Antimicrobial activity of Zatacin against bacterial diarrheal pathogens

Mahboubi, M.[1*], Falsafi, T.[2], Torabi Goodarzi, M.[3]

[1]*Department of Microbiology, Biology Center, Medicinal Plant Center of Barij, Kashan, Iran*

[2]*Department of Biology, Faculty of Basic Sciences, Alzahra University, Vanak, Tehran, Iran*

[3]*Razi Vaccine & Serum Research Institute (Central Area branch), Arak, Iran*

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Correspondence
Mahboubi, M.
Department of Microbiology, Biology Center, Medicinal Plant Center of Barij, Kashan, Iran
Tel: +98(86) 44465112
Fax: +98(86) 44465187
Email: mahboubi1357@yahoo.com

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Abstract:

BACKGROUND: Calf diarrhea is an important disease that is caused by different pathogens including bacteria, virus and parasites and is associated with economic losses. **OBJECTIVES:** In this study, we evaluated the antibacterial activities of Zatacin (*Z. multiflora* aerial part ethanol extract), colistin, co-Trimoxazole and enrofloxacin against clinical isolates of *Salmonella* sp, *E. coli* and *Campylobacter* sp isolated from diarrheic calves. **METHODS:** Disc diffusion method and microbroth dilution assay were used for antimicrobial evaluation. **RESULTS:** In disc diffusion method, the antibacterial activity of Zatacin increased dose dependently. The sensitivity of different isolates of *E. coli*, *Salmonella* sp and *Campylobacter* sp to Zatacin was almost the same. The antibacterial activity of Zatacin was lower than that of enrofloxacin but it was higher than co-Trimoxazole and colistin. The means of MIC values of Zatacin for *E.coli* were higher than that of *Campylobacter* sp and *Salmonella* sp but its means of MBC values for *E. coli* were lower than that of two other bacteria. **CONCLUSIONS:** Zatacin can be used as an antimicrobial agent in treatment of infectious causes of calf scours instead of antibiotics with undesired adverse effects on animal and humans.

Introduction

Salmonella sp, *Campylobacter* sp and *Escherichia coli* are the specific bacterial agents of dairy and beef calf diarrhea. Diarrhea in dairy and beef cattle is the leading cause of mortality and morbidity in cattle industries. Diarrhea is the result of increase in bacterial colonization 5 to 10000-fold in duodenum, jejunum and ileum of cattle (Isaacson et al., 1978). Increase in colonization of small intestine has been associated with impaired glucose, xylose and fat absorption (Youanes and Herdt, 1978). Diarrhea is caused in all species of domestic animals including young animals, pregnant and lactating animals and bacteremia, acute septicemia, abortion, arthritis and respiratory diseases may occur.

The main therapies in calf diarrhea are fluid therapies, antibiotics and nursing care. The use of antibiotics in treatment of the disease carries the risk of bacterial flora and developing drug resistance to important anti-bacterial agents such as colistin, enrofloxacin and co-trimoxazole.

Zatacin is a natural herbal product made from ethanol extract of *Zataria multiflora* Boiss aerial part for treatment of calf diarrhea. *Z. multiflora* is an aromatic plant belonging to Labiatae family and has been traditionally used for treatment of infectious diseases. There are many researches on their pharmacological activity as its traditional uses include antinociceptive effects (Hosseinzadeh et al., 2000; Jaffary et al., 2004; Ramezani et al., 2004), anti-inflammatory effects (Hosseinzadeh et al., 2000, Ashtaral-Nakhai et al., 2007), immune stimulation (Shokri et al., 2006), antioxidant activity (Sharififar et al.,2007; Babaie et al., 2007), and their antimicrobial activities (Misaghi and AkhondzadehBasti, 2007; Sharififar et al., 2007; Fazeli et al.,2007; Moosavy et al., 2008; Khosravi et al., 2008; Mahboubi and Ghazian Bidgoli, 2010). The aim of this investigation was to evaluate the in vitro antimicrobial activities of Zatacin against clinical isolates of *Escherichia coli*, *Salmonella* sp. and *Campylobacter* sp from diarrhea in calves.

Materials and Methods

Disc antibiotics: Co-Trimoxazole (Sulpha/Trimethoprim) COT 25 mcg (23.75/1.25 mcg); Enrofloxacin EX 10 mcg, Colistin (Methane Sulphonate) Cl 10 mcg; Co-Trimoxazole Ezy MIC Strips (COT 0.016-256 mcg ml^{-1}), Enrofloxacin powder, Colistin Ezy MIC Strip (Cl 0.016-256 $mcgml^{-1}$) were provided by HiMedia Laboratories Pvt. Ltd.

