infected groups was estimated daily after the beginning of the treatment regime. Briefly, upon collection of fecal samples from each cage, 1 g of feces was dissolved in 1 mL sterile saline (0.9% NaCl). Next, 100 µL aliquots were serially diluted (tenfold) in sterile saline. The numbers of viable *Salmonella* spp. per gram of feces were then determined by plating 10 µL from each dilution on duplicate XLD agar plates, which were subsequently incubated overnight at 37°C. Typical colonies were then identified and counted on the plates. Furthermore, the actual count was estimated using the following formula.

$$\text{CFU/g} = 10^{-3} \times 1/\text{dilution} \times \text{average number of colonies on two plates}$$

As well as, fecal samples from the other neutral control and EEP control groups were tested daily to ascertain that mice were still free from *Salmonella* infection along the experiment period.

Analysis of selected hematological and serum biochemical parameters: After 30 days of treatment, all mice were anesthetized by chloroform vapor, and blood samples were collected into two tubes, one with anticoagulant (EDTA) and one plain tube, respectively, using the cardiac puncture method according to Gatsing *et al*. (2005). The portion aliquoted to the EDTA-containing tube was used for estimation of hematological parameters, while the other portion (plain tube) was used for estimation of selected serum parameters, including albumin, alanine aminotransferase (ALT), urea, and creatinine after separation of serum. The evaluation of serum parameters was performed using designated test kits (Spectrum Diagnostic Kits, Egypt), according to manufacturer's instructions, and for estimation of hematological parameters, an automated veterinary hematology analyzer (VETSCAN®, HM5) was used. The anesthesia and blood sampling of animals were performed by laboratory personnel under the researcher supervision.

Histopathological analysis: After a terminal procedure of cardiac puncture and collection of blood samples in fully anesthetized mice, all mice were dissected, and liver samples were removed carefully, fixed in 10% buffered neutral formalin solution, and subjected to hematoxylin and eosin staining according to Gamble (2008) for further microscopic evaluation and imaging.

Statistical analysis: Means of triplicate data of the estimated optical densities were presented as (means ± standards deviations) using Microsoft Office Excel 2013. For the *in vivo* experiment, statistical analysis was performed using IBM SPSS Statistics 20. A *p-value* < 0.05 was considered to indicate a statistical significance. Testing of differences of treatment and infection categorical variables between the first four groups was performed using two-way analysis of variance (ANOVA), and one-way ANOVA to test differences of treatment type variable between the three infected groups (groups 3–5). The implemented statistical analysis was designed in order to account the proposed research questions. The questions aimed to 1) Identify if the infection and EEP-treatment have effects on the tested haematological and biochemical parameters. 2) Investigate the difference between the used EEP and cefotaxime treatments on the tested haematological and biochemical parameters. Therefore, two-way ANOVA and one-way ANOVA were performed to account two aims, respectively.

Results

Propolis extract yield and chemical composition of EEP: The weight of crude ethanolic extract of propolis was 13.0g and the yield percentage was 43.33%. Chromatographic analysis of EEP has enabled peak identification as shown in (Fig. 1) for the 43 compounds listed in Table 1. The majority of the identified compounds were flavonoids, aromatic acids, and aromatic acid esters. The main identified flavonoids compounds were 5-hydroxy-7-methoxyflavanone, 4H-1-benzopyranone, 2,3-dihydro-5,7-dihydroxy-2-phenyl-, (S)-, 5-hydroxy-7-methoxy-2-phenyl-,4H-1-benzopyranone,2-(3,4-dihydroxyphenyl)-6,8-DI-á-D glucopyranosyl-5,7-dihydroxy-, 5-hydroxy-4',7-dimethoxyflavanone, and 3,7,3',4'-tetrahydroxyflavone. The predominating acids and their esters were n-hexadecanoic acid, octadecanoic

acid, tetradecanoic acid, dodecanoic acid, 9-octadecanoic acid, fumaric acid-hexadecyl pentenyl ester, and cholanoic acid, 3,12-dihydroxy-, (3à,5á,12à)-.

In vitro antibacterial efficacy of EEP against Salmonella spp. strains

Growth inhibition: Ethanolic extract of Bulgarian propolis exhibited antimicrobial activity against all 20 tested MDR *Salmonella* spp. isolates. For all tested isolates, bacterial counts were lower than the counts observed before incubation (<5 ×10^5 CFU/mL).

Minimum inhibitory concentration (MIC) of EEP and bacterial viability assay: The details for each strain are presented in Tables 2–4. The EEP MIC range was determined to be at ≤0.012–6.250 mg/mL (mean, 1.294 ± 1.557) (Table 5).

Minimum bactericidal concentration (MBC): The initial MBC was recorded as the lowest EEP concentration showing no change in color, when the color of the culture control row matched that of the PH endpoint control; Final readings were recorded after incubation of inoculated plates and confirmation of no obvious growth of the corresponding dilution in comparison with the obvious growth of culture control inoculated plates. The MBC ranged from 1.563 to 12.50 mg/mL (mean, 4.531 ± 2.678). (Table 5, Fig. 2).

