The Pharma Innovation Journal 2021; 10(8): 1172-1176

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(8): 1172-1176

www.thepharmajournal.com
Received: 01-05-2021
Accepted: 08-06-2021

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In-vitro and *In-vivo* evaluation of antibacterial activity of ethanolic and aqueous leaf extract of *Murraya koengii* in mice

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Abstract
Infectious diseases are the second leading cause of death worldwide, killing almost 50,000 people every day. The world wide emergence of multidrug resistant *Escherichia coli* and many other β- lactamase producers has become a major therapeutic problem (Khan *et al.*, 2004) [3]. The development of bacterial resistance to presently available antibiotics has necessitated the search for the new antibacterial agents. Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. In rural and backward areas of India, several plants are commonly used as herbal medicine for treatment of infectious diseases. *Murraya koengii* Linn commonly found plants were screened for potential antibacterial activity. The present study was conducted to evaluate the *In-vitro* and *In-vivo* evaluation of antibacterial activity of ethanolic and aqueous leaf extract of *Murraya Koengii* in mice. *In-vitro* activity of ethanolic and aqueous leaf extract of *Murraya koengii* at concentration of 50, 100 and 200mg/ml was tested against *E. coli* and *Staphylococcus aureus* using agar well diffusion method and was compare with penicillin and gentamicin. Ethanolic leaf extracts showed higher inhibitory activity compared to aqueous extract at 50 and 100mg/ml concentration. MIC value of *Murraya Koengii* was found to be 2.5 and 5mg respectively. Ethanolic extract of *Murraya koengii* effectively inhibited growth of *E. coli and staphylococcus aureus* and significantly reduced the mouse mortality.

Keywords: Antibacterial, *Murraya koengii*, *E. coli*, *Staphylococcus aureus*, mice

Introduction
Infectious diseases are the leading cause of death world-wide, killing almost 50,000 people every day. Infections due to a variety of bacterial etiologic agents such as pathogenic *Escherichia coli, Pseudomonas aeuriginosa, staphylococcus aureus spp, salmonella spp, vibrio cholerae, shigella spp, Klebshiella spp, Campylobacter* spp are most common. (Parmar and Rawat 2012) [3]. Antibacterial agents provide the main basis for the therapy of microbial (bacterial and fungal) infections. The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens and has further complicated the treatment of infectious diseases. The world wide emergence of multidrug resistant *Escherichia coli* and many other β- lactamase producers has become a major therapeutic problem (Khan *et al.*, 2004) [3].

Plants form an integral part of life in many indigenous communities as a readily and cheaply available alternative to allopathic medicines. Such plants have been found to cure urinary tract infections, gastrointestinal disorders, respiratory diseases and cutaneous infections (Somchit *et al.*, 2003) [4], caused by bacteria often known to resist various classes of conventional antibiotics. Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Traditional medicine is in practice for many centuries by a substantial proportion of the population.

Murraya koenigii Linn member of *Rutacea* family is deciduous to semi green aromatic tree found throughout India and mainly cultivated for its aromatic leaves. Leaves are used as condiment in the preparation of curry powder, pickle, chutney, sausages and seasonings and also used in Ayurvedic medicine. Traditionally it is used as analgesic, febrifuge, stomachic, carminative and for the treatment of dysentery and skin eruptions. The leaves are rich in monoterpenoids and sesiquiterpenoids which exhibit antifungal activities. Minor furano-coumarins are also reported from seeds (Malwal and Serin, 2011). It is reported to possess anti-diabetic, antioxidant, anti-inflammatory, hepatoprotective and hypolipidemic activities (Das *et al.*, 2012) [1]. Considering this present study was planned to evaluate the *in-vitro* and *in-vivo* antibacterial activity of *Murraya koengii*.

Material and Methods

Collection of plant material and Preparation of extract

The leaves of *M. koenigii* L and *was* collected from their natural habitat from Shirval village, Satara, Maharashtra. The plant materials were identified by Botanist from Pune University. Each plant samples were carefully dried in laboratory at room temperature. All the plant samples were separately pulverized to fine powder with mixer grinder and are kept in air tight container at room temperature. The powdered leaves of *Murraya koenigii Linn* was extracted with ethanol using Soxhlet extraction apparatus and extractability percentage was determined as per the method suggested by Rosenthaler (1930) [6].

