Yuzuncu Yil University
Journal of Agricultural Sciences
(Yüzüncü Yıl Üniversitesi Tarım Bilimleri Dergisi)
https://dergipark.org.tr/en/pub/yyutbd

ISSN: 1308-7576
e-ISSN: 1308-7584

Research Article

In-vitro and *In-vivo* Antimicrobial Properties of Pomegranate (*Punica granatum* L.) Peel Extract Genotypes Against Bacterial Vaginosis in Bovine

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Article Info

Received: 26.08.2022
Accepted: 29.11.2022
Online published: 15.12.2022
DOI: 10.29133/yyutbd.1167392

Keywords

Antibacterial effect,
Punica granatum L.,
Vaginal drug

Abstract: Drug resistance against bovine bacterial vaginosis (BV) in common treatments needs new therapeutic agents from other sources. Many plants demonstrate antimicrobial properties that could control pathogenic microorganisms. Uterus infections in bovine are associated with calving difficulty, retained placenta, and overgrowth of pathogenic microorganisms in the reproductive tract. This study examined the antibacterial effects of pomegranate peel extracts against various bacterial vaginosis in bovine. Methanolic and aqueous extracts of different pomegranate peels were prepared. An antibiogram test was performed against nine various bacterial vaginosis of bovine. Then, inhibitory concentration values were determined for pomegranate peel extracts (100, 200, and 400 mg/ml). In *in-vivo* observation, the greatest inhibition zone activated pink pomegranate peel extracts (8 mg/ml) prepared by methanolic extracts. The serial dilution tests indicated that the bactericidal effect of high-concentration methanolic extract was more than those of low-concentration types. Experimental treatments in *in-vitro* observation constituted including (treated, T) with 180 cows that encountered difficulty in birth and non-including (control, C) with 30 cows with no treatments after vaginal problems. The effect of various pomegranate peel extract (PPE) concentrations increased during the treatment days. All the treated Holstein cows with nearly 400mg/ml of PPE gel (both methanolic and aqueous extracts methods) were recovered after 4 days. The results of this study showed the PPE gel effectiveness in the pharmaceutical industry.

To Cite: Ziya Motalebipour, E, Pirestani, A, 2022. *In-vitro* and *In-vivo* Antimicrobial Properties of Pomegranate (*Punica granatum* L.) Peel Extract Genotypes Against Bacterial Vaginosis in Bovine. *Yuzuncu Yil University Journal of Agricultural Sciences,* 32(4): 825-834.
DOI: https://doi.org/10.29133/yyutbd.886006

1. Introduction

Drug resistance to animal pathogens has needed new therapeutic agents from other sources. The antimicrobial property of traditional plans based on medicines has been revisited over the last decade (Meléndez and Capriles, 2006). Many plants demonstrated antimicrobial properties, which could have pivotal activity against bacteria, allowing them to deal with pathogenic microorganisms (Meléndez and Capriles, 2006). Chemical compounds include flavonoids, antioxidants, and anthocyanin in many plants such as pomegranate (*Punica granatum* L.) which has an antibacterial role in bacterial diseases (Prashanth et al., 2001; Holetz et al., 2002).

Punica granatum L. is considered a member of the Punicaceae (Lythraceae) family, which consists of two species *Punica granatum* L. and *Punica protopunica* Balf. The *Punica granatum* L. species typically referred to as pomegranate and used in folklore medicine to cure various diseases across countries (Ricci et al., 2006; Lansky and Newman, 2007; Jurenka, 2008; Kahramanoglu, 2021). The known compounds of the *Punica* genus are Ellagitannins, Galoutanins, Anthocyanins, Flavonoids, Sterols, Terpenoids, Tannin, Polyphenols, Alkaloids, Organic Acids, B1, B2, and C vitamins (Dong et al., 2011). It is used in traditional medicine due to its anti-inflammatory and antimicrobial effects in Asia and Mediterranean Europe (Heber et al., 2006).

Various parts of pomegranate, such as fruit peel and seed, demonstrated the broadest antibacterial activity (Prashanth et al., 2001, Dahham et al., 2010; Ismail et al., 2012). Pomegranate peels are important in traditional medicine due to containing strong phenolic, flavonoid, and anthocyanin compounds. Essential oils in pomegranate peels contain a wide range of tannins (Reddy et al., 2007), anthocyanins, and flavonols (Naz et al., 2007). The latest scientific research studies among different *P. granatum* L. varieties (Black, White, and Red skin fruit) showed that black skin fruit has the highest amount of phenolic compounds, total anthocyanin, and antioxidant properties (Bayati and Asadi-Gharneh, 2019).