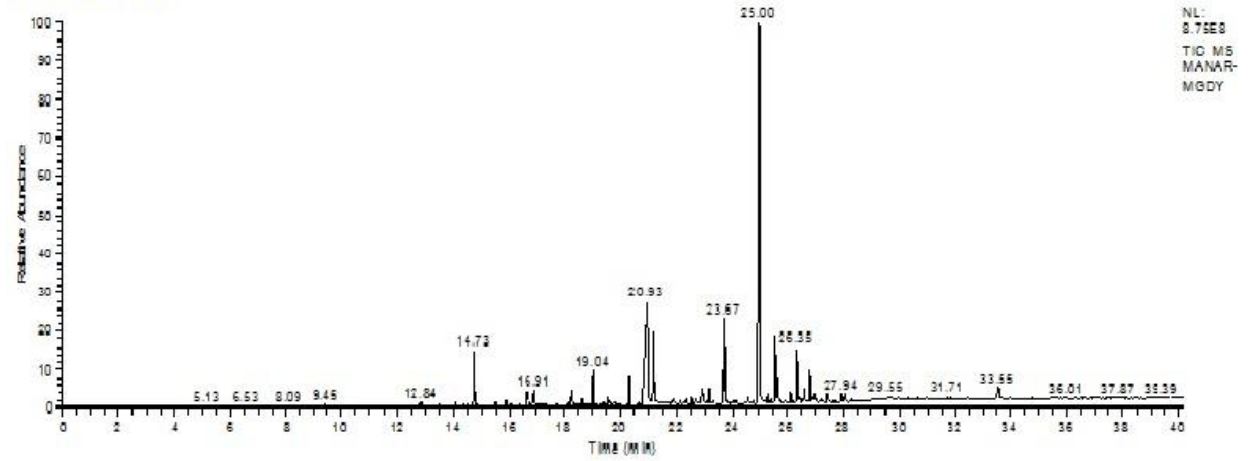

Figure 1 Gas chromatography mass spectroscopy peaks of identified compounds in EEP

Table 1 Percentage, retention time and structure of compounds identified in Bulgarian EEP by GC-MS.

C. peak	Compound name	C.C.	M. Weight	Molecular Formula	R.T	Area %
1	1-Tetradecene	F	196	C14H28	12.84	0.28
2	2,4-Bis(1,1-Dimethyl ethyl)- Phenol	F	206	C14H22O	14.73	3.25
3	Dodecanoic acid	A	200	C12H24O2	15.48	0.45
4	1-Hexadecene	F	224	C16H32	15.89	0.52
5	2-Naphthalene methanol,1,2,3,4,4a,5,6,7-octahydro-à,à,4a,8-tetramethyl-, (2R-cis)-	F	222	C15H26O	16.60	0.86
6	Longifolen aldehyde	F	220	C15H24O	16.66	0.78
7	2-(4A,8-Dimethyl-1,2,3,4,4A,5,6,7 Octahydro-2Naphthalenyl)-2-Propenol	F	220	C15H24O	16.72	0.25
8	2-((2S,4aR)-4a,8-Dimethyl-1,2,3,4,4a,5,6,7 octahydronaphthalen-2-yl)propanol	F	222	C15H26O	16.91	1.65
9	Tetradecanoic acid	A	228	C14H28O2	18.27	1.59
10	3-Octadecene, (E)-	F	252	C19H38	18.65	0.64
11	Phenol, 4-(1-Pentenyl)-, (E)-	F	162	C11H14O	19.04	2.27
12	2-(4A,8-dimethyl-1,2,3,4,4A,5,6,7-octahydro-2-naphthalenyl)-2-propenol	F	220	C15H24O	19.59	0.62
13	Methyl 14-Methylpentadecanoate	E	270	C17H34O2	20.33	1.68
14	n-Hexadecanoic acid	A	256	C16H32O2	20.93	14.52
15	Hexadecanoic acid, Ethyl ester	E	284	C18H36O2	21.16	4.13
16	1-Heptatriacotanol	F	536	C37H76O	21.86	0.54
17	2-Nonadecanone	F	282	C19H38O	22.33	0.78
18	Octadecanoic acid, Methyl ester	E	298	C19H38O2	22.52	0.25
19	9-Octadecanoic acid	A	282	C18H34O2	22.66	0.61
20	Octadecanoic acid	A	284	C18H36O2	22.89	1.70
21	Octadecanoic acid, Ethyl ester	E	312	C20H40O2	23.15	0.98
22	Benzaldehyde, 3,4-Dimethoxy-2-(1-Methylethyl)-	F	208	C12H16O3	23.67	4.92
23	Dodecanoic acid, 4-pentenyl ester	E	268	C17H32O2	23.73	3.12
24	8-Octadecenal	F	266	C18H34O	24.12	0.29
25	9-Octadecenamide, (Z)-	F	281	C18H35NO	24.58	0.64
26	5-hydroxy-7-methoxyflavanone	F	270	C16H14O4	25.00	32.78