Bacterial strains

Standard strain of Gram positive bacteria *(Staphylococcus aureus)* and Standard strain of Gram negative *(Escherichia coli)* was used for antibacterial studies & was obtained from MTCC, Chandigarh (No.2940 & 739) & Department of Veterinary Microbiology, KNPVC, Shirval.

Drugs

Gentamicin susceptibility test discs 10mcg/disc ('Gentamicin Antibiotic disk) Batch No.0000183307 and manufactured by Sterile Specialties India HI Media Laboratories Pvt. Ltd. purchased from Omkar traders was used for Antibacterial sensitivity test.

Penicillin susceptibility test discs 10 units/disc ('Penicillin antibiotic disk) batch No. 0000182528 manufactured by Sterile Specialties India, HI Media Laboratories Pvt. Ltd. purchased from an Omkar traders was used for Antibacterial sensitivity test.

Preparation of Inocula

The bacterial strains were maintained in nutrient agar (HI media) at 35 ^{0}C anaerobic conditions. All the organisms were sub cultured every 2 weeks before testing. The bacterial inoculums were prepared & cultivated on Nutrient broth for 12h at incubator temperature of 37 ^{0}C. The microbial cultures were serially diluted (10 fold increment) in sterile broth to obtain the cell suspension of 1.0 x 10^5 CFU/ml. To standardize the inoculums density for test, $BaSo_4$ turbidity standard equivalent to 0.5 McFarland standards was used. A 0.5 McFarland standard was prepared as described in NCCLS of Sulphuric acid was prepared by adding 1ml concentration Sulphuric acid to 99ml of water & mixed well. A 1.175% w/v solution of barium chloride was prepared by dissolving 2.35gm of dehydrated barium chloride ($Bacl_2.H_20$) in 200ml of distilled water. To make the turbidity 0.5ml of the barium chloride solution was added to 1% 99.5ml Sulphuric acid & mixed well. A small volume of those turbid solutions was transferred to a screw capped tube of the same type as used for preparing control inoculate & stored in the dark at room temperature. Inocula were obtained from an overnight agar culture of the test organism. Inoculum prepared by taking top of each colony was touched with a sterile loop and growth was transferred into a tube containing 4 to 5 ml normal saline. The broth culture achieved the turbidity of the 0.5 McFarland standards. This result suspension containing approximately 1-2 x10^8CFU/ml. The turbidity of actively growing broth culture was adjusted with sterile broth to turbidity comparable to that of the 0.5 McFarland standards.

Table 1: *In-vitro* antibacterial activity of *Murraya koenigii L*

Sr. No.	Antibacterial activity	Antibacterial activity of different dose		
		50 µg/ml	100 µg/ml	200 µg/ml
	Gentamicin + E. Coli	Alcoholic leaf extract of *M. koenigii Linn+ E. Coli*	Alcoholic leaf extract of *M. koenigii Linn + E. Coli*	Alcoholic leaf extract of *M. koenigii Linn+ E. Coli*
	Gentamicin + E. Coli	Aqueous leaf extract of *M. koenigii Linn + E. Coli*	Aqueous leaf extract of *M. koenigii Linn + E. Coli*	Aqueous leaf extract of *M. koenigii Linn + E. Coli*

The antibacterial activity of ethanolic & aqueous extract of *Murraya koenigii (Linn)* leaves was tested against E. coli and *S. aureus* by agar-well diffusion method as described by Perez *et al.*, 1990 and Bauer *et al.*, 1966 with minor modifications. MIC of *Murraya koenigii* was determined by broth dilution method described by Miles and Mistra method. Nutrient broth was used to determine MIC.

Experimental Design

The mice of either sex with average weight 15-20gm procured from Institute of Bioscience, Pune, were used in present investigation. The experiment protocol was approved by (IAEC) Institutional animal ethic committee of the college. The experimental protocol met the guidance as per the recommendation of the committee for the purpose of Control & Supervision on Experiments on Animals (CPCSEA), Ministry of social justice & Empowerment, Govt. of India, New Delhi. Test animals were housed in a polypropylene cage covered with a stainless steel wire mesh and a paddy husk bed, with adequate provision for feed and water. Test animals were maintained on commercial feed manufactured by Amrut Laboratory Animal feed, Pranav Agro Ltd., Sangli. The mice were housed in clean polypropylene cages, under controlled environments conditions temperature (18-25°C), relative humidity (50-70%), 12:12 light: dark cycle and other micro and macro environment conditions as suggested by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India..

