

Inhibitory Effect of Hydroalcoholic Extract of Mint Plant on *Escherichia Coli* Isolated from Lambs and Goat Kids with Diarrhea in Kazeroun

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Abstract

In spite of advances in the management and prevention techniques as well as strategies for diarrhea treatment, it is still a very common and costly disease affecting the small ruminants. The present study aimed at determining the prevalence of *Escherichia coli* (E.coli) in under 1 month old goat kids and lambs with diarrhea in some livestock husbandries in Kazeroun city using culture method and utilizing herbal medicines instead of antibiotics. It has been done according to increasing use of antibiotics and consequently, an increase in antibiotic resistance and different susceptibility to E.coli in different areas in the world. In order to investigate the effect of hydroalcoholic extract of mint plant on goat kids and lambs diarrhea, 100 lambs and 100 goat kids with diarrhea were collected from sheep husbandries in Kazeroun. The enrichment media, selenite F Broth and MacConkey Agar (Mac) were used for isolation. In this study, all isolated strains showed resistant against antibiotics as ampicillin and tetracycline, and were susceptible to antibiotics such as streptomycin, gentamicin, nalidixic acid, cefixime, ciprofloxacin, nitrofurantoin and chloramphenicol. The halo diameter formed by hydroalcoholic extract of mint was 10 mm, demonstrating a low susceptibility of this bacterium to the mint extract. Generally, the findings revealed that the hydroalcoholic extract of the mint had antimicrobial properties and would be a good alternative to chemical drugs.

Keywords: mint, *Escherichia coli*, diarrhea, lamb, goat kids, Kazeroun

Introduction

Despite the advances in the management and prevention techniques and strategies for diarrhea treatment, it is still a very common and costly disease which affects the small ruminants. A study conducted in a sheep testing centre in America indicated that diarrhea was responsible for 46% of lambs' fatality. Diarrhea in goat kids and lambs is a multi-factorial and complex disease including animal, environment, nutrition, and infectious agents. The four main causes of diarrhea in these animals include *E.coli*, rotaviruses, cryptosporidium strains and salmonella strains among which *E.coli* diarrhea is more prevalent.

E. coli is a natural and dominant bacterial flora in the mammals and birds' gastrointestinal tract, but most of their strains cause infection in human urinary system and their pathogenic strains are very important in digestive

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diseases of human and animals. This bacterium is responsible to develop respiratory diseases in the poultry, which can lead to significant sepsis-related deaths.

E.coli is the most important cause of diarrhea in lambs and kids in the first three weeks of their lives. White diarrhea in lambs and kids is due to specific serotypes of *E.coli* which is regarded very important in the first week of life, but still important in lambs and kids of 2-3 weeks. Although enterotoxigenic strains of *E.coli* (ETEC) cause diarrhea in lambs and kids by producing different toxins, the most important issue in pathogenicity in these strains is their colonization ability that is possible due to the presence of sticky elements such as F165, F41, and K99.

The most important prevalent sticky agent which is recognized on *E.coli* strains involved in lambs and kids diarrhea is the factor K99 that links bacteria to epithelial intestinal cells. Factor K99 can be linked to its receptor *N*-Glycolylneuraminic acid (Neu5Gc). This composition is present at the surface of intestinal tract of lamb and kids and is considered as a receptor for the factor K99. Since in some cases, *E.coli* strains have been isolated from the feces of seemingly healthy lambs and kids, the presence of disease.

In recent years, commercially available anti-microbial drugs have been used to control infectious diseases. The excessive use of antibiotics has led to the development of multiple drug resistance in most bacterial pathogens. The increased drug resistance is regarded as the most important barrier to the successful treatment of infectious diseases and pathogen control of microbial agents. The development of drug resistance in bacterial pathogens and also increased consumer interest in safe and healthy foods has led to the emergence of new and natural antimicrobial agents. The aim of this study was to determine the prevalence of *E.coli* in diarrhea of lambs and kids under one month old in a number of livestock husbandries in Kazeroun city using culture method. The research was carried out considering predisposing factors is also effective in developing the the increasing use of antibiotics and consequently the increased antibiotic resistance and different susceptibility to *E.coli* in different parts of the world, as well as the use of herbal drugs instead of antibiotics.