27	Fumaric acid, hexadecyl pentenyl ester	E	408	C25H44O4	25.29	0.52
28	9,12,15-Octadecatrienioc acid, (2-Phenyl-1,3-Dioxolanyl) Methyl ester	E	440	C28H40O4	25.40	0.28
29	4H-1-Benzopyranone, 2,3-dihydro-5,7-dihydroxy-2-phenyl-, (S)-	F	256	C15H12O4	25.55	4.27
30	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	E	330	C19H38O4	25.64	1.80
31	Hexadecanoic acid, phenylmethyl ester	E	346	C23H38O2	26.12	0.62
32	4H-1-Benzopyranone, 5-hydroxy-7-methoxy-2-phenyl-	F	268	C16H12O4	26.35	3.41
33	Benzyl (E)-ferulate	E	284	C17H16O4	26.46	0.30
34	Benzo[3,4]Phenanthro [2,1-B]Thiophene	F	284	C20H12S	26.61	0.99
35	Phenethyl palmitate	E	360	C24H40O2	26.80	1.62
36	4H-1-Benzopyranone,2-(3,4-Dihydroxyphenyl)-6,8 -DI-á-D-Glucopyranosyl-5,7-Dihydroxy-	F	610	C27H30O16	26.87	0.46
37	5-Hydroxy-4',7-dimethoxyflavanone	F	300	C17H16O5	26.97	0.96
38	Indazolo [2,3-A]Quinoline, 2,3,8,9-Tetramethoxy-	F	338	C19H18N2O4	27.24	0.52
39	3,7,3',4'-Tetrahydroxyflavone	F	286	C15H10O6	27.43	0.96
40	Cholanoic acid, 3,12-Dihydroxy-, (3à,5á,12à)-	A	392	C24H40O4	27.94	0.58
41	5'-(2-Thienyl)-2,2':3',3"-Terthiophene	F	330	C16H10S4	28.02	0.33
42	Phenethyl stearate	E	388	C26H44O2	28.07	0.50
43	Octadecyl3-(3,5-Ditertbutyl -4-hydroxyphenyl)Propanoate	E	530	C35H62O3	33.55	1.32

C. peak: compound peak; **C.C:** compound category; **M. Weight:** molecular weight; **R.T:** retention time; **F:** flavonoids; **A:** aromatic acids and **E:** aromatic esters.

Table 2 Antibacterial effect of Bulgarian EEP against *S.* Paratyphi A, *S.* Ferruch, *S.* Durham (No. 1-2) and *S.* Mississippi (No. 1-4) isolates

Bulgarian EEP serial dilutions' concentration (mg/ml)	Antibacterial effect (Inhibition percentage)							
	***S.* Paratyphi A**	***S.* Ferruch**	***S.* Durham (Isolate No. 1)**	***S.* Durham (Isolate No. 2)**	***S.* Mississippi (Isolate No. 1)**	***S.* Mississippi (Isolate No. 2)**	***S.* Mississippi (Isolate No. 3)**	***S.* Mississippi (Isolate No. 4)**
25.00	99.81	94.44	98.91	98.61	99.85	95.83	94.76	96.333
12.50	99.53	89.62	93.93	91.08	92.23	92.57	89.81	92.17
6.250	99.43	72.44	75.98	61.20	57.95	90.26	70.17	75.67
3.125	86.22	71.65	74.18	20.57	27.31	42.04	43.86	45.79
1.563	79.19	69.58	42.59	19.37	22.51	36.22	34.61	29.67
0.781	68.04	37.94	18.12	16.39	20.26	33.75	25.96	20.87
0.391	67.38	36.49	15.69	14.87	19.39	16.50	22.00	17.13
0.195	63.91	35.36	12.64	14.81	18.81	6.11	22.00	14.26
0.097	58.66	19.77	7.45	14.24	11.90	3.88	21.21	13.53
0.048	55.10	9.91	7.32	8.54	8.50	3.51	21.09	13.32
0.024	43.48	4.86	6.86	7.09	5.16	0.21	17.51	11.92
0.012	12.91	2.50	6.79	5.25	1.23	0.17	11.86	9.99

MIC (Minimum inhibitory concentration) of Bulgarian EEP was identified as the lowest EEP concentration inhibiting at least 20% of bacterial growth.

Table 3 Antibacterial effect of Bulgarian EEP against *S.* Mississippi (No. 5-10) isolates

Bulgarian EEP serial dilutions' concentration (mg/ml)	Antibacterial effect (Inhibition percentage)					
	***S.* Mississippi (Isolate No. 5)**	***S.* Mississippi (Isolate No. 6)**	***S.* Mississippi (Isolate No. 7)**	***S.* Mississippi (Isolate No. 8)**	***S.* Mississippi (Isolate No. 9)**	***S.* Mississippi (Isolate No. 10)**
25.00	99.19	96.22	95.69	97.76	94.26	99.92
12.50	94.61	94.29	91.12	90.02	89.45	92.69
6.250	71.30	48.27	72.96	62.14	70.38	73.63
3.125	29.73	15.04	40.08	35.85	37.94	39.10
1.563	18.09	13.27	33.15	26.43	22.26	23.91
0.781	16.90	12.23	31.08	24.15	19.80	21.65
0.391	13.57	11.50	27.70	18.04	19.55	17.41
0.195	12.44	9.25	19.23	16.08	19.52	16.56
0.097	7.86	5.78	16.23	15.76	7.11	14.62
0.048	6.68	3.46	15.23	13.01	7.07	11.07
0.024	6.57	2.33	12.81	8.95	6.87	1.60
0.012	6.41	0.32	7.35	8.02	4.32	0

MIC (Minimum inhibitory concentration) of Bulgarian EEP was identified as the lowest EEP concentration inhibiting at least 20% of bacterial growth.