Table 2: *In-vivo* antibacterial activity of Murraya koengii ethanolic extract of leaf in mice

Sr. No.	No. of animals in each group	Group
1.	6	Group I: Disease control.
2.	6	Group II: Positive control (infected with bacterial suspension *E. coli* 1x10^9 CFU/ml).
3.	6	Group III Positive control (infected with bacterial suspension *S. aureus* 1x10^9 CFU/ml).
4.	6	Group IV: Treatment group infected with *E. coli* 1x10^9 CFU/ml received 5mg/kg ethanolic extract of *Murraya koenigii* Linn.
5.	6	Group V: Treated group infected with *S. aureus* 1x10^9 CFU/ml received 5mg/kg ethanolic Extract of *Murraya koenigii* Linn.
6.	6	Group VI: Treated group infected with *E. coli* 1x10^9 CFU/ml received 0.5mg/100gm Gentamicin

Results

The ethanolic extract of *Murraya koenigii Linn* at the concentration of 50µl, 100 µl and 200 µl (mg/ml) showed inhibitory activity against *Staphylococcus aureus*, zone of diameter were 15.66 ± 0.88, 17.33 ± 0.66 and 17.33 ± 0.66mm respectively. while the antibacterial effect of ethanolic extracts of *Murraya koenigii Linn* against the standard strains of *staphylococcus aureus* at dose rate of 50mg, 100 mg and 200 mg showed inhibitory activity with zone of diameter 15.66 ± 0.33, 16.66 ± 0.66 and 21.33 ± 0.66mm respectively. Penicillin was used as standard drug for *in-vitro* antibacterial activity against *staphylococcus aureus*, showed *23.33* ± 0.66mm zone of diameter. Ethanolic extract of *Murraya koenigii Linn* at 50mg showed less inhibition 15.66 ± 0.88mm zone of diameter *as* compare at dose rate of 100 and 200mg showed 17.33 ± 0.66 and 17.33 ± 0.66mm zone of diameter respectively against *Staphylococcus aureus.*

The inhibitory effect of ethanolic extract leaf of *Murraya koenigii* Linn against *E. coli* at the concentration of 50, 100, 200mg/ml showing 17.00 ± 0.57, 18.00 ± 1.15 and 18.66 ±1.33mm zone of diameter respectively. The ethanolic leaf extract at all concentration showed intermediate degree of inhibitory effect. While the antibacterial effect of ethanolic extracts of *Murraya koenigii Linn* at different concentration (50, 100, 200mg) against the standard strains of E. coli showed 17.33±0.66, 18.66±1.15 and 21.33±0.66mm respectively. The ethanolic leaf extract at 200mg showed highest degree of inhibition with increase dose increased inhibition. The ethanolic extract at 50 and 100mg showed intermediate zone of inhibition. Thus ethanolic extract is found to be good bioactive compound to inhibit growth of *E. coli* at higher concentration than lower concentration. Gentamicin was used as reference antibiotic for comparative study, gentamicin showed inhibitory effect with 30.66±0.66mm zone of diameter

The MIC values of Ethanolic leaf extract of *Murraya koenigii* Linn against the standard strains of *Staphylococcus aureus* and *E. coli* were 2.5mg and 2.5mg respectively.

The *in-vivo* antimicrobial efficacy of MKEE in Mice was determined by Itelima and Agina (2014). All rats were found negative for *E. coli* and *S. aureus* in feces before inoculation and treatment with ethanolic leaf extract of *Murraya koenigii* Linn and antibiotic (Penicillin and gentamicin).

