An Investigation into the Inhibitory Effect of Hydroalcoholic Extract of *Zataria multiflora Boiss*(Shirazi thyme)on *Escherichia Coli* isolated from calves with Diarrhea in Kazerun

Hosseinizadeh-Ghasemi, A.[1];Nowroozi-Asl, A.[2*];Fattahi, H.[3]

[1]Young and Elite Research Club, Kazerun Branch, Islamic Azad University, Kazerun, Iran; [2]Department of Clinical Sciences, Faculty of Veterinary Medicine, Kazerun Branch, Islamic Azad University, Kazerun, Iran; 3Department of Pathobiology, Faculty of Veterinary Medicine, Kazerun Branch, Islamic Azad University, Kazerun, Iran.

[*]**Corresponding Author:** A. Nowroozi-Asl, Department of Clinical Sciences, Faculty of Veterinary Medicine, Kazerun Branch, Islamic Azad University, Kazerun, Iran, *E-mail:nowroozia@yahoo.com*

[DOR: 20.1001.1.2717378.2017.1.3.3.3]

Abstract

Diarrhea in calves is one of the most prevalent diseases the veterinarians encounter in practice. Diarrhea is responsible for major economic damages in livestock husbandries. *Escherichia coli* (*E.coli*) has been regarded as the main factor that causes calves diarrhea in their first three weeks of life. White diarrhea in calves is caused by certain serotypes of *E.coli*. The excessive use of antibiotics has led to the multiple drug resistance in most bacterial pathogens. The development of drug resistance in pathogens has led to the emergence of new and natural antimicrobial agents.This descriptive cross-sectional study was carried out to investigate the effect of hydroalcoholic extract of Shirazi thyme on 100 samples of calves with diarrhea collected from traditional and industrial livestock husbandries crops in Kazerun during February and March 2016 and April 2017. The enrichment media, Selenite F Broth and Mac Conkey Agar (Mac) were used. To evaluate the lactose fermentation and to determine the identity of isolated bacteria, Eosin Methylene Blue agar(EMB) was used.The antibiogram test also demonstrated that isolated strains were susceptible to most antibiotic groups used in the treatment of diarrhea. In this study, all isolated strains showed resistant against antibiotics as ampicillin and tetracycline, and were susceptible to antibiotics such as streptomycin, gentamicin, nalidixicacid,cefixime, ciprofloxacin, nitrofurantoin and chloramphenicol.The halo diameter formed by hydroalcoholic extract of Shirazi thyme was 8mm,demonstrating a low susceptibility of this bacterium to the hydroalcoholic extract of Shirazi thyme. In general, the findings indicate that the hydroalcoholic extract of Shirazi thyme has few antimicrobial properties and much more studies are required to achieve better results.

Keywords: hydroalcoholic extract, Shirazi thyme, diarrhea, calf, *Escherichia coli*, Kazerun

Introduction

Diarrhea in calves has been an important serious problem and is one of the major causes of economic losses in each husbandry due to their fatality, their inevitable treatment costs and a reduction in calves' growth (Bulgin et al.,1982). Neonatal calf diarrhea is not associated with only an agent but it is a multi-factorial disease.The infections factors are mainly related to *E.coli*,*Salmonella sp*.,rotaviruses and coronaviruses,some protozoan organisms such as cryptosporidium and coccidia parasite(Sharififar et al.,2007). The *E.coli* are the most prevalent cause of diarrhea in calves in the first three weeks of their lives(Ouahouo et al.,2004).

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(Kalemba and Kunicka,2003; Palmer et al.,2001).Concerning the increasing use of antibiotics and consequently the increased antibiotic resistance and different susceptibility to *E.coli* in different parts of the world, the aim of the present study was to determine the prevalence of *E.coli* in diarrhea of calves in a number of traditional and industrialized animal husbandries in Kazerun city through the culture method,utilizing hydroalcoholic extract of Shirazi thyme instead of antibiotics.

Shirazi thyme (*Zataria multiflora Boiss*) is the only species of the genus *Zataria* belonging to mint family (Lamiaceae)found in the southern Iran, Afghanistan and Pakistan and has medicinal and edible properties.Thymus is another genus in the family Lamiaceae (Labiatae)(Shaiq Ali et al.,2000).

This shrub is the only species of Iranian native Thyme which is grown in Isfahan, Lorestan, Khuzestan, Fars, Bushehr, Hormozgan,Baluchestan, Khorasan and Yazd (Karman et al.,2001).

Shirazi thyme extract restricts intrinsic immunity(Islam et al.,2007),inhibiting the growth of some micro organisms such as fungi and bacteria (Shaiq Ali et al.,2000).