Table 4 Antibacterial effect of Bulgarian EEP against *S.* Stanleyville, *S.* Kottbus, *S.* Bonariensis, *S.* Allerton and *S.* Enteritidis (No. 1-2) isolates

Bulgarian EEP serial dilutions' concentration (mg/ml)	Antibacterial effect (Inhibition percentage)					
	***S.* Stanleyville**	***S.* Kottbus**	***S.* Bonariensis**	***S.* Allerton**	***S.* Enteritidis (Isolate No. 1)**	***S.* Enteritidis (Isolate No. 2)**
25.00	99.67	97.27	95.78	96.93	90.50	94.46
12.50	99.29	76.65	91.03	92.11	88.86	91.46
6.250	89.89	75.08	72.10	73.02	88.23	72.00
3.125	64.44	71.54	38.02	38.43	87.08	42.15
1.563	29.58	63.83	19.65	29.61	66.07	25.47
0.781	21.25	59.03	14.07	22.09	53.19	16.35
0.391	14.81	58.22	10.28	21.48	33.04	12.48
0.195	9.82	39.89	9.61	20.59	31.92	9.52
0.097	9.07	32.54	5.98	20.27	27.80	8.76
0.048	5.73	27.03	5.70	18.78	18.74	8.54
0.024	0.79	23.75	4.82	16.95	18.63	7.09
0.012	0.74	22.54	2.07	7.56	13.77	5.10

MIC (Minimum inhibitory concentration) of Bulgarian EEP was identified as the lowest EEP concentration inhibiting at least 20% of bacterial growth.

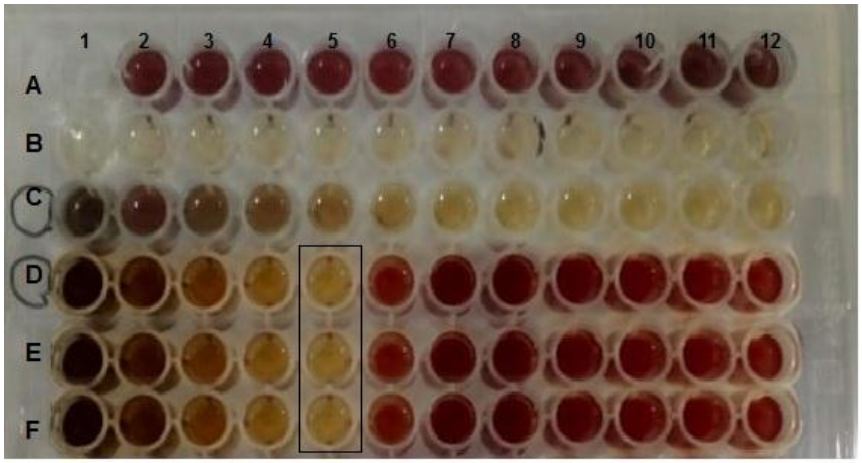

Figure 2 Microtiter plate for bacterial viability assay. Yellow color is evidence for bacteria death and pink color of formazen dye is evidence for viable bacteria. Wells number (D5, E5 and F5) considered end point at initial reading of MBC. Well number (A1) left empty blank well according to (ELX-800) plate reader instructions. Row number (A) culture control row contains 100 µLof sterile Müller Hinton broth and 100 µL of sub-culture broth. Row number (B) positive control row containing 100 µL ciprofloxacin antibiotic and 100 µL of sub-culture broth. Row number (C) negative control row containing 100 µL EEP and 100 µL of sterile Müller Hinton broth. Rows number (D, E and F) are test rows for each isolate in triplicate.

Table 5 Overall antibacterial effect of Bulgarian EEP on tested MDR *Salmonella spp.* isolates (MIC, MBC, and Inhibition percentage at the calculated MIC)

Salmonella isolates	Antibacterial activity of Bulgrian EEP			
	Inhibition% at which MIC calculated	**MIC (mg/ml)**	**MBC (mg/ml)**	**MBC/MIC Ratio**
S. ParatyphiA	43.48	0.024	3.125	130.21
S. Ferruch	35.36	0.195	1.563	8.02
S. Durham (Isolate No. 1)	42.59	1.563	3.125	1.99
S. Durham (Isolate No. 2)	20.57	3.125	3.125	1.00
S. Mississippi (Isolate No. 1)	20.26	0.781	3.125	4.00
S. Mississippi (Isolate No. 2)	33.75	0.781	3.125	4.00
S. Mississippi (Isolate No. 3)	21.09	0.048	6.250	130.2
S. Mississippi (Isolate No. 4)	20.87	0.781	1.563	2.00
S. Mississippi (Isolate No. 5)	29.73	3.125	6.250	2.00
S. Mississippi (Isolate No. 6)	48.27	6.250	6.250	1.00
S. Mississippi (Isolate No. 7)	27.70	0.391	3.125	7.99
S. Mississippi (Isolate No. 8)	24.15	0.781	1.563	2.00
S. Mississippi (Isolate No. 9)	22.26	1.563	1.563	1.00

S. Stanleyville	21.25	0.781	6.250	8.00
S. Mississippi (Isolate No. 10)	21.65	0.781	3.125	4.00
S. Kottbus	22.54	≤0.012	6.250	520.0
S. Bonariensis	38.02	3.125	6.250	2.00
S. Allerton	20.27	0.097	6.250	64.43
S. Enteritidis (Isolate No. 1)	27.80	0.097	6.250	64.43
S. Enteritidis (Isolate No. 2)	25.47	1.563	12.50	7.99
Average activity (mean±SD)	-	1.294±1.557	4.531±2.678	-

The MBC/MIC ratio: The estimated MBC/MIC ratio established that the antibacterial potency of Bulgarian EEP varied from bacteriostatic to bactericidal. The bacteriostatic and bactericidal effects were exhibited by 9 and 11 of the tested *Salmonella* spp. isolates, respectively (Table 5); when ratio ≤4 the agent was classified as bactericidal and bacteriostatic when the ratio >4.