Materials and Method

Sampling

In this research, 100 samples of lambs and 100 samples of goat kids with diarrhea were collected from some sheep husbandries in Kazeroun in a standard condition. The swabs were inserted into the tubes containing Selenite F Broth and transferred to a microbiology laboratory at Islamic Azad University, Kazeroun branch.

Isolation and purification of *E.coli*

After completion of sampling, the enrichment of samples was done on Selenite F Broth and isolation on MacConkey Agar (Lab.M) containing 0.05 mg/l Cefixime (Oxoid company), and 2.5 mg/l potassium tellurite (Oxoid) at 37 ° C for 24 hours. To evaluate the lactose fermentation and to determine the identity of isolated bacteria, Eosin Methylene Blue agar (Merck, Germany) was used (Duarte Moreira et al., 2014; Hussain et al., 2010).

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Preparation of hydroalcoholic extract of mint plant

In this research, the hydroalcoholic extract of mint plant was utilized. To prepare the extract, the leaves were first dried and powdered, then 50 g of powder was mixed with 450 ml of 80% methanol and water. After filtering, using a laboratory Soxhlet extractor, the extraction was performed at a microbiology laboratory in Islamic Azad University, Kazeroun. The extract concentration was 500 mg / ml, and different required doses were prepared by adding normal saline.

Tube dilution method

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined using dilution method in the tube. To determine MIC, a series of 12 test tubes were used for extraction. 9 test tubes were used to test different dilutions of each extract, one tube as positive control (containing diluted extract plus culture medium) and a tube as negative control (containing microbial suspension plus culture medium) (Van Wyk and Wink, 2004; Aridogan et al., 2002; Dophne et al., 2004; Kazem Alvandi et al., 2011).

Results

Of the total samples in this study, 39 samples (19.5%) were related to male lambs and 61 (30.5%) were related to female lambs. 39 samples (19.5%) were male goat kids and 61 (30.5%) were female goat kids (Chart 1).

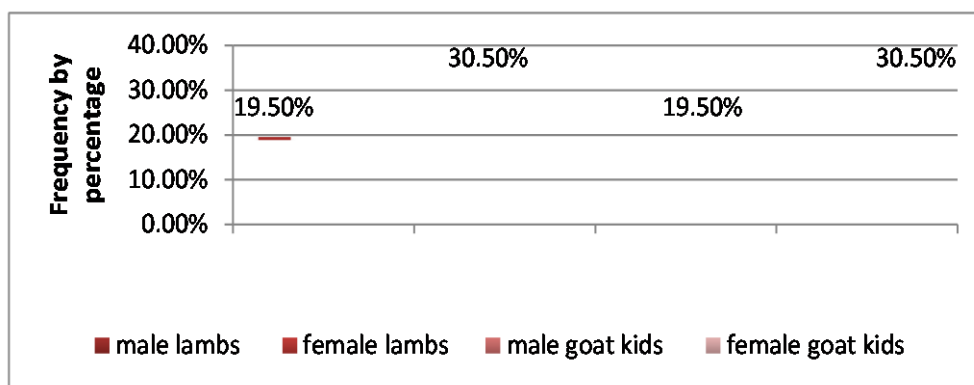


Chart 1. The sample population rate (lambs and goat kids)

Absolute and relative frequency distribution of the population under study based on age has been presented in Table 1 as follows.