Materials and Method

Sampling

[DOR: 20.1001.1.2717378.2017.1.3.3.3]

In this research, 100 samples of faeces swabs from calves (under one month of age) with clinical diarrhea were collected from some husbandries in Kazerun. The swabs were inserted into the tubes containing Selenite F Broth and transferred to a microbiology laboratory at Islamic Azad University, Kazerun 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 (Lotfollahzadeh et al.,2005; Rivas et al.,2003; Rota et al.,2008).

Preparation of hydroalcoholic extract of Shirazi thyme

10 g of thyme crushed leaves were added separately to 100 ml hydroethanol 80% and distilled water and placed in a rotary extractor for 72 hours. The solvent mixture and the plant werethen separated by a filter. The initial extract was derived from a soxhlet extractor, the solution was slowly evaporated for 1 hour at 80 ° C and concentrated extract was obtained.

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 11 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)(Breuer et al.,2001;Cavar et al.,2008;Kim et al.,2005;Yaltirak et al.,2009).

Results and Data Analysis

Of the total samples in this study, 36 samples (36%) were related to male calves and 64 (64%) were related to female calves (Chart 1).

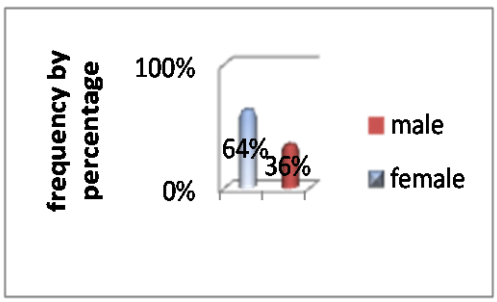

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Chart 1. The sample frequency distribution of the population based on gender in male calves and female calves

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

Age (Day)	0-5	6-10	11-15	16-20
Frequency	17 (17%)	33 (33%)	35 (35%)	15 (15%)

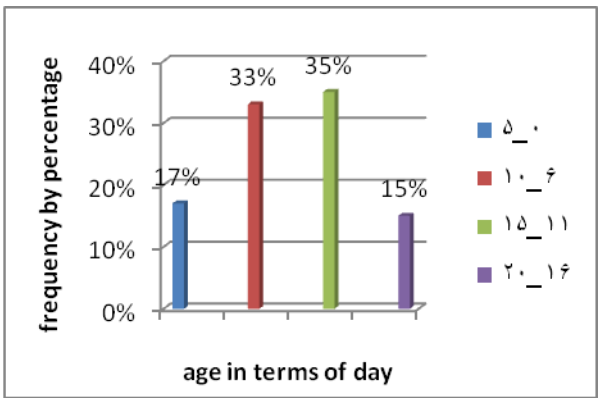

Chart 2.The Frequency by percentage of sample population in terms of age(in male calves and female calves)

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

Table 2. Absolute and relative frequency distribution of the population based on gender in calves

Age(day)	male	famale
0-5	5(13.89%)	12(18.75%

		)
6-10	10(27.77%)	**23(35.93%)**
11-15	14(38.89%)	**21(32.81%)**
16-20	7(19.44%)	**8(12.5%)**
Total	36	64

Table 3. The frequency distribution of *E. coli* population based on month and gender in calves

Month	**Male**	**female**
February-17	4(25%)	11(40.75%)
March-17	7(43.75%)	10(37.04%)
April-17	5(31.25%)	6(22.23%)

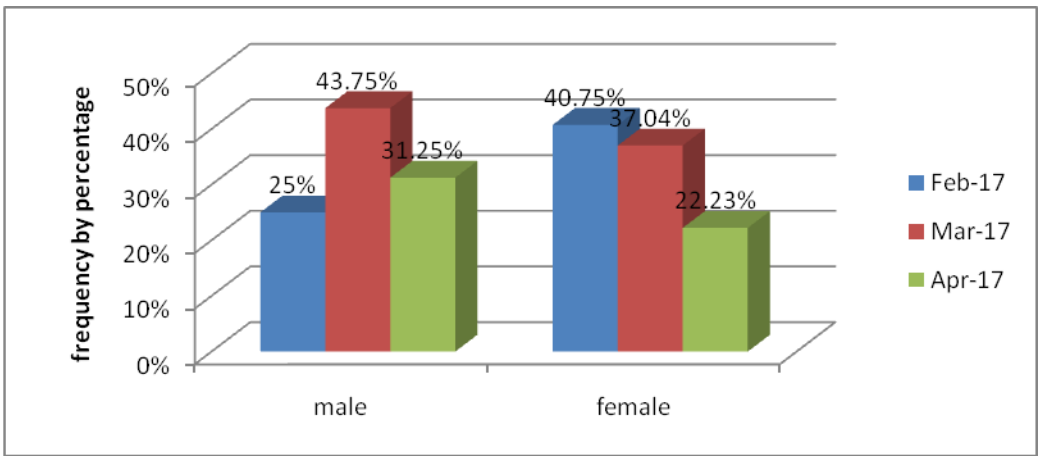

Chart 3.Comparion of *E.coli* contamination rate based on month and gender in male calves and female calves