In vivo antibacterial efficacy of EEP against Salmonella spp. Strains

Therapeutic efficacy: Experimental infection in mice was verified by detecting a steady increase in the viable number of *S.* Enteritidis recovered from the feces within the first 2 days of infection. EEP administration was found to be associated with significant decreases in the viable count of *S.* Enteritidis recovered from feces (Fig. 3). Moreover, *S.* Enteritidis shedding of in feces stopped between the fourth and sixth days of the treatment period; a similar result was observed for mice treated with cefotaxime. The number of viable *S.* Enteritidis also decreased in the feces of infected untreated control animals, although shedding lasted for 2 days longer than in the other infected and treated groups.

Effect of EEP treatment on hematological and serum biochemical parameters: The evaluated hematological and serum parameters have provided evidence of the well-being of the neutral group. Meanwhile, alterations were observed in the infected control and test groups as compared with the neutral and EEP control groups (Table 6).

The altered parameters included band neutrophil counts, eosinophil counts and serum ALT levels, while no evidence of significant changes were observed for other parameters. Regarding the effect of infection, significant increases in band neutrophil counts (P = 0.024), eosinophil counts (P = 0.037), and ALT levels (P = 0.016) were observed in both infected and non-infected groups, while differences between the infected groups or between both non-infected groups were observed. Furthermore, there were no significant differences in any of the parameters between the three infected groups with regard to the effect of treatment (Table 7).

Effect of EEP treatment on liver histology: Histopathological lesions were found in both the control group of *S.* Enteritidis infected mice and the EEP-treated infected group; specifically, inflammatory and degenerative lesions were observed in the liver. The changes were more prominent in the infected non-treated group in comparison with the treated one. Severe acute inflammatory response with heavy infiltration of mononuclear cells, dilatation of the hepatic sinusoids, thrombosis and perivascular cuffing with inflammatory cells was only observed in the negative control group of *S.* Enteritidis infected mice (Figs. 4A–B). Congestion of hepatic vessels was noted in both the EEP–treated group and the non-treated infected control group, but these changes were milder in the treated group (Figs.4A–C). However, only mild degenerative changes in the hepatocytes were observed in the group treated with EEP (Figs. 4C–D).

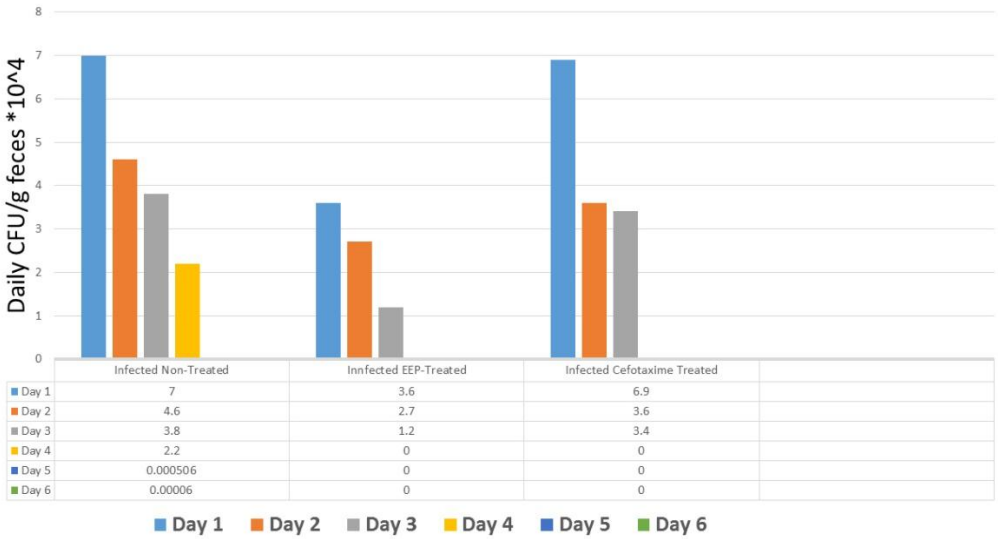

Figure 3 *Salmonella* Enteritidis fecal load in the three infected groups following treatment (CFU/g)

Table 6 Hematological and selected serum biochemical parameters of the first four tested groups