The effect of pomegranate extract feeding on animal health has been reported in previous studies. Those effects included modifying the microbial ecology of broiler chickens (Perricone et al., 2020) and the microbiome in mice (George et al., 2019). There are also studies on the effects of concentrated pomegranate extract on lactating cow rations (Jami et al., 2012) and pomegranate extract feeding on health, growth, and nutrient digestion in calves (Oliveira et al., 2010). However, the antimicrobial effect of pomegranate peel extract has been scarcely investigated in animal diseases.

Reproductive potentials, milk production, treatment expenses, and culls in infertile animals are notably affected by uterine infection (Lewis, 1997; Ross, 2002). In dairy cows, calving problems, retained placenta, and an abundance of pathogenic microorganisms in the reproductive tract lead to uterus infections (Coleman et al., 1985). Uterine pathogens, which cause clinical endometritis and severe endometrial inflammation, include *Escherichia coli*, *Staphylococcus aureus, and Klebsiella pneumoniae* sub. *pneumonia*, *Pseudomonas aeruginosa*, *Corynebacterium glutamicum*, *Salmonella enterica* subsp. *Enterica*, *Proteus mirabilis*, *Bacillus licheniformis*, and *Streptococcus agalactiae* (Sheldon et al., 2002; Williams et al., 2005; Carneiro et al., 2016).

To the best of our knowledge, the effect of pomegranate peel extraction on vaginosis diseases has not been adequately tested in animals. This study comprehensively reports the antibacterial effect of pomegranate peel extraction on vaginosis bacteria. Therefore, we aimed to find a vaginal gel that can be used against vaginosis bacteria in Holstein cows.

2. Material and Methods

2.1. Sample collection and extract preparation

Three pomegranates (*Punica granatum* L.) genotypes with different peel colors: black skin varieties of Yazd, white skin varieties of Abrandabad Yazd, and pink skin varieties of Shahreza were collected from Isfahan Agricultural and Natural Resources Research Center, Iran (Figure 1). Peels were first prepared and dried for a week at room temperature in the dark. Then, the dried peels were separately blended for 2 min.

Each powder was divided into two groups. The first group (5 g of pomegranate peel powder) was dissolved in 30 ml of distilled water. The second group was dissolved in 30 ml methanol (99.9%), shacked for 4 h, and then centrifuged (Universal 320 R, Iran) at 12000rpm for 10 min at 5°C. Each solution was filtered, followed by reduced concentration by an otary evaporation method. The concentrates were dried in an oven (FGiran, Iran), and the dried extract was stored at −20°C.

Figure 1. The pomegranate (*Punica granatum* L.) genotypes with different peel colors: Black peel (A), White peel (B), and Pink peel (C).

2.2. Microorganisms and growth conditions

Table 1 lists microorganisms used to evaluate the antimicrobial activity of aqueous extract (AE) and methanolic extract (ME). The reference microorganisms were obtained from the Iranian Research Organization for Science and Technology (IROST). The nine animal vaginal bacterial strains were used as the test organisms.

2.3. Formulating the topical herbal gel

Pomegranate herbal gel was made using the affected pomegranate peel extract. The gel base was prepared by applying lubricant gel (Jardines and Alcoba, 2020). The affected pomegranate peel extracts were added to the gel base and mixed well.

2.4. Antimicrobial activity (*in-vitro*) of extracts

The antimicrobial tests were implemented based on Kirby- Bauer standard method (Bauer, 1966) using a modified agar well diffusion method (Okunji et al., 1990). Various concentrations (50, 100, 200, and 400 mg/ml) of pomegranate peel extract were used in the current study. Moreover, 20 µl of each concentration was added to each well. Therefore, each well contained 1, 2, 4, and 8 mg of pomegranate peel extract. *Escherichia coli*, *klebsiella pneumoniae* subsp. *pneumonia*, *Salmonella enterica* subsp. *Enterica*, *Proteus mirabilis*, *Bacillus licheniformis*, and *Staphylococcus aureus* were grown at 35 ºC for 18-24 h by inoculating in Nutrient Agar (NA, Merck, Germany). In addition, *Streptococcus agalactiae*, and *Corynebacterium glutamicum* were inoculated in Brain heart infusion (BHI, Merck, Germany). *Pseudomonas aeruginosa* was inoculated in Tryptic soy agar (TSA, Merck, Germany). One hundred µl of each 1/100 dilution of McFarland 0.5 bacterial culture was added to the plate and was uniformly spread on the surface. Petri dishes with 10 ml of each bacterial culture were prepared. The wells (for each extract and antibiotic concentrations in triplicate) were planted, and then the plates were kept in an incubator at 37°C for 24 h, while *Bacillus licheniformis* was kept at 30°C.