Bacterial strains: *Salmonella* sp (12 clinical isolates), *Campylobacter* sp (12 clinical isolates) and *Escherichia coli* (12 clinical isolates) were isolated from clinical cases of diarrhea in newborn calves. The bacterial isolates were confirmed by biochemical tests. All strains were cultured on nutrient agar and incubated in suitable condition at 37 ºC overnight. 1-2 colonies of each strain were dissolved in normal saline and turbidity was adjusted to 0.5 McFarland spectrophotometrically (Optical density 600 was 0.6).

Disc diffusion assay: The antibacterial activities of Zatacin and antibiotic discs (co-trimoxazole, enrofloxacin, colistin) were evaluated by disc diffusion assay. The above bacterial suspensions were cultured on Muller Hinton Agar by sterile swab and blank discs or antibiotic discs were put on cultured plates. The different dilutions of Zatacin were put on blank discs (5, 10, 15 and 20 µl). The plates were incubated at 37 ºC overnight and then the diameters of inhibition zone in millimeter (in triplicate) were measured. Inhibition zone diameters were expressed as millimeter ±standard deviation (mm±SD) (Mahboubi et al., 2015).

Antibacterial activity of Zatacin by microbroth dilution assay and E-test: One milliliter of Zatacin contained 0.63 mg phenolic compounds (thymol and carvacrol). A serial concentration of Zatacin was prepared in purified water (15-0.015 mg ml^{-1}). 100 µl of each serial dilution was added to each well of 96 micro plates. Bacterial suspension was diluted in broth medium (Muller Hinton Broth or *Brucella* broth) to reach the 1×10^6 $CFUml^{-1}$. 100 µl of diluted bacterial suspension was added to each well. The plates were shaken for 30 min and then incubated at 37 ºC overnight. Then, the first well with no growth was determined as MIC value, and the well that showed no growth on solid media as MBC value. 100 µl of tetrazolium chloride (0.02%) was added to each well to confirm the results. All experiments were done in triplicate. The

Table 1. The inhibition zone diameters (mm) of Zatacin against calf diarrheal pathogens by disc diffusion assay. * = µl/disc,** based on the observed means at level 0.05, there were seven subsets, a was the most sensitive compound followed by b,c,…..

	E. coli		*Salmonella*		*Campylobacter*		total
	Means±SE	Min-Max	Means±SE	Min-Max	Means±SE	Min-Max	Means**
Zatacin* (5)	7.57±0.25	6.1-10.4	6.8±0.14	6.1-8.6	6.2±0.05	6.1-8.1	6.74^{g}
Zatacin* (10)	10.5±0.43	6.45-14.8	10.9±0.39	7.1-18.8	9.2±0.16	6.1-17.6	10.2^{f}
Zatacin* (15)	14.02±0.6	0.1-20.15	16.2±0.34	12.1-20.9	14.6±0.47	8.1.19.90	14.9^{d}
Zatacin* (20)	17.9±0.67	11.5-22.1	20.6±0.39	16.7-25.4	19.9±0.3	15.4-24.8	19.6^{b}
Colistin	16.5±0.19	14.8-19.1	16.1±0.45	12.6-21.8	16.9±0.36	11.7-21.4	16.6^{c}
co-Trimoxazole	15.1±0.06	6.1-30.7	15.7±2.01	6.1-30.6	9.53±1.09	6.1-33.9	13.02^{e}
Enrofloxacin	28.5±1.2	12.3-35.2	29.2±0.58	23.1-34	28.2±0.78	13.2-32.9	28.6^{a}

Table 2. The antimicrobial activity of antimicrobial agentsagainst clinical isolates of calf diarrhea by micro-broth dilution assay and E-test. The reported MIC and MBC values have been expressed as the means±SE of MIC or MBC against clinical isolates.

	Zatacin (mg/ml)		Co-Trimoxazole(µg/ml)		Enrofloxacin(µg/ml)		Colistin(µg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	0.60±0.1	1.1±0.05	1.48±1.26	1.48±1.26	1.25±0.45	2.3±0.9	0.85±0.06	0.85±0.06
Salmonella	0.445±0.0	5.2±0.5	4.2±3.9	8.4±4	0.29±0.05	0.5±0.12	1.2±0.12	1.2±0.12
Campylobacter	0.445±0.0	2.2±0.4	192±63	192±63	0.88±0.4	1.7±0.9	1±0.17	1±0.17

results were analyzed by SPSS 17.0 (SPSS for Windows; SPSS Inc, Chicago, IL) and expressed as means±SE (Mahboubi et al., 2015). The MIC values of enrofloxacin were determined by micro broth dilution while the MIC values of colistin and co-trimoxazole were determined by disc diffusion method with their E- Strips.