The presence of diarrhea in mice after inoculation and treatment with extract and antimicrobial agents were presented in Table. Group I disease control does not receive any bacterial suspension and treatment, was administered on normal saline. However 100% of the infected non treated group *E. coli* $1x10^9$CFU/ml (group II) and 60% of the infected antibiotic treated group (group VI) manifested the symptom of watery diarrhea 2nd day after inoculation with *E. coli.* Group II positive control infected with suspension of *E.coli* $1x10^9$CFU/ml showed diarrhea from 3rd day of inoculation and remain upto 10 days. Group IV treatment group infected with *E. coli* $1x10^9$CFU/ml and received 2.5mg/kg of ethanolic leaf extract of *Murraya koenigii* Linn showed diarrhea on 3rd day and diarrhea subsides on 5th day of treatment. Group IX infected with *E.coli* $1x10^9$CFU/ml and treated with standard antibiotics Gentamicin @ 4mg/kg body weight showed diarrhea on 3rd day and stopped on 5th day. Group III positive control infected with $1x10^9$CFU/ml of *Staphylococcus aureus* showed diarrhea on 2nd day. Group VI treatment group infected with suspension of *Staphylococcus aureus* $1x10^9$CFU/ml and received 2.5mg/kg of ethanolic leaf extract of *Murraya koenigii* Linn showed diarrhea on 3rd day and stopped diarrhea on 5th day. Group IX infected with *Staphylococcus aureus* $1x10^9$CFU/ml and treated with Penicillin (Ampicillin) @ 10,000IU/kg body weight showed diarrhea on 2nd day and subsides on 6th day. The result revealed that there was a significant interaction between treatment and time ($p>0.05$) over the course of the study. However when comparing treatment groups at specific observation days, the proportion of albino mice showing diarrhea in infected antibiotic treated group was significantly higher than infected non treated groups on 5, 6th day. None of the mice group suffered from bloody diarrhea. It was observed that there was more reduction in the number of mice defecating watery stool over time among the infected non treated group of mice than the infected antibiotic treated group. Thus, the defecation of watery diarrhea by the rats lasted between 7 to 8 days in group II and group III and 5 to 6 days in group IV, V. The groups of the infected animals treated with *M. Koengii,* penicillin and gentamicin stopped shedding *E. coli* and *S. aureus* at quantifiable concentration levels at days 5-5, 6-6, 5 and 6 days respectively Variation was also apparent in the amount of E. coli and S. aureus shed in feces among the various mice groups. Thus, during the course of experiments the concentration of the organisms (*E. coli and S. aureus*) in feces of the positive animals in groups II to IX ranged between 3 $x10^3$-$8x10^3$CFU/g, 1 $x10^3$-5 $x10^3$ CFU/g, 2 $x10^3$-4 $x10^3$ CFU/g,$1x10^3$-$4x10^3$CFU/g, $2x10^3$ - $4x10^3$CFU/g, 3 $x10^3$-5 $x10^3$CFU/g, $2x10^3$ -$4x10^3$CFU/g and $2x10^3$ -$5x10^3$CFU/g respectively Statistical analysis of the results showed that there was a significant time effect, but no significant treatment effect among some of the infected mice groups treated with the *M. Koengii* and *H. Rosa-sinensis* plant extract. However, significant difference ($p< 0.05$) was observed in treatment effects among the *E. coli* infected non-treated group of animals and those treated with the plant extracts. While, no significant difference () was in treatment effects among *S. aureus* infected non-treated group of animals and those treated with the plant extracts. Among the mice that suffered from diarrhea and abnormalities such as general weakness with slow movement, loss of appetite and loss of weight were observed in them. No pathological changes were observed in other mice groups all through the course of the experiment.

Discussion

Infectious diseases are the leading cause of death world-wide, killing almost 50,000 people every day. Infections due to a variety of bacterial etiologic agents such as pathogenic *Escherichia coli, Pseudomonas aeruginosa, staphylococcus aureus spp, salmonella spp, vibrio cholerae, shigella spp, Klebsiella spp, Campylobacter* spp are most common. (Parmar and Rawat 2012) [3]. Previous studies described the antimicrobial activity of ethanolic and Methanolic leaf extract of *Murraya koenigii* L. against *E. coli* and *Staphylococcus aureus* with zone of diameter 16mm, 11mm and 14mm respectively (Argal *et al.,* 2011) [7], Methanolic and ethanolic extract of *Murraya koenigii* L. showed higher zone of inhibition i.e. 16mm and 16mm against *E. coli* compared to 11mm and 14mm against *Staphylococcus aureus*. Hence solvent extract has found more antibacterial activity compared to aqueous extract for tested microorganism. Khuntia and Panda (2011), also reported similar observation, petroleum ether extract of *Murraya koenigii* L. at 5mg/ml and 10mg/ml against *E. coli* and *Staphylococcus aureus* showed 20mm &