The calves diarrhea samples were cultured on mechanical culture medium, the bacterial growth was observed on 70 plates while pathologic bacteria did not grow on 30 plates.Then, these bacteria were cultured on EMB,in which 43 (16 males and 27 females) colonies in a bright

metallic green color were grown after 24 hours of incubation at 37℃, indicating the presence of *E.coli* bacteria.

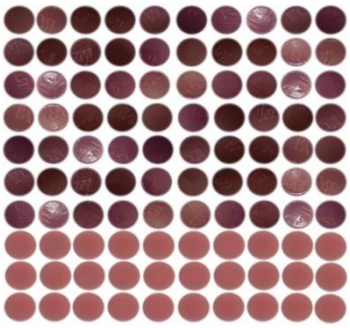

Figure 1. Culture of calves diarrhea samples on a mechanical culture medium

Figure 2. Culture of calves 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 Shirazi thyme plant are presented in Table 4. The lowest antibacterial concentration of hydroalcoholic extract of Shirazi thyme on *E.coli* was found as 12.5 mg/mL (Table 2).

Table 4. The result of MIC test in different tubes

Tubes No.	Extract Concentration	Extract of Shirazi thyme
1	50	+

2	**25**	**+**
3	**12.5**	**+**
4	**6.25**	**−**
5	**3.125**	**−**
6	**1.562**	**−**
7	**0.781**	**−**
8	**0.390**	**−**
9	**0.195**	**−**
10	**0.097**	**−**

Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) is the minimum concentration of extract that prevents bacterial growth up to 99.9%. The evaluation of MBC test was carried out for concentrations 12.5, 25, and 50 mg/mL and the results showed a progressive growth of the bacteria.

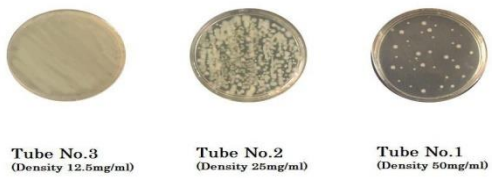

Figure 3. The results of MBC on hydroalcoholic extract of Shirazi thyme

Agar diffusion with disk

The results for the diameter of the growth inhibition zone are shown in Table 3. The hydroalcoholic extract of Shirazi thyme on the growth of *E. coli* showed a low inhibitory effect (8 mm). All antibiotics used on *E. 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 5.The results of antibiotic gram

Antibiotic	**Halo Diameter in Shirazi Thyme Plates**
Cefixime	20±1/52mm
Chloramphenicol	22±1/46mm
Nitrofurantoin	25±1/31mm
Streptomycin	15±1/15mm
Tetracycline	0±./5mm
Nalidixic acid	23±1/63mm
Ampicillin	0±./4mm
Ciprofloxacin	40±2/54mm
Gentamicin	30±2/08mm
Shirazi Thyme Extract	8±./67mm

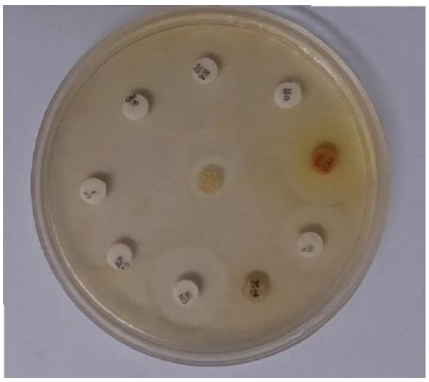

Figure 4. Results of diffusion disk for Shirazi thyme extract

Discussion

In the present study, using 100 samples of calf diarrhea isolated from animal husbandries around Kazerun through culture method, 43 samples (43% of the cases) were *E.coli*. Most calves were Holstein cross bred and were kept for milk production (lactation).