Results

The antibacterial evaluation of Zatacin (5, 10, 15 and 20 µl/disc) against 36 clinical isolates of *E. coli*, *Salmonella* sp and *Campylobacter* sp. in comparison with colistin, co-trimoxazole and enrofloxacin by disc diffusion method showed that the measured inhibition zone diameters for higher concentration of Zatacin against all strains were larger than the lower concentrations. On the other hand, the antibacterial activity of Zatacin increased dose dependently. The sensitivity of different isolates of *E. coli*, *Salmonella* sp and *Campylobacter* sp. to Zatacin was almost the same but higher concentrations of Zatacin had larger inhibition zone diameters against clinical isolates of *Salmonella* sp. The inhibition zone diameters of enrofloxacin on clinical isolates of *E. coli*, *Salmonella* sp and *Campylobacter* sp. were larger than the inhibition zone diameters of Zatacin even in concentration of 20 µl/disc, while the inhibition zone diameters of co-trimoxazole on clinical isolates of *E. coli* and *Salmonella* sp were equal to the inhibition zone diameters of Zatacin (15 µl/disc). The inhibition zone diameters of co-trimoxazole on clinical isolates of *Campylobacter* sp were comparable to 10 µl/disc Zatacin. The inhibition zone diameters of colistin on 36 clinical isolates of *E. coli*, *Salmonella* sp. and *Campylobacter* sp. were comparable to 15 µl per disc Zatacin (Table 1).

Antimicrobial activity evaluation of Zatacin against the clinical diarrheal bacteria by micro broth dilution assay revealed that the mean of MIC values for different clinical isolates of *E. coli* were higher than this amount for *Salmonella* sp. and *Campy-*

lobacter sp. The mean of MBC for clinical isolates of *Salmonella* sp were two-fold toward *Campylobacter* sp (2.15 versus 5.2 mg/ml), while the mean of MBC value for clinical isolates of *E. coli* was 1.13 mg/ml. In other words, with respect to MIC and MBC values of Zatacin against different clinical isolates of bacteria, Zatacin had a higher bactericidal effect against clinical isolates of *E. coli*, followed by *Salmonella* sp and *Campylobacter* sp (Table 2).

Discussion

Calf diarrhea is the most important cause of death in dairy and beef calves. *E. coli* is the single most important cause of bacterial diarrhea in most newborn calves and is transmitted from environment. *Salmonella* sp infects the calves at six days of age or older. Antibiotic treatment of infected calves with *salmonella* sp damages the *Salmonella* organism and releases a toxin that will poison the animal, resulting in endotoxic shock. The administration of electrolyte solutions and antimicrobial agents plays an important role in treatment of calf diarrhea. Regardless of the appearance of the antimicrobial resistant bacteria among calves, the use of some antibiotics has been prohibited in food producing animals due to the occurrence of non-dose related anemia and concerns regarding the mutagenicity or carcinogenicity of related product in humans (Constable, 2004). The aim of this study was to evaluate the in vitro efficacy of herbal preparation with the commercial name of Zatacin (BarijEssence Pharmaceutical Co. Kashan, Iran) against bacterial diarrhea infection. Zatacin is made from the ethanol extract of *Z. multiflora* flowering aerial part. Some studies have evaluated the antimicrobial effects of *Z. multiflora* ethanol extract against different kinds of microorganisms (Owlia et al., 2006; Fazeli et al., 2007). It has been reported, *Z. multiflora* aerial parts ethanol extract (80%) with 21% extract yield exhibited the antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris* and *Shigella flexeneri* with MIC values 0.4% (v/v), while *S. typhi* was less sensitive than others with MIC value 0.8% (Fazeli et al., 2007).

The antimicrobial activity of *Z. multiflora* ethanol extract against *S. aureus* ATCC 25923 was %25 (Owlia et al., 2006). Therefore, hydro alcoholic extract of *Z. multiflora* aerial parts exhibited the antibacterial activity against Gram positive and Gram negative bacteria.

Furthermore, *Z. multiflora* aerial part ethanol extract with LD_{50} 3.47 g/kg has shown the anti-inflammatory effect against acute and chronic inflammation (Hossein zadeh et al., 2000).

In this investigation we evaluated the antibacterial activity of *Z. multiflora* aerial part ethanol extract (Zatacin) against clinical isolates of *E. coli*, *Salmonella* sp. and *Campylobacter* sp from calf diarrheal infection. In disc diffusion method, the inhibition zone diameters of Zatacin were lower than that of enrofloxacin, while Zatacin (20 µl/disc) had higher inhibition zone diameters than that of colistin and co-Trimoxazole. *Campylobacter*sp and *Salmonella* sp had higher inhibition zone diameters for Zatacin than that of *E. coli*. Also, in micro broth dilution assay, with respect to the means of MIC values, clinical isolates of *E. coli* had less sensitivity to Zatacin than *Campylobacter* sp and *Salmonella*sp (0.601 versus 0.445 mg/ml), but in evaluating the MBC values, *E. coli* exhibited more sensitivity to

Zatacin than *Campylobacter*, and *Salmonella* sp.