2.5. Antimicrobial activity (*in-vivo*) of extracts

Two hundred and ten Holstein cows (2 or 3 years) were housed at the Alian husbandry in a shaded corral with free access to water in the spring of 2020. The experiment site was a semi-open indoor springboard that was in contact with the open air from all sides. Holstein cows were divided at the onset of the experiment into two groups, which were individually fed with a typical total mixed ration (TMR). Experimental treatments consisted of the including group (treated, T) with 180 cows that had problems at birth and the non-including group (control, C) with 30 cows with no treatments after vaginal problems. The treated cows received methanolic and aqueous PPE vaginal gel from 1 to 10 days after parturition. Vaginal gel performance was evaluated during the trial period from the beginning to the end of the experiment.

2.6. Data analysis

Statistical analysis was done by SPSS computer software (version 25; SPSS, USA, 2017). The data were evaluated in terms of mean ± SEM for each pomegranate peel extract. Analysis of variance (ANOVA) followed by Duncan's multiple range test was used to evaluate the impact significance of factors such as peel color, extraction method, and extract concentration. Differences considered significant in a p-value were less than 0.05. The homogeneity of variance test was used to examine factor effectiveness in each case (2 and 3 factors) and determine group homogeneity

3. Results

3.1. *In-vitro* observation

The results of *in-vitro* antibacterial activity of aqueous and methanolic extracts determined by the mean of inhibition zone diameters are presented in Table 1. The table summarizes the inhibition zone of various bacteria by different pomegranate peel extracts. Among the extraction methods (aqueous and methanolic), the methanolic extract was the most active with 25 mm of mean inhibition diameter. In our study, the type of pomegranate peel (white, pink, black) did not significantly increase the inhibition halos. Comparing inhibition zone means in extractions indicates notable effects of pomegranate peel extract with discs containing 8 mg pomegranate peel extract. *Corynebacterium glutamicum*, *klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Streptococcus agalactiae,* and *Bacillus licheniformis* showed the highest sensitivity (250.5 ± mm, Figure 2). The lowest sensitivity was illustrated by *Salmonella enterica* subsp. *enterica* (9± 0.0 mm). There was a significant difference between high- and low-concentration pomegranate peel extract ($P<0.05$). Furthermore, 8 mg of extraction was generally higher than those of other concentrations. There was no significant difference between aqueous and methanolic peel extract, though the efficacy of methanol peel extraction was more ($P<0.05$).

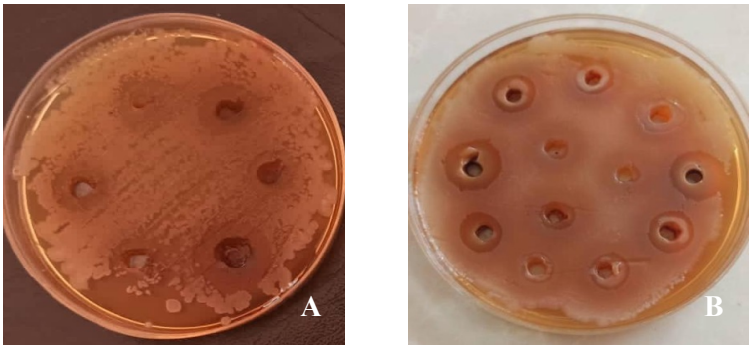

Figure 2. The antimicrobial inhibitory zone diameters (mm) of aqueous (A) and methanolic (B) extraction in different pomegranate peel extracts against *Streptococcus agalactiae.*

Table 1. Comparison of the inhibitory zone diameters (mm) in different pomegranate (*Punica granatum* L.) peel extract concentrations against vaginosis bacteria in bovine