Table 1. Absolute and relative frequency distribution of the population based on age

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Age (Day)	0-3	4-6	7-9	10-12
Frequency	61 (30.50%)	80 (40%)	43 (21.50%)	16 (8%)

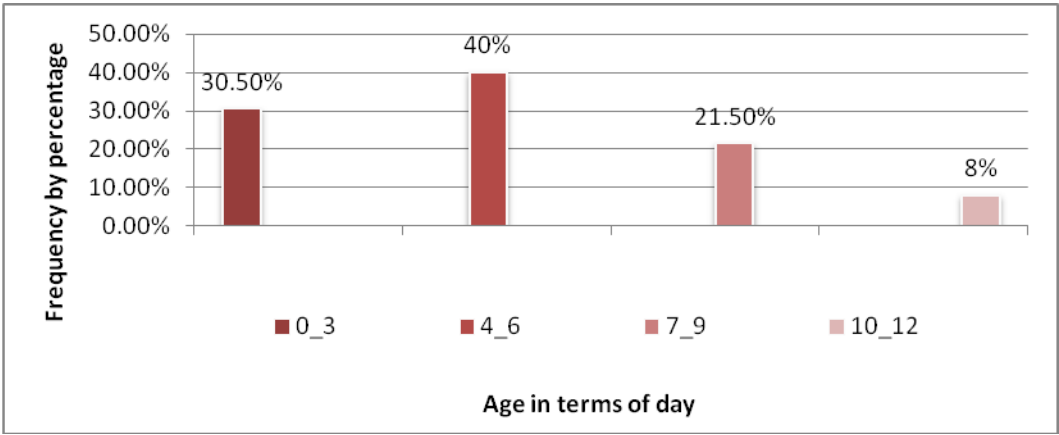


Chart 2.The Frequency of sample population in terms of age

The diarrhea samples were cultured on mechanical culture medium, and the bacterial growth was observed on 153 plates while pathologic bacteria did not grow on 47 plates. After culturing on the MacConkey Agar, smears were prepared and stained by hot staining method. Under the microscope, all samples were gram negative cocci. Then, the bacteria were cultured on EMB, *Eosin Methylene Blue agar* (Merck, Germany), in which 73 colonies in a bright metallic green color were grown after 24 hours of incubation at 37°C, indicating the presence of *E. coli* bacteria. Moreover, 80 colorless colonies were observed; performing TSI and IMViC tests, it was revealed that bacteria were *Enterobacteriaceae* of total coliform bacteria.

