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Original article

The Effect of *Boswellia* Vaginal Gel on Oxidative Stress and Expression of Apoptotic Biomarkers in Vaginal Discharge of Women With Vaginitis

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Abstract

Background and objectives: *Boswellia serrata* is an important medicinal plant with strong antioxidant activity. The present study was designed to evaluate the effects of *Boswellia* vaginal gel on apoptosis and oxidative damage in vaginal mucosal cells of women with vaginal candidiasis. **Methods:** Ninety-five women with vaginal candidiasis were enrolled in the clinical trial and received the gel for seven consecutive nights. The clinical symptoms of the disease including vaginal secretion, pain, itching, unpleasant odor, and dyspareunia were recorded. Vaginal discharges were collected before and after treatment for the assessment of malondialdehyde (MDA) and total antioxidant capacity (TAC) using TBAR and FRAP methods, respectively. Expression of Bax, Bcl2, and Caspases-3 genes was surveyed by RT-PCR. **Results:** Gel therapy significantly decreased the frequency of pain, burning, itching, dyspareunia and secretion compared to the baseline ($p<0.001$). *Boswellia* vaginal gel treatment significantly improved TAC values (from 1.27 ± 0.41 µM/mL to 4.69 ± 0.53 µM/mL; $p<0.001$) and decreased MDA values (from 31.47 ± 6.69 nM/L to 13.85 ± 3.72 nM/L; $p<0.001$). The therapy caused a significant decrease in Bax and Casp3 expression, as well as Bax/Bcl2 ratio by 2.34-fold ($p=0.018$), 2.86-fold ($p=0.002$), and 12.72-fold ($p<0.001$), respectively. In contrast, BVG treatment significantly enhanced the expression of Bcl2 expression by 5.42- fold ($p<0.001$). **Conclusion:** Vaginal candidiasis is remarkably linked to oxidative stress, reduction of the antioxidants and vaginal mucosal cells apoptosis. *Boswellia* vaginal gel has potential role to improve vaginitis symptoms by elevating antioxidants capacity, mitigating oxidative stress, as well as down-regulating of apoptotic factors.

Keywords: antioxidant; apoptosis; *Boswellia*, oxidative stress; vaginal candidiasis

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Introduction

Vaginitis, also called vulvovaginitis, is an inflammation or infection of the vagina that is associated with several complications and symptoms such as urinary tract infections, preterm labor, pelvic inflammatory disease, abnormal vaginal discharge, odor, irritation, and itching, or burning [1,2]. Annually, almost 10 million visits to clinics are due to vaginal discharge worldwide [3]. Vaginitis can be divided into two main groups, including

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infectious and non-infectious vaginitis.
Non-infectious vaginitis is often caused by factors such as allergic reaction, foreign body, chemical irritation, desquamative vaginitis and lichen planus [4]. In contrast, pathogens such as *Candida albicans, Trichomonas vaginalis and* bacterial vaginosis are reported as the main causes of infectious vaginitis [1]. The mentioned infections are usually treated with appropriate medications properly. Vulvovaginal candidiasis is a main type of vaginitis that refers to vaginal and vulval symptoms caused most frequently by *Candida albicans*. vaginitis affects women's life quality by disruption of sexual activity, itching and vulvovaginal discomfort together with adverse effects on pregnancy and increasing the rate of sexually transmitted disease (STD) and pelvic inflammatory disease (PID) [5]. Understanding of the pivotal mechanisms behind vaginitis is of utmost important in its treatment. Oxidative stress (OS) that occurs as a result of over production of free radicals has been widely reported as possible mechanism for vaginitis development [6]. Although low concentration of ROS are reported to be essential for physiological functions, they can damage intra cellular macromolecules at high concentrations [7,8]. Chromosomal aberrations, altered gene expressions, mutations and single and double-strand DNA breaks induced by ROS can trigger apoptosis [8,9]. For this reason, antioxidant supplementation has been proposed as a helpful strategy to mitigate oxidative damage in vaginitis [10].
Apoptosis is a form of programmed cell death that is modulated by a wide range of biomolecules such as Bax, Fas, P53, and Bcl2 [11]. While Bax protein triggers cells apoptosis, Bcl2 protein prohibits apoptosis through inhibiting the release of cytochrome C from mitochondria. Therefore, higher level of Bax protein to Bcl2 protein is frequently correlated to cells death or apoptosis. Caspases family, such as Caspases-3, -8 and -9 are important mediators of apoptotic proteins that cause cells apoptosis through their protease activity [11]. Therefore, caspases along with Bax and Bcl2 play essential roles in the regulation of apoptosis through the intrinsic pathway. Since oxidative stress is considered as one of the main factors for the induction of apoptosis, we hypothesize that antioxidant supplementation may decrease apoptosis in vaginitis.
Boswellia is an important traditional medicine plant that currently represents an interesting topic for pharmaceutical research. Some studies revealed its antioxidant and inflammatory properties [12,13]. More recently, we formulated a *Boswellia* vaginal gel using the extract of *Boswellia* oleogumresin [14]. *Boswellia serrata* is an important traditional medicinal plant with strong antioxidant activity. In the present study, we decided to evaluate the level of biomarkers of oxidative stress (e.g. total antioxidant and malondialdehyde levels) and expression of Bax, Bcl2, and Caspases-3 in vaginal discharge of women with vaginal candidiasis.

Material and Methods

Ethical considerations

This open label clinical trial study was approved by the Ethics Committee of the Shahid Beheshti University of Medical Sciences (ethical code IR.SBMU.RETECH.REC.1399.1008) and also registered at the Iranian Center for Clinical Trials (IRCT code No. 20200701047977N1).
We followed the declaration of Helsinki and considered ethics in our research. All patients entered the study based on their willingness and they signed a written informed consent form.