				Inhibition zone (mm)					
				Aqueous extract			Methanolic extract		
No	Microorganism	PTCC*	Sample Concentration (mg/disc)	Black Peel	White Peel	Pink Peel	Black Peel	White Peel	Pink Peel
1	***Escherichia coli***	1860	1	6.5±0.5	1±0.5	0±0	13±2	8.5±0.5	5±0
			2	10.5±0.5	7.5±0.5	0±0	12.5±0.5	12±0	10±0
			4	15.5±0.5	10±0	5.5±0.5	10±0	15±0	11±1
			8	21±1	15.5±0.5	12.5±0.5	12±0	16±1	16±1
2	***Staphylococcus aureus***	1431	1	5.5±0.5	0±0	0±0	6.5±1.5	4±0.9	10±0
			2	9.5±0.5	0±0	9.5±0.5	7.5±0.5	7.5±0.5	14±1
			4	14.5±0.5	5.5±0.5	15±0	13.5±1.5	12.5±2.5	16±1
			8	17.5±0.5	9.5±0.5	17.5±0.5	14±1	14.5±2.5	22.5±0.5
3	***klebsiella pneumoniae***	1290	1	0±0	0±0	0±0	0±0	0±0	0±0
			2	0±0	5±0	0±0	0±0	0±0	9±0
			4	5±0	7±0	11±1	5.5±0.5	7.5±0.5	14±1
			8	7.5±0.5	10±0	20.5±0.5	9.5±0.5	9.5±0.5	25±0
4	***Pseudomonas aeruginosa***	1310	1	0±0	0±0	0±0	0±0	0±0	5±0
			2	0±0	0±0	0±0	7±0	6±1	10±0
			4	6±1	8.5±0.5	6±1	10±0	10.5±1.5	11.5±0.5
			8	17.5±2.5	12.5±2.5	15±0	10±0	12.5±2.5	24.5±0.5
5	***Corynebacterium glutamicum***	1532	1	0±0	0±0	0±0	0±0	0±0	0±0
			2	5±0	5±0	0±0	5.5±0.5	0±0	5±0
			4	8±0.9	9.5±0.5	15±0	10±0	0±0	13±1
			8	11±1	13±2	21±1	13.5±1.5	17.5±0.5	25±0
6	***Salmonella enterica subsp. enterica***	1787	1	0±0	0±0	0±0	0±0	0±0	0±0
			2	0±0	0±0	0±0	0±0	0±0	0±0
			4	0±0	0±0	0±0	0±0	5.5±0.5	0±0
			8	7.5±0.5	5.5±0.5	6±1	10±0	9.5±0.5	9±0
7	***Proteus mirabilis***	1776	1	6.5±1.5	0±0	0±0	0±0	5±0	5.5±0.5
			2	9±0	9±1	0±0	6±0	6.5±0.5	11±1
			4	8.5±1.5	9±1	5±0	11±1	10.5±4.5	15±0
			8	10.5±0.5	13.5±1.5	14±1	14.5±0.5	19.5±0.5	20±0
8	***Bacillus licheniformis***	1331	1	5±0	0±0	0±0	0±0	0±0	5.5±0.5
			2	5±0	0±0	10±0	0±0	0±0	10±0
			4	6±0	5.5±0.5	11.5±0.5	5.5±0.5	6±0	13.5±0.5
			8	13±1	10±0	20.5±0.5	12.5±0.5	10±0	25±0
9	***Streptococcus agalactiae***	1768	1	0±0	0±0	0±0	0±0	0±0	5±0
			2	0±0	0±0	0±0	5±0	0±0	10.5±0.5
			4	13.5±1.5	7.5±0.5	15±0	8±0	6±1	14±1
			8	19±1	14±1	20.5±0.5	11±1	18.5±1.5	25±0

*PTCC: Persian Type Culture Collection (PTCC is a member of the World Federation for Culture Collections and the UNESCO Microbial Resources Centers Network).

Means and standard deviations are considered for n = 3. For inhibition bacteria, the experimental values of pomegranate peel extract concentrations within each row are shown, which show significant differences ($p < 0.05$).

3.2. *In-vivo* observation

The PPE vaginal gel results are shown in Table 2. The post-treatment with pomegranate peel extraction gel in both treatment groups (Aqueous and Methanolic extract) produced similar results. In the current study, slightly more and less than the effective concentration in *in-vitro* results were evaluated in the *in-vivo* experiment. The recovery of various concentrations (6, 8, and 10 mg/ml) in PPE gel increased during the days. The results *in-vivo* confirmed those of *in-vitro*. In these experiments, the untreated Holstein cows remained infected until the end.