Some species of *E.coli* are naturally found in the intestines of animals and humans. There are about hundreds of species of *E.coli*, most of which are harmful. This bacterium enters the intestines and binds to the intestinal mucosal cells and begins to multiply, and while it is increasing in number, they all begin to release toxin.Toxins caused by bacteria,damage the intestinal mucosa and cause severe abdominal pain and diarrhea. Some *E.coli* serotypes are pathogenic. The isolation of *E.coli* from calves that experience 24 hours of antibiotic treatment, indicates the in effectiveness of antibiotics administered on *E.coli* bacteria or much more damage to the normal microbial flora and the provision of conditions for the colonization of *E.coli* bacteria.Antibiogram is thus necessary for the appropriate and effective treatment of diarrhea *E. coli*. It seems some of the differences are related to the differences in the prevalence of *E.coli* diarrhea in these areas.More over,the sensitivity and specificity of the methods used are also different.

Similar studies in some other areas have reported comparable results with the present research findings. In 1988 in north western Spain, Blanco and his colleagues studieda total of 289 colonies isolated from diarrheic calves and reported that 20.5% of samples were *E.coli* (Fukushima and Seki.,2004).In Japan, Fukushima and some other researchers (2004) investigated 605 cattle (caws and calves) faeces,using culture and PCR method, to search for Shiga toxin-producing *Escherichia coli* (STEC).As they reported, 31 of samples were infected with *E.coli* (Kargar et al.,2006).

Different cases of *E.coli*-induced diarrhea have been reported in Iran. In a study by Shams and his colleagues conducted through using mPCR in Fars province, 5.3% were reported as *E. coli* diarrhea(Sharififar et al.,2007).On the other hand,using agglutination method in Babol and Ghaemshahr, Lotfollahzadeh, together with his colleagues, reported this rate as 1.07% (Nahaei et al.,2007). It seems that some of the disparities are related to the differences in the prevalence rate of *E. coli* diarrhea in these areas.More over,the sensitivity and specificity of the methods used are also different.

[DOR: 20.1001.1.2717378.2017.1.3.3.3]

The tendency to utilize natural antimicrobials, herbs and spices, which are also regarded as traditional food additives and flavors, is increasing (Sindambiwe et al.,1999).This study aimed at determining the minimum inhibitory and bactericidal concentration of hydroalcoholic extract of Shirazi thyme on *E.coli* bacteria and also investigating the effects of these concentrations by this bacterial strain.

The antibiogram test also indicated that the isolated *E.coli* were susceptible to most antibiotic groups used in the treatment of diarrhea. This drug susceptibility was found to be less frequently in traditional animal husbandries compared to the industrial ones.It might be due to the uncontrolled and constant use of a certain drug or the lack of therapeutic doses of antibiotics. Bolgin and colleagues(Goudarzi et al.,2006) also observed that *Salmonella sp*. and *E.coli* were resistant to certain drugs in laboratory conditions, especially those used to treat calves diarrhea. In this study *E.coli* isolated from dairy farms from around Kazerun was not susceptible to tetracycline and ampicillin.

Breuer's(2001) studies in America (Gandomi et al.,2009), Radu(2001) in Malaysia, Kim(2005), Salder (Nagy and Fekete,2005) in Korea, Kargar (2005) in Jahrom, Nahaie (2007) in Tabriz also reported isolated strains resistance to ampicillin which were in line with findings of this present study.On the other hand, kanamycin resistance in 1996 in Japan(Nagy and Fekete.,2005),erythromycin resistance in 2005 in Korea(Nagy and Fekete.,2005)and penicillin resistance in 2005 in Jahrom have been reported,the reason for this disparities could be due to the climatic conditions.

Shirazi thyme vulgaris (*Zataria multiflora*) has a medicinal,spice use belonging to the family Laminaceae, which is native to Iran, Pakistan and Afghanistan (Sindambiwe et al.,1999).

This plant known as Shirazi thyme as been used as a flavoring in many Iranian foods and has antimicrobial and antioxidant effects (Shams et al.,2010).The antimicrobial activity of different species of thyme is related to the presence of its phenolic compounds, such as thymol, carvacrol (Saei-Dehkordi et al.,2010; Shams et al.,2010) and gamma-terpinene (Saei-Dehkordi et al.,2010).According to the studies conducted by Saei dehkordi and his colleagues, gram-negative bacteria are the most resistant and yeasts are the most sensitive microorganisms against the antimicrobial activity of Shirazi thyme, and gram-positive bacteria show a moderate sensitivity to that (Shafiee and Javidnia.,1997).ParsaeiMehr and some other researchers (2009), for instance,

investigated the effects of various concentrations of *Zataria multiflora* and nisin essential oil, alone and in combination,on alpha-hemolysin production and entero toxin production of *Staphylococcus aureus* in laboratory culture (Rojhan.,2000).