19mm zone of diameter and 20mm & 21mm zone of inhibition respectively. The MIC values of *Murraya koenigii* Linn *Linn* was determined for microorganisms *(Staphylococcus aureus* and *E.coli)* that were found to be sensitive during the agar well diffusion assay. The MIC was determined by broth dilution method described by Miles and Mistra method (1938) [9]. Nutrient broth was used to determine MIC. The MIC values of Ethanolic leaf extract of *Murraya koenigii* Linn against the standard strains of *Staphylococcus aureus* and *E.coli* were 2.5mg and 2.5mg respectively. Similar observations were reported by Saini *et al* (2013) [10] who observed that methanolic and aqueous leaf extract *Murraya koenigii* Linn showed antimicrobial activity against *E. coli* 7.05 and 3.22mm zone of diameter and against *S. aureus* 10.64 and 8.09mm zone of diameter respectively, MIC of leaf extract *Murraya koenigii* Linn against *E.coli and S. aureus* were 2.5mg/ml and 0.312mg/ml respectively. According to Malval M. and Sarin R (2011) findings MIC values of methanolic leaf extract *Murraya koenigii* Linn against *E. coli* and *S. aureus* were 0.321mg/ml and 0.078mg/ml respectively. The aqueous leaf extract does not showed any inhibitory effect against both strains.

There were no *in-vivo* studies references available for the ethanolic extract of *Murraya koenigii* Linn against *E. coli* and *S. aureus.* Similar studies was also conducted by Sylvie Lea Wansi *et al* (2014) who studied antimicrobial and antidiarrhoeal properties of Pentadesma butyracea stem bark methanolic extract against *shigella flexneri.* MEPB inhibited the bacterial growth in a dose dependent manner. The extract at the dose of 125, 250 and 500 mg/kg effectively reduced shigella density from the seventh day of therapy and beyond; the percentage of reduction of *shigella flexneri* density was respectively 69.03%, 75.54% and 80.37% compared to the value administered. Thus, the dose 500 mg/kg of the extract appeared to be more active than ciprofloxacin (70.63%) compared to the negative control.

Table 3: Antibacterial susceptibility test of plant extract on *E. coli*

Sr. No.	Bacterial suspension	Concentration mg/well	*Murraya koenigii L.*	
			Ethanolic	Aqueous
1.	*E. coli*	50	17.000^{b} ±0.573	10.333^{b} ± 2.094
		100	18.000^{b} ±1.157	10.333^{b} ± 2.094
		200	18.666^{b} ±1.333	10.333^{b} ± 2.094
		Gentamicin	30.666^{a} ±1.157	30.666^{a} ± 0.666

Table 4: Antibacterial susceptibility test of plant extract on *S. Aureus*

Sr. No.	Bacterial suspension	Concentration mg/well	*Murraya koenigii L.*	
			Ethanolic	Aqueous
1.	*S. aureus*	50	15.666^{b} ± 0.889	10.000^{b} ± 0.000
		100	17.333^{b} ± 0.666	10.333^{b} ± 0.333
		200	17.333^{b} ± 0.666	10.333^{b} ± 0.333
		Penicillin	23.333^{a} ± 0.666	22.666^{a} ± 0.666

Table 5: MIC of *Murraya koenigii L.* on *E. coli* strain

Sr. No.	*E. coli* (CFU/ml)	Concentration of *Murraya koenigii L.* (mg)	Growth observed
1	1×10^6	10	No growth observed
2	1×10^6	5	No Growth observed
3	1×10^6	2.5	No Growth observed
4	1×10^6	1.25	Growth observed
5	1×10^6	0.62	Growth observed
6	1×10^6	0.32	Growth observed
7	1×10^6	0.015	Growth observed
8	1×10^6	0.007	Growth observed
9	1×10^6	_	Growth observed