As a result, the 10mg/ml concentration showed the best inhibitory activity (Figure 3). All the treated Holstein cows with 500mg/ml concentration of PPE gel, which included methanolic and aqueous extract pomegranate peel, recovered after 3±1 and 4±1 days, respectively. There was no notable difference between 6 and 8 mg/ml concentrations in terms of their actions. All the treated Holstein cows with 8 mg/ml concentration of methanol PPE gel recovered after 6±1, while those treated with aqueous PPE gel recovered after 7±1 days. More than 95% of Holstein cows with 6 mg/ml concentration of methanolic and aqueous

PPE gel recovered after 7±1 and 8±1 days, respectively. The inhibitory activity of all concentrations was excellent. Furthermore, taking PPE gel in Holstein cows had no recurrence after the treatment.

Table 2. Recovery time of different pomegranate peel extract concentrations in *in-vivo* conditions

	Methanolic extraction	**Aqueous extraction**
The concentration of PPE gel (mg/ml)	**Number of days of use until recovery**	**Number of days of use until recovery**
6	7±1	8±1
8	6±1	7±1
10	3±1	4±1

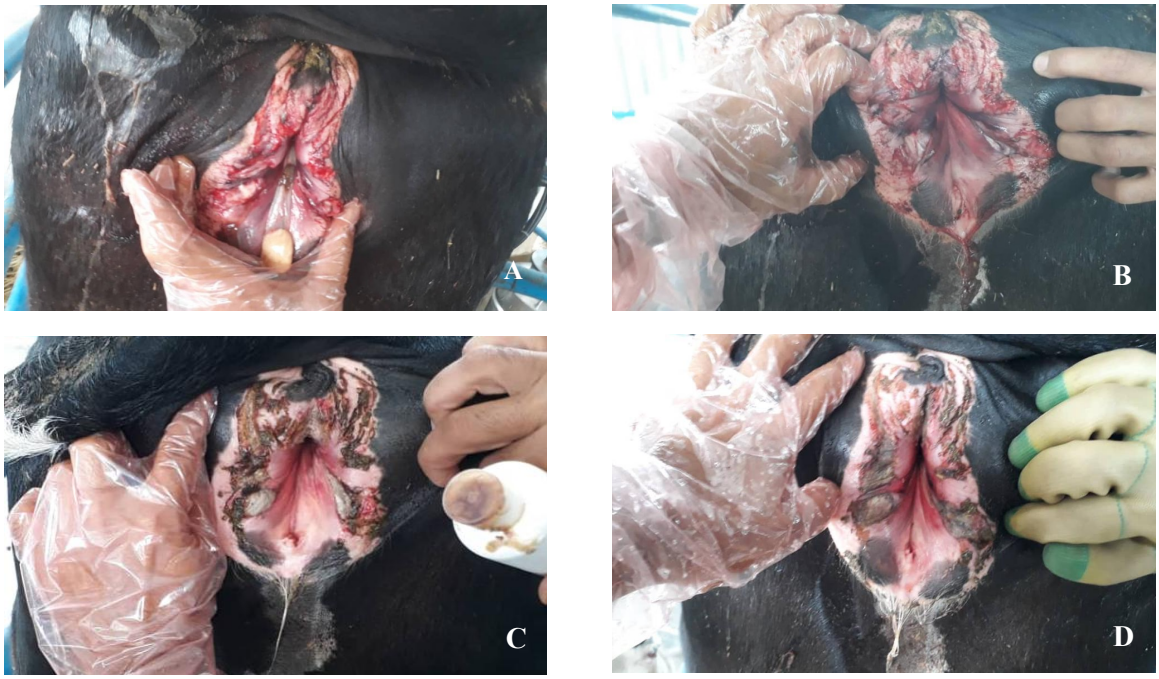

Figure 3. The effect of methanol PPE gel treatment on Holstein cow which passed difficulty in birth. The recovery increased by the gel used during the days. (A: The first day of using the gel; B: The second day of using the gel; C: The third day of using the gel; D: The fourth day of using the gel).