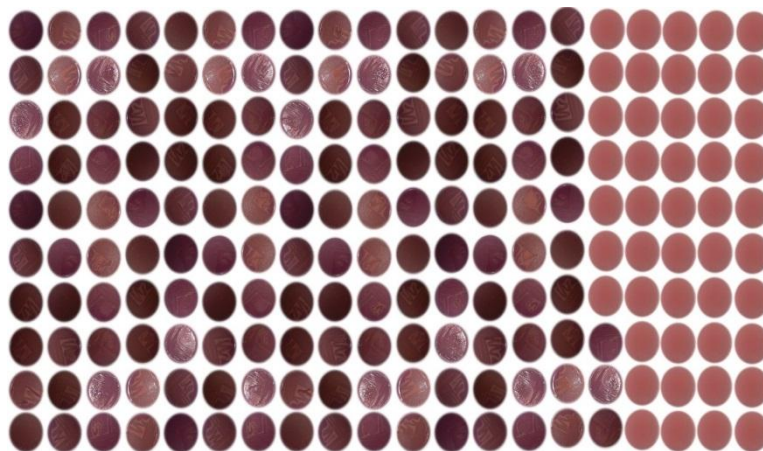


Figure 1. Culture of diarrhea samples on a mechanical culture medium

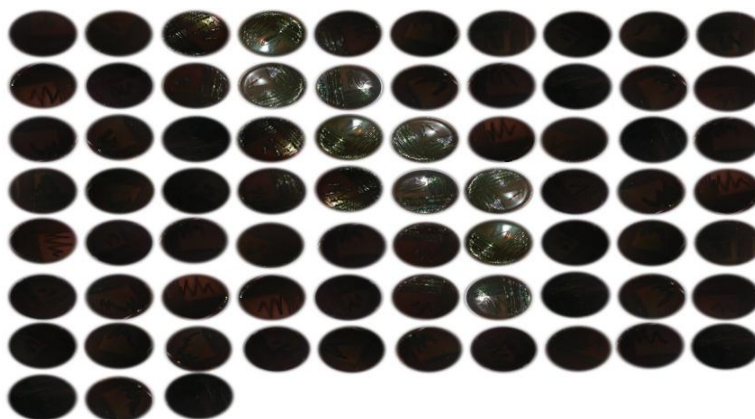


Figure 2. Culture of diarrhea samples on EMB

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is the minimum concentration that can prevent the bacterial growth up to 90%. The results obtained from the minimum inhibitory concentration of hydroalcoholic extract of mint plant are presented in Table 2. The lowest antibacterial concentration of mint extract on *Escherichia coli* was found as 62.5 mg (Table 2).

Table 2. The result of MIC test in different tubes

Tubes No.	Extract Concentration	Mint Extract
1	250	+
2	125	+
3	62.5	+
4	31.25	-
5	15.625	-
6	7.812	-
7	3.906	-
8	1.953	-
9	0.976	-
10	0.488	-

Minimum bactericidal concentration(MBC)

The minimum bactericidal concentration(MBC) is the minimum concentration of extract that prevents bacterial growth up to 99.9%. Since the results obtained for hydroalcoholic mint extracts were positive (+) for mint in tubes 1, 2, and 3, the evaluation of MBC test was not possible.

Agardiffusionwith disk

The results for the diameter of the growth inhibition zone are shown in Table 3. The diameter of the disks used for the hydroalcoholic extract of mint was 10 mm. The hydroalcoholic extract of the mint on the growth of *E. coli* had an inhibitory effect (10 mm). All antibiotics used on *Escherichia coli* bacteria were effective except for ampicillin and tetracycline which showed resistance. The highest effect belonged to ciprofloxacin and gentamicin (40 and 30 mm) and the least effect was related to streptomycin (15 mm).

Table 3.The results ofantibiogram

Antibiotic	Halo Diameter in Mint Plates
Cefixime	20mm
Chloramphenicol	22mm
Nitrofurantoin	25mm
Streptomycin	15mm
Tetracycline	0mm
Nalidixic acid	23mm
Ampicillin	0mm
Ciprofloxacin	40mm
Gentamicin	30mm
Mint Extract	10mm



Figure 3.Results of diffusion disk formint extract

Discussion

The present study was conducted considering the lambs and goat kids diarrhea and important concern given to the public hygiene and animal husbandry economy and also the treatment of the diarrhea infections. It aimed at determining the frequency of *E.coli* in lambs and goat kids with diarrhea in some livestock husbandries in Kazeroun using culture method. With an increase in antibiotic consumption and subsequently the increase in resistance against antibiotics and also different susceptibility to *E.coli* in different areas in the world, herbal drugs are recommended instead of antibiotics use. For this purpose, the effect of hydroalcoholic extract of mint plant was investigated on lambs and goat kids with diarrhea. As the samples, 100 lambs and 100 goat kids with diarrhea were collected from some sheep husbandries in Kazeroun and were put under careful examination. Of the total samples, 39 samples (19.5%) were related to male lambs and 61 (30.5%) belonged to female lambs, 39 samples (19.5%) were male goat kids and 61 (30.5%) were female goat kids. Regarding the results obtained and the forms filled on the history of each animal, it was shown that the highest prevalent degree of diarrhea in males belonged to the lambs in the age group 4-6 days (52.38%) and for female was related to the goat kids in the age group 0-3 days (52.46%).

The frequency of *E.coli* as the cause of diarrhea in male and female lambs were 16 (38.09%) and 21 (36.20%) and in male and female goat kids were 11 (28.20%) and 25 (40.98%), respectively. These frequencies were not consistent with some earlier studies carried out by some other researchers in different regions. Several studies have been conducted on *E.coli* indicating the presence of this strain in animals especially in lambs and also the infection incidence of this strain in the animals' first week of life.