Table 6: MIC of *Murraya koenigii L.* on *S. aureus* strain

Sr. No.	*S. aureus* (CFU/ml)	Concentration of *Murraya koenigii* L. (mg)	Growth observed
1	1×10^6	10	No growth observed
2	1×10^6	5	No Growth observed
3	1×10^6	2.5	No Growth observed
4	1×10^6	1.25	Growth observed
5	1×10^6	0.625	Growth observed
6	1×10^6	0.315	Growth observed
7	1×10^6	0.156	Growth observed
8	1×10^6	0.078	Growth observed
9	1×10^6	_	Growth observed

Table 7: Effects of treatment with MKEE & HREE on fecal shedding of *E. coli* ($CFUg^{-1}$) by mice

Group	Days					
	1st	2nd	3rd	4th	5th	6th
Group II: Positive control (infected with bacterial suspension *E. coli* $1x10^9$ CFU/ml).	3×10^3	4×10^3	7×10^3	7×10^3	8×10^3	8×10^3
Group IV: Treatment group infected with *E. coli* $1x10^9$ CFU/ml received 2.5mg/kg ethanolic extract of *Murraya koenigii* Linn.	2×10^3	3×10^3	3×10^3	4×10^3	3×10^3	1×10^3
Group VI Treated group infected with *E. coli* $1x10^9$ CFU/ml received 4mg/kg Gentamicin	3×10^3	4×10^3	4×10^3	5×10^3	4×10^3	2×10^3

Table 8: Effects of treatment with MKEE & HREE on fecal shedding of *S. aureus* ($CFUg^{-1}$) by mice

Group	Days					
	1st	2nd	3rd	4th	5th	6th
Group III Positive control (infected with bacterial suspension *S. aureus* $1x10^9$ CFU/ml).	1×10^3	3×10^3	3×10^3	4×10^3	5×10^3	5×10^3
Group IV: Treated group infected with *S. aureus* $1x10^9$ CFU/ml received 2.5mg/kg ethanolic extract of *Murraya koenigii* Linn.	3×10^3	3×10^3	4×10^3	5×10^3	4×10^3	3×10^3
Group VI: Treated group infected with *S. aureus* $1x10^9$ CFU/ml received 10,000 IU/kg BW penicillin	2×10^3	4×10^3	4×10^3	4×10^3	3×10^3	2×10^3

Table 9: Diarrhoea observed in E. coli infested groups during course of treatment:

Group	Days					
	1st	2nd	3rd	4th	5th	6th
Group II: Positive control (infected with bacterial suspension *E. coli* $1x10^9$ CFU/ml).			Diarrhea	Diarrhea	Diarrhea	Diarrhea
Group V: Treatment group infected with *E. coli* $1x10^9$ CFU/ml received 2.5mg/kg ethanolic extract of *Murraya koenigii* Linn.			Diarrhea	Diarrhea	Solid	Solid
Group VI Treated group infected with *E. coli* $1x10^9$ CFU/ml received 4mg/kg Gentamicin			Diarrhea	Diarrhea	Solid	Solid

Table 10: Diarrhoea on *S. aureus*

Group	Days					
	1st	2nd	3rd	4th	5th	6th
Group III Positive control (infected with bacterial suspension *S. aureus* $1x10^9$ CFU/ml).		Diarrhea	Diarrhea	Diarrhea	Diarrhea	Diarrhea
Group VII: Treated group infected with *S. aureus* $1x10^9$ CFU/ml received 2.5mg/kg ethanolic extract of *Murraya koenigii* Linn.			Diarrhea	Diarrhea	Solid	Solid
Group VIII: Treated group infected with *S. aureus* $1x10^9$ CFU/ml received 10,000 IU/kg BW penicillin		Diarrhea	Diarrhea	Diarrhea	Semisolid	Solid

Conclusion

The present study has concluded, hat the inhibitory effect of the antibiotic drug against the organism *in-vivo* seemed to be less effective than the effect of the plant extract; hence, 60% of the rat had the symptoms of diarrhoea. The reason for the less effectiveness of the antibiotic as compared to the plant extracts could be attributed to the fact that the antibiotic drug inhibited the competitive microorganism in the gut more than *E. coli* O157: H7 strain. This condition enables the proliferation of *E. coli* O157: H7 in the gut and also enhances the development of disease condition such as watery diarrhoea. This suggestion was supported by Jin-Hyung *et al.* (2011) who reported that most antibiotics often eradicate intestinal commensal bacterial more than the pathogenic bacteria.

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