Parameter/ Unit	Animals' groups				*P-value*		
	Group (1) Neutral control	Group (2) EEP control	Group (3) Negative control	Group (4) Test group			
	[Non-Infected/ Non-Treated]	[Non-Infected/ EEP-Treated]	[Infected /Non-Treated]	[Infected/EEP-Treated]	Infection effect	Treatment effect	Infection*Treatment interaction effect
RBCs count (106/µl)	5.56±0.813	6.07±0.614	7.18±0.813	5.70±0.813	0.431	0.541	0.216
PCV (%)	32.00±4.11	31.00±3.11	37.50±4.11	30.50±4.11	0.530	0.320	0.453
Hb content (g/dl)	14.59±0.956	11.75±0.781	13.86±0.956	11.02±0956	0.498	0.106	0.305
WBCs count (103/µl)	13.90±2.52	17.41±2.52	18.87±2.52	17.60±2.52	0.550	0.426	0.349
MCV (fl)	54.53±1.67	54.77±1.27	52.40±1.67	53.78±1.67	0.337	0.614	0.725
MCH (pg)	18.80±0.41	18.74±0..31	17.93±0.41	17.98±0.41	0.050	0.993	0.891
MCHC (g/dl)	37.90±0.98	34.96±0.74	35.28±0.98	34.75±0.98	0.146	0.800	0.210
Lymphocytes(103/µl)	8.28±0.01	11.67±0.00	9.97±0.01	10.99±0.01	0.861	0.104	0.742
Monocytes (103/µl)	1.98±0.003	2.07±0.002	3.44±0.003	2.51±0.003	0.157	0.182	0.702
Eosinophils (103/µl)	0.44±0.002^{A}	0.95±0.001^{A}	1.66±0.002^{B}	1.25±0.002^{B}	0.037*	0.488	0.528
Basophils (103/µl)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-	-	-
Segmented NQ (103/µl)	2.78±0.004	2.15±0.003	2.82±0.004	2.16±0.004	0.668	0.150	0.554
Band NQ (103/µl)	0.42±0.001^{A}	0.57±0.001^{A}	0.98±0.001^{B}	0.69±0.001^{B}	0.024*	0.325	0.237
Albumin (g/dl)	2.73±0.21	3.21±0.15	3.20±0.25	3.16±0.16	0.300	0.289	0.217
ALT (µ/l)	98.00±11.43^{A}	118.33±0.33^{A}	138.33±9.33^{B}	123.60±7.23^{B}	0.016*	0.282	0.103
Urea (mg/dl)	49.67±4.75	57.00±3.68	68.00±5.82	56.50±3.36	0.071	0.652	0.059
Creatinine (µ/l)	0.48±0.07	0.48±0.05	0.46±0.09	0.49±0.06	0.954	0.818	0.762

Data were tabulated as mean±SEM and results were significant at (p value<0.05); p value with asters (*) is of statistical significance. Means within the same row with different letters are statistically significant different, the other with common letters are not statistically different, and there is no evidence of significance in other variables without letters.

Table 7 Hematological and selected serum biochemical parameters of the three infected groups

Parameter/ Unit	Animals' groups			*P-value*
	Group (3) Negative control	Group (4) Test group	Group (5) Positive control	
	[Infected /Non-Treated]	[Infected/EEP-Treated]	[Infected /cefotaxime treated]	
RBCs count (106/µl)	7.18±0.813	5.70±0.813	5.70±1.03	0.460
PCV (%)	37.50±4.11	30.50±4.11	30.50±5.92	0.531
Hb content (g/dl)	13.86±0.956	11.02±0956	10.73±1.41	0.156
WBCs count (103/µl)	18.87±2.52	17.60±2.52	18.25±2.32	0.163
MCV (fl)	52.40±1.67	53.78±1.67	53.23±0.69	0.692
MCH (pg)	17.93±0.41	17.98±0.41	18.47±0.27	0.768
MCHC (g/dl)	35.28±0.98	34.75±0.98	38.17±0.09	0.150
Lymphocytes(103/µl)	9.97±0.01	10.99±0.01	10.48±2.00	0.206
Monocytes (103/µl)	3.44±0.003	2.51±0.003	2.98±4.67	0.433
Eosinophils (103/µl)	1.66±0.002	1.25±0.002	1.46±0.002	0.238
Basophils (103/µl)	0.00±0.00	0.00±0.00	0.00±0.00	-
Segmented NQ (103/µl)	2.82±0.004	2.16±0.004	2.49±0.002	0.566
Band NQ (103/µl)	0.98±0.001	0.69±0.001	0.84±0.001	0.351
Albumin (g/dl)	3.20±0.25	3.16±0.16	3.34±0.24	0.796
ALT (µ/l)	138.33±9.33	123.60±7.23	132.67±6.60	0.056
Urea (mg/dl)	68.00±5.82	56.50±3.36	46.33±4.67	0.050
Creatinine (µ/l)	0.46±0.09	0.49±0.06	0.47±0.07	0.955

Data were tabulated as mean±SEM and results were significant at (p value<0.05) and there no evidence of significance in tested parameters between three infected groups.