4. Discussion

4.1. The effect of PPE treatment in *in-vitro* condition

The current study showed the antibacterial activity of pomegranate peel extracts against vaginosis bacteria in animals. Our experiments confirmed the antibacterial activities in reaction to bacterial strains. Mean inhibition zone values indicate that pomegranate peel extracts (aqueous and methanolic) exert a more powerful effect on Gram-positive than Gram-negative bacteria ($P<0.05$). Similar results were obtained by Ismail et al. (2016), Casquete et al. (2015), and Naziri et al. (2012). The substantial antimicrobial effect of the pomegranate peel extracts is possibly due to the presence of polyphenols, flavonoids, and tannins (Al-Zoreky, 2009; Miguel et al., 2010; Alexandre et al., 2019). The antimicrobial impacts may be related to the chemical structure of the phenolic compounds. Phenolic compounds contain lipophilic natures that enter the pathogen cell layer, interfere with the enzymes responsible for energy and protein generation within the microorganism's cells, and subsequently cause cell death (Kantachumpoo and Chirapart, 2010). In our

study, varying amounts of antibacterial activity were observed depending on the pomegranate peel extract types, which could be, in turn, attributed to the compounds' chemical compositions. Among different compounds, only a few may have antibacterial activity (Elshafie et al., 2021). Medical centers can employ pomegranate wastes, including peel, which show great antioxidant capacities.

4.2. The effect of PPE treatment in *in-vivo* condition

The results showed that vaginal gel consisting of pomegranate peel extracts was effective against various bacterial vaginosis in Holstein cows without any serious side effects or relapse. The present study is a pioneering attempt in cattle domains that examines the antibacterial activity in *P. granatum* peel extract in reaction to bacterial vaginosis. We ascribe the antibacterial activity to metabolic toxins or broad-spectrum antibiotics in animals. Interestingly, previous research has attributed metabolites of herbs, such as alkaloids, tannins, and sterols, to antimicrobial activity (Machado et al., 2002; Voravuthikunchai et al., 2005). Moreover, the increase in oxygen-free radicals and lipid peroxidation could enhance antibacterial activities, destructing microorganisms' walls (Al-Saimary et al., 2002).

Despite the improvements in medical technologies, the strength of diseases is currently high. Many studies showed that medical drugs extracted from natural products are more successful than those manufactured in equal portions. These chemicals are used in the chemical-medical drug industry from low to heavy intensity (Nisar et al., 2018). In addition, despite the effectiveness of metronidazole and clindamycin treatments in infected animals, there still appear to be certain shortcomings in this respect (Joesoef et al., 1999; Yudin et al., 2003; Klebanoff et al., 2004).

In the present study, we focused on *P. granatum* peel extract effects, which modify the plasma membrane permeability, raise protein concentrations in a cell, and eventually destroy the cell (Kantachumpoo and Chirapart, 2010). Previous studies have mainly examined the antibacterial activity in *P. granatum* derivatives (Dahham et al., 2010; Ismail et al., 2012), and such effects on the *in-vivo* conditions have remained rather unclear.

The findings presented in this paper could help develop fresh and research-supported antibacterial therapies in cattle. Further research is also warranted investigating how pomegranate peel extracts and the affected compounds prevent bacteria growth.

Conclusion

In general, the results of this study show that vaginal gel consisting of pomegranate peel extract is effective in treating vaginosis diseases in cattle and could be used as an alternative option to chemical drugs. As noted, the pomegranate peel extract indicates antibacterial, antifungal, and anti-inflammatory capacities. Thus, further studies should probe into peel compound effects on bacterial vaginosis treatments.

Abbreviation

BV: bovine bacterial vaginosis, T: treated, C: control, PPE: pomegranate peel extract, AE: aqueous extract, ME: methanolic extract, IROST: Iranian Research Organization for Science and Technology, NA: Nutrient Agar, BHI: Brain heart infusion, TSA: Tryptic soy agar, TMR: total mixed ration, SPSS: Statistical Package for the Social Sciences, ANOVA: Analysis of variance, PTCC: Persian Type Culture Collection.

Acknowledgments

The authors are thankful to the Director of the Transgenesis Center of Excellence, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran, and Medicinal plants research center, Isfahan (Khorasgan) Branch, Islamic Azad University, Isfahan, Iran for providing all the research facilities during this study. This work was supported by the Technology Incubator Center of Medicinal Plant of Isfahan (Khorasgan) Branch, Islamic Azad University.

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