In a study in Kashmir, Vani and his colleagues (2013) stated that 5% of the lambs with diarrhea had ETEC-induced diarrhea, and that no ETEC strain was observed in the healthy lambs (Wani et al., 2003).

In a study conducted on sheep with diarrhea, Shabana (2014) reported that 2.2% of the samples were infected with ETEC strain (Shabana, 2014). In this study investigating the lambs and goat kids with diarrhea, the incidence of pathogenic agents as K99 fimbriae and F41 and STa enterotoxin in enterotoxigenic *E.coli* were 18.04% and 15.78%, while 12.03% of the samples had all three genes together.

The study by Chen and other researchers (2004) in East China showed that the frequency of K99 and F41 in enterotoxigenic *E. coli* was 5% (Chen, 2004). The results obtained by Naciri and his colleagues (1999) in France showed this prevalence as 6.1%. It seems that a part of these differences is related to the natural flora of the ruminants' digestive tract, which increases the amount of these microorganisms in the samples. Moreover, the sensitivity and specificity of the methods used are also different. Some other studies conducted by other researchers in the field show a higher frequency percentage compared to this study and studies mentioned above.

The mint is a plant that has antimicrobial properties (Tassou et al., 2000). The mint plant consists of more than 25 species, including *menthapiperita*, and *menthaspicata* (Hussain et al., 2010). The mint extract contains

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various compounds such as Pulegone, Menton, Poelon, Alpha-Pinene, and Terpinolene (Eidi et al., 2009). The type and percentage of compounds in mint extract are related to factors such as geographical condition, and environmental conditions as temperature, humidity, and light cycle (Ultee et al., 1999).

In the present study, all isolated strains showed resistance against antibiotics as ampicillin and tetracycline, and were susceptible to streptomycin, gentamicin, nalidixic acid, cefixime, ciprofloxacin, nitrofurantoin, and chloramphenicol. The present study indicated that the halo diameter formed by hydroalcoholic extract of mint was due to the low impact of this extract on the *Escherichia coli*.

The use of extracts and essences derived from plants that exhibit antibacterial activities has been considered important issues in recent years (Chitsaz et al., 2007; Naghdi-Badi and Malekizadeh-Tafti, 2003; Naseri, 2003). The germicidal effects of the hydroalcoholic extract of mint plant on *E. coli* are low but it has good bacteriostatic effects.

The minimum bactericidal concentration and the minimum inhibitory concentration of hydroalcoholic extract of mint indicate that the mint extract has low antimicrobial activity against the bacteria under study.

In general, antimicrobial active compounds of extracts are terpenes as eugenol, thymol and carvacrol with a phenolic nature. Particularly, the antimicrobial property of the mint extract examined in Broth and in a Model Food System showed that this property was due to menthol and ketones like isomenthol and careen (Sivropoulou et al., 1996; Tassou et al., 2000).

The results of this study showed that the antibacterial effects of the mint plant were not significant. These findings were in line with those studies conducted in this area. Nourizadeh and his colleagues (2004) investigated the antibacterial effects of peppermint, licorice, oregano, chamomile and thyme on *Helicobacter pylori* (*H. pylori*) using disk diffusion and agar dilution methods. As the findings revealed, the most important effect was related to the peppermint. The MIC for different strains was 200-350 µg/ml, and the halo diameter of the inhibition zone of growth was 12-17 mm (Nourizadeh et al., 2004).

In a study investigating antimicrobial activity of mint extract using agar gel diffusion, Padmini and other researchers (2010) reported that mint extract had a significant effect on *Salmonella typhi* and *Pseudomonas aeruginosa*.

Conclusion

In this study, the MIC and MBC of mint extract were determined to be between 62.5 and 250 mg/ml, respectively. The results showed a low inhibitory and bactericidal effect of the mint plant extract on *Escherichia coli*.

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