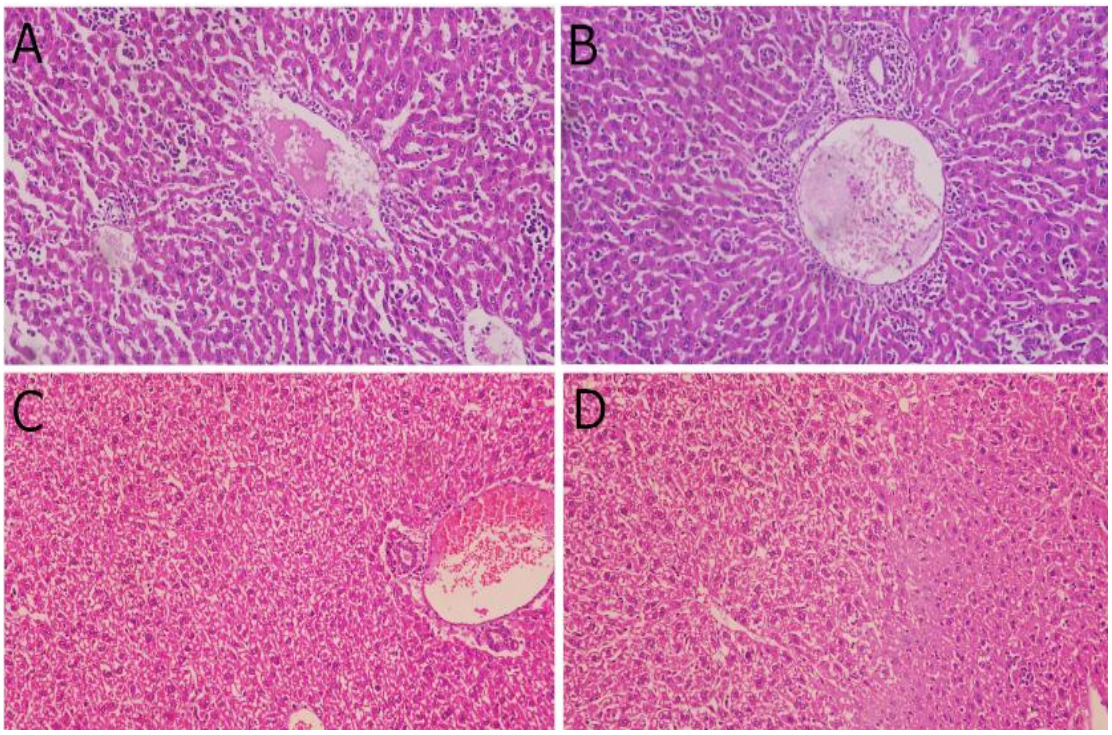

Figure 4 **(A, B).** Microphotographs of histopathology lesions from *S.* Enteritidis infected mice group.
C, D). Microphotographs of histopathology from *S.* Enteritidis infected mice which are treated with propolis extract.
(A) Liver is showing severe congestion and thrombosis of hepatic central vein, hepatic sinusoids are widely dilated and associated with severe and diffuse infiltrations of mononuclear inflammatory cells, between the hepatic cords (H&E, 200X)
(B) Perivascular cuffing of congested portal vein and bile ductules with mononuclear inflammatory cells, heavy infiltrations of inflammatory cells are observed also in hepatic parenchyma (H&E, 200X)
(C) Moderate dilatation and congestion of portal vein with mild degenerative changes of the hepatocytes (H&E, 100X)
(D) Mild to moderate degenerative changes of hepatocytes (H&E, 200X).

Discussion

Salmonella's antimicrobial resistance (AMR) is of major concern to public health and veterinary medicine (D'Aoust *et al*., 1992). The key findings provide insights into of the emergence of antimicrobial resistance salmonellosis in sheep and goats, which may not respond to conventional antimicrobial therapy and increases the need for a new effective curative approach to controlling this problem.

Phytochemical alternatives may help reduce demands for improved conventional antibiotics and are considered essential to the potential success of animal husbandry by preventing and combating bacterial infections in animal populations (Hoelzer *et al.,* 2018). Propolis is well known for its antimicrobial activity against a wide range of microorganisms (Pobiega *et al.,* 2017, Przybyłek and Karpinski, 2019), and its antibacterial activity against resistant *Salmonella* spp. was investigated in this study.

The chemical composition of propolis relies on geographical and climatic factors, local flora, and genetic diversity of the bees producing it (Bankova, 2005). The chemical composition is complex, and more than 300 constituents have been identified to date (Zhang *et al.,* 2014). The chemical analysis of 70% Bulgarian EEP by GC-MS was performed to give an indication about the major compounds present in it. The main identified flavonoids compounds were 5-hydroxy-7-methoxyflavanone, 4H-1-benzopyranone, 2,3-dihydro-5,7-dihydroxy-2-phenyl-, (S)-, 5-hydroxy-7-methoxy-2-phenyl-,4H-1-benzopyranone,2-(3,4-dihydroxyphenyl)-6,8-DI-á-D glucopyranosyl-5,7-dihydroxy-, 5-hydroxy-4',7-dimethoxyflavanone, and 3,7,3',4'-tetrahydroxyflavone. The predominating acids and their esters were n-hexadecanoic acid, octadecanoic acid, tetradecanoic acid, dodecanoic acid, 9-octadecanoic acid, fumaric acid-hexadecyl pentenyl ester, and cholanoic acid, 3,12-dihydroxy-, (3à,5á,12à)-. These findings are in line with a previous study identifying the main components of ethanolic extract of propolis (EEP) samples as flavonoids, aromatic acid esters, aromatic alcohols, aromatic acids, aliphatic carboxylic acids, terpenes, and aliphatic carboxylic acid esters (Ayhan TEMİZ *et al.,* 2011).

The antibacterial activity of EEP was determined against all 20 isolates, and inhibitory activity was observed at a starting concentration of 25 mg/mL in 90.50%–99.92% of all the strains. The MIC range was ≤0.012–6.250 mg/mL (mean, 1.294 ± 1.557), and the MBC ranged from 1.563 to 12.50 mg/mL (mean, 4.531 ± 2.678). The antibacterial activity of EEP on the tested strains varied from bactericidal to bacteriostatic depending on the strain; antimicrobials were classified as bactericidal if the MBC/MIC ratio was < 4 and as bacteriostatic if the ratio was > 4 (French, 2006). Similarly, the bactericidal activity of Brazilian propolis against *S.* Enteritidis and *S.* Typhimurium was recently reported (Orsi *et al.,* 2005). The variation in antibacterial effect observed in the present study is in line with the previous studies reporting that propolis exhibits a bacteriostatic effect against different genera of bacteria and may have bactericidal effects at high concentrations (Mirzoeva *et al.,* 1997). The

antimicrobial action of Bulgarian EEP should be further elucidated, but may be attributable to the presence of flavonoids and acids as flavonoids have bactericidal effects rooted in the metabolic disruption in ion channels as a consequence of an impaired phosphorylation/dephosphorylation process (Farnesi *et al*., 2009), and acids confer structural and functional damage to the microbial cell membrane (Mirzoeva *et al*., 1997).