According to the information derived,the antibacterial properties of *Zataria multiflora* in different infections have been evaluated in vitro and clinical tests on various animal species and also in some human cases. These studies have shown that Shirazi thyme demonstrated antimicrobial effect on various gram-positive and gram-negative bacteria including *Streptococcus agalactiae* (Andrews et al.,2004*), Streptococcus pyogenes* (Bagamboula et al.,2004), *Staphylococcus aureus* (Andrews et al.2004; Blanco.1988), *Salmonella typhimurium* (Blanco et al.,1988),*Listeria monocytogenes*(Mousavi et al.,2009),*Pseudomonas aeruginosa* (Bagamboula et al.,2004), and *E.coli* (Andrews et al.,2004; Bagamboula CF et al.,2004; Mousavi et al.,2009). In the recent study, antimicrobial activity of *Zataria multiflora* was investigated on *E.coli* isolated from calves with diarrhea. The results showed that the extracts of these plants on the culture medium of *E.coli*,especially at concentrations 50 and 25 mg / ml, had moderate inhibitory effects.In some other studies,the investigation into the antimicrobial activity of *Zataria multiflora* on *E.coli* isolated from mastitis in caws (Andrews et al.,2004) and entero haemorrhagic (Ayepola and Adeniyi. Journal of Applied Sciences Research.2008) also indicated the antibacterial effect of these plants on the growth of *E.coli*.Therefore, it appears that Shirazi thyme, irrespective of the origin of *E. coli* bacteria, can act as an inhibitor for *E. coli* growth in different species. Considering that the most important active ingredients in these plants are thymol and caracrole (Aksoy et al, Archives of Oral Biology 2006; Bart, Int. J. Food Mic. 2004), it seems that the antibacterial properties of these plants is related to the effects of these compounds.

Rahnema and his colleagues(2008)investigated the antimicrobial effects of Shirazi thyme essential oil, nisin, and in combination against *Listeria monocytogenes* in the brain heart infusion broth (BHI).The results showed that the Shirzi thmey essentioal oil alone had antimicrobial effects on *Listeria monocytogenes*(MIC:9.5,MBC:19µg/ml).The effect of *Zataria multiflora* essential oil and nisin reduced MIC and MBC (MIC: 2.1, MBC: 4.2 µg / ml). Shirazi thyme essential oil has inhibitory effects on *Listeria monocytogenes*. These effects increased noticeably in conjunction with nisin (Parsaeimehr et al.,2010; J. Ethnopharmacol.2008).

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Generally, the higher the amount of phenolic substances in the essential oil, the greater is its antibacterial properties against the pathogens. The antimicrobial activity of various species of thyme is related to its phenolic compounds, such as carvacrol and thymol, and then linalool and paracymene (Zahraie et al.,2005).

The acidity nature of the hydroxyl group in thymol and carvacrol and the participation of the hydroxyl group in the formation of hydrogen bonds is a significant reason for the antimicrobial activity of these compounds (Moaveni,2009). In general, the antimicrobial activity of the essential oils derived from the reaction between their active groups (e.g. Hydroxyl group) and microorganism cell components (ISIRI 6806/1: 2005; Moaveni,2009).

Probably the mechanism of the effect of these phenolic compounds includes the following: disorders in cytoplasmic membrane function, disturbance in protonic movement and electrical current, and coagulation of cellular contents (Dakhili et al.,2006; Park et al.,2002).Prevention of toxins production and activity by plant extracts can be performed indirectly as a result of a disorder in factors including transcription and translation or through direct toxins deactivation.

This multiple natural effect of plant extracts makes them much more preferable than many other common antimicrobials, affecting on only one single target. On the other hand, the naturalness of plant extracts has led consumers to prefer them against chemical and synthetic antimicrobials (Erdogrul,2002).

Conclusion

Most of these studies have been carried out in vitro culture medium. Therefore, conducting and continuing these studies in vivo models and investigating the impact of effective compounds in extracts on bacteria in order to create novel strategies for biological control of these bacteria without antibiotics use seems to be necessary. It is because antibiotics disrupt the bacterial ecosystem and the emergence of antibiotic-resistant strains that are of major concern in public health.

Due to the abundant and indigenous nature of the Shirazi thyme in Iran and its easy and inexpensive access and also consumers' great acceptance, and on the other hand, because it leads to bacterial death in very low concentrations and that it inhibits the production of toxin from this bacterial strain, this study can be an introduction to the use of this extract in order to provide an

[DOR: 20.1001.1.2717378.2017.1.3.3.3]

affordable and cost-effective source, and ultimately, an attempt to achieve antimicrobial properties.

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[DOR: 20.1001.1.2717378.2017.1.3.3.3]