Z. multiflora contains alkanes, fatty acids, phytosterols, hydroxycinnamic acid, flavonoids, tannins, resins and saponins (Sajed et al., 2013). Furthermore, Zatacin has been standardized by thymol and carvacrol. Thymol and carvacrol as terpenoid compounds of *Z. multiflora* essential oil have high antimicrobial activity (Mahboubi and Ghazian Bidgoli, 2010). The higher antibacterial activity of thymol than carvacrol was reported against *E. coli* (Bassolé et al., 2010). Thymol disrupts the outer and inner membranes and interacts with membrane proteins and intracellular targets (Xu et al., 2008), periplasmic enzymes (Juven et al., 1994). The synergistic activity of thymol and carvacrol has been reported (Zhou et al., 2007). Therefore, the antimicrobial activity of Zatacin against bacterial infections involved in calf diarrheal diseases may be related to the components that are found in *Z. multiflora* aerial parts ethanol extract and especially to thymol and carvacrol or synergistic effects of compounds. Regardless of its antimicrobial activity, Zatacin may have antinociceptive (Hosseinzadeh et al., 2000; Jaffary et al., 2004; Ramezani et al., 2004), anti-inflammatory (Hosseinzadeh et al., 2000, Ashtaral Nakhai et al., 2007), and immune modulating effects (Shokri et al., 2006).

Conclusion: Therefore, Zatacin may be used as a natural antibacterial agent in treatment of calf diarrheal diseases that are caused by Gram negative bacteria such as *E. coli*, *Salmonella* sp or *Campylobacter* sp instead of chemical antibiotics with no reported side effects for human and calves. Furthermore, it is essential to demonstrate its efficacy in farms.

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فعالیت ضدمیکروبی زاتاسین در مقابل پاتوژن‌های باکتریایی عامل اسهال گوساله

محدثه محبوبی[1*] طاهره فلسفی[2] مجید ترابی گودرزی[3]

۱) گروه میکروبیولوژی، مرکز علوم زیستی، مرکز تحقیقات گیاهان دارویی باریج، کاشان، ایران
۲) گروه علوم زیستی، دانشکده علوم پایه دانشگاه الزهرا تهران، تهران، ایران
۳) موسسه سرم سازی و واکسن سازی رازی (ناحیه مرکزی)، اراک، ایران

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چکیده

زمینه مطالعه: اسـهال گوساله بیماری مهمی است که توسـط انواع مختلفی از پاتوژن‌های باکتریایی، ویروسی و انگلی ایجاد می‌شود که با ضرر و زیان اقتصادی همراه می‌شود. **هدف:** در این مطالعه ما فعالیت ضدمیکروبی زاتاسین (عصاره اتانولی اندام هوایی آویشن شیرازی)، کولیستین، کوتریموکسازول و انروفلوکساسین را در مقابل ایزوله‌های بالینی سالمونلا، شرشیا کلی و کمپیلوباکتر جدا شده از گوساله‌های بیمار مبتلا به اسهال بررسی کردیم. **روش کار:** برای ارزیابی فعالیت ضدمیکروبی از روش دیسک دیفیوژن و میکروبراث دایلوشن استفاده شد. **نتایج:** در روش انتشار در محیط آگار، فعالیت ضدباکتریایی زاتاسین به طریق وابسته به دوز افزایش یافت. حساسیت ایزوله‌های بالینی مختلف سالمونلا، شرشیا کلی و کمپیلوباکتر به زاتاسین یکسان بود و فعالیت ضدباکتریایی زاتاسین از انروفلوکساسین پایین‌تر بود ولی این تأثیر بالاتر از کوتریموکسازول و انروفلوکساسین بود. متوسط مقادیر MIC برای زاتاسین در مقابل *E. coli* از کمپیلوباکتر و سـالمونلا بالاتر بود، ولی متوسـط مقادیر MBC زاتاسین در مقابل ایزوله‌های بالینی *E. coli* از دو باکتری دیگر کمتر بود. **نتیجه‌گیری نهایی:** زاتاسین می‌تواند به عنوان یک عامل ضدمیکروب در درمان عوامل عفونی اسهال گوساله به جای انواع آنتی بیوتیکی دارای عارضه جانبی برای حیوان و انسان مورد استفاده قرار گیرد.

واژه‌های کلیدی: گوساله، اسهال، آویشن شیرازی، زاتاسین، ضدباکتری

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*) نویسنده مسؤول: تلفن: ۴۴۴۶۵۱۱۲ (۸۶)۹۸+ نمابر: ۴۴۴۶۵۱۸۷ (۸۶)۹۸+ Email: mahboubi1357 @yahoo.com