Furthermore, experimental infection of mice with a MDR *S.* Enteritidis strain was carried out to evaluate the therapeutic efficacy of Bulgarian EEP on MDR-related salmonellosis. A marked increase in the counts of the recovered *S.* Enteritidis from feces was observed in the first 2 days following infection. A difference in recovered counts was observed between the cefotaxime-treated and EEP-treated mice. However, in both groups, bacterial shedding has stopped on the same day. However, the discontinuation of *Salmonella* shedding occurred in treated groups on the fourth day of treatment, the therapeutic regime lasted for 30 days in order to improve the hematological, biochemical, and histopathological parameters because propolis not only has a therapeutic effect but also it has antioxidant and ameliorative effects on toxicity induced by *Salmonella* infection in mice (Kalia *et al*., 2016).

Hematological and serum biochemical parameters were evaluated to identify any effect of salmonellosis and Bulgarian EEP, respectively. A significant increase in band neutrophil counts, eosinophil counts, and serum ALT levels was observed, properly reflecting infection; however, no other differences were observed between infected and non-infected groups. The observed rise in ALT is supported by previous studies reporting that salmonellosis results in hepatic injury and release of liver enzymes into the blood (Hasbun *et al*., 2006). The significant increase in band neutrophil counts due to inflammation has resulted in increased neutrophils demand and left-shift neutrophilia because of the release of immature band neutrophils into the circulation (Rosenfeld and Dial, 2010), and significant eosinophilia in response to infection effect is in the line with the previous study which reported typhoid fever causing hematological disturbances, including eosinophilia (Abro *et al*., 2009). Along with the comparison of the control negative group with the positive control and test groups, the effect of Bulgarian EEP and cefotaxime showed no significant differences between these, but verified therapeutic effect of EEP and cefotaxime. This suggests that the values might be restored slowly to near-normal values or this could be due to the investigated parameters were tested only once after 30 days and differences might be determined if they were tested daily or weekly. Histopathological analysis was applied to investigate the ameliorative effect of Bulgarian EEP in case of *S.* Enteritidis infection. The histopathological lesions induced by *S.* Enteritidis infection revealed inflammatory response in the liver, which is in line with the previous studies (Tala *et al*., 2015), including congestion and inflammatory cell infiltrations. Our findings revealed that in the Bulgarian EEP-treated group (test group), the inflammatory response was markedly reduced and had almost disappeared. This could be attributed to the direct antibacterial action of propolis extract (Kalia *et al*., 2012) in addition to its anti-inflammatory and tissue damage repair actions (Castaldo and Capasso, 2002, Kalia *et al*., 2015), which aid to ameliorate the inflammation severity induced by *S.* Enteritidis infection. The degenerative changes in the hepatocytes found in Bulgarian EEP-treated group were determined to be mild and reversible. After 30 days of treatment, there were mild and prominent histopathological lesions exerted on liver tissue of infected- EEP treated and infected non-treated groups, respectively. However, the shedding of *S.* Enteritidis stopped within the fourth and sixth days of treatment in the infected EEP-treated group and lasted 2 days longer in the other infected groups. The persistence of histopathological changes in liver in spite of absence of *S.* Enteritidis in fecal matter may be attributed to *S.* Enteritidis was not completely eliminated and persisted in liver for longer time or could be still present in the fecal matter at non-detectable count by conventional bacteriological examination. So that, it is preferable to quantify the bacterial load by quantitative real time polymerase chain reaction (qrt-PCR). As well as, detection and quantification of *S.* Enteritidis in liver tissue. In conclusion, Bulgarian EEP showed significant antibacterial effects on MDR *Salmonella*, both *in vivo* and *in vitro*, with variable bacteriostatic to bactericidal effects and may be considered as a relevant therapeutic agent for control of MDR salmonellosis. In conclusion, the current study demonstrated a potential antibacterial effect and therapeutic potential of Bulgarian propolis on clinically recovered antibiotic-resistant *S.* Enteritidis from diarrheic goat. Besides, estimation of MIC and MBC which acted as indicators for antimicrobial efficacy and potency of Bulgarian EEP. Whereas, the determined effective antibacterial concentration of Bulgarian EEP was 1.294 ± 1.557 mg/mL, and the reported potency was variable from bacteriostatic to bactericidal. The reported efficacy and potency are essential in research and development phase of a new commercial product. Regarding the current study limitations, a significant effect of treatment type on hematological and serum biochemical parameters in treatment groups may be reported if the animals were sampled and parameters were estimated daily or weekly. It is preferable to estimate count of *S.* Enteritidis in liver tissue of animals and in feces by qrt-PCR. Also, the curative effect was studied only in a mouse model therefore, further studies are needed to test EEP in food animal models, so as to design appropriate formulations and propolis dose recommendations for farm animal therapy with the view of limiting the emergence of such antibiotic-resistant strains. The other study limitations; comparative studies on antibacterial efficacy of different propolis types including local Egyptian propolis and other antimicrobial alternatives against the tested isolates are needed.

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