Chemicals

RNX-Plus Kit (SinaClon; RN7713C, Iran); revert aid reverse transcriptase (Thermo science, Germany) and random hexamer primers (Thermo science, Germany); FRAP assay kit (Sigma-Aldrich-MAK369 MSDS, USA); TBAR kit (Cayman, USA). Carbomer and triethanolamin were provided from Merck (Germany). Propylene glycol was purchased from Dr. Mojallali (Iran).

Plant material

Boswellia was purchased from a herbal market in Tehran and identified at the Herbarium of the Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran with code 454-HMS.

Plant derived gel

To prepare 100 g of *Boswellia* gel, 2 g of powdered *Boswellia* was dissolved in water/ propylene glycol solvent (80:20) on a shaker for 24 hours. The resulting extract was then filtered. Carbomer 940 (1 g) was dissolved in 70 mL

water and then mixed with *Boswellia* extract. Triethanolamine was gently applied to form the gel. The gel's pH was adjusted around [14].

Study design

A clinical-trial study involving 95 women with vaginal candidiasis was conducted at Firoozgar Hospital (Tehran, Iran) from October 2020 to March 2021. All patients underwent clinical evaluations prior to enrolment in the study. Vaginitis was diagnosed by a specialist according to the laboratory tests after developing by the symptoms of pain, itching, unpleasant odor, secretion and dyspareunia. Vaginal candidiasis was diagnosed via vaginal discharge sample mixed with 10% potassium hydroxide (KOH) and examined by microscope with observation of fungal hyphae.

After patients selection based on inclusion criteria, a questionnaire was provided in which the age and clinical symptoms of the disease (vaginal discharge, pain, itching, unpleasant odor, and dyspareunia) were recorded. Then vaginal discharges were collected from the lateral sides of the vaginal wall using a swab for the measurement of oxidative stress biomarkers and gene expression analysis. The consort diagram for the study is summarized in Figure 1.

Inclusion criteria

The inclusion criteria were a negative urinary tract infection (UTI), Pap smear and HPV DNA detection.

Exclusion criteria

Pregnancy, receiving antibiotic therapy at least two weeks prior the study, diagnosis with a sexually transmitted disease, chronic systemic disease, and acute infections were the exclusion criteria for the study.

Treatments

Recently, we formulated *Boswellia* vaginal gel [14]. In this study we used the *Boswellia* vaginal gel 2% for treatment of vaginal candidiasis. Patients used the gel vaginally for seven consecutive nights. Forty-eight hours after the final treatment, patients were referred to the hospital and clinical symptoms of the disease, were recorded by the specialist. Vaginal discharges were again collected from the lateral sides of the vaginal wall using a sterilized swab and parameters of oxidative stress and gene expression analysis were assessed.

Oxidative stress biomarkers

Vaginal discharges were centrifuged at 1800 g for 10 min. the supernatants were used for the measurement of oxidative stress biomarkers and the pellets were applied for gene expression analysis. The total antioxidant capacity (TAC) of supernatants was measured by ferric reducing of antioxidant power (FRAP) method according to a previous study by Benize et al., [15]. Malondialdehyde (MDA) level in supernatants was measured using the thiobarbituric acid (TBA) method [16].

Gene expression analysis

The total RNA of pellets was extracted using RNX-Plus kit. To consider the quantity and quality of extracted RNAs, a Nanodrop ND-1000 spectrophotometer (Thermo Sci., Newington, USA) was applied. Revert aid reverse transcriptase and random hexamer primers were applied for cDNA synthesis at 42 ℃ for 1 h. A Rotor Gene 6000 (Corbett Research, Australia) thermocycler in 40 cycles was applied for amplifications. Each reaction included 4 μL master mix and 100 nM primers. Primer sequences are shown in Table 1.

The levels of mRNA were normalized relative to the amount of GAPDH mRNA. The relative expression of studied genes was calculated using $2^{-\Delta Ct}$ method.

Statistical analysis

Samples size was calculated using a standard formula (with α=0.05, 1-β=0.9, effect size=1 and r=3). All data are presented as means±SD. Crosstabs and Chi-Square tests were used to compare the percentage or frequency of parameters between before and after the study. A simple paired t-test was used to compare the mean of all data before and after the study. Data were analyzed using SPSS software (version 19); $p<0.05$ was considered as significant.

Table 1. Primers sequence of the studied genes

Genes	Forward	Reverse
Bax	5′-GAGGATGATTGCTGATGTGGATA-3′	5′-CAGTTGAAGTTGCCGTCTG-3′
Bcl2	5′- GGAGCGTCAACAGGGAGATG-3′	5′-ACAGCCAGGAGAAATCAAACAGA-3′
Caspase 3	5′- AAGCCGAAACTCTTCATCATTCA-3′	5′- GCCATATCATCGTCAGTTCCAC-3′
GAPDH	5′-AAGTTCAACGGCACAGTCAAGG-3′	5′-CATACTCAGCACCAGCATCACC-3′

Table 2. Comparison of the clinical outcomes before and after therapy in patients

	Before	After	p-Value
Pain	61 (64.2%)	4 (4.2%)	<0.001
Burning	81 (85.3%)	5 (5.3%)	<0.001
Itching	82 (86.3%)	3 (3.2%)	<0.001
Dyspareunia	63 (66.3%)	1 (1.1%)	<0.001
Secretion	93 (97.9%)	4 (4.2%)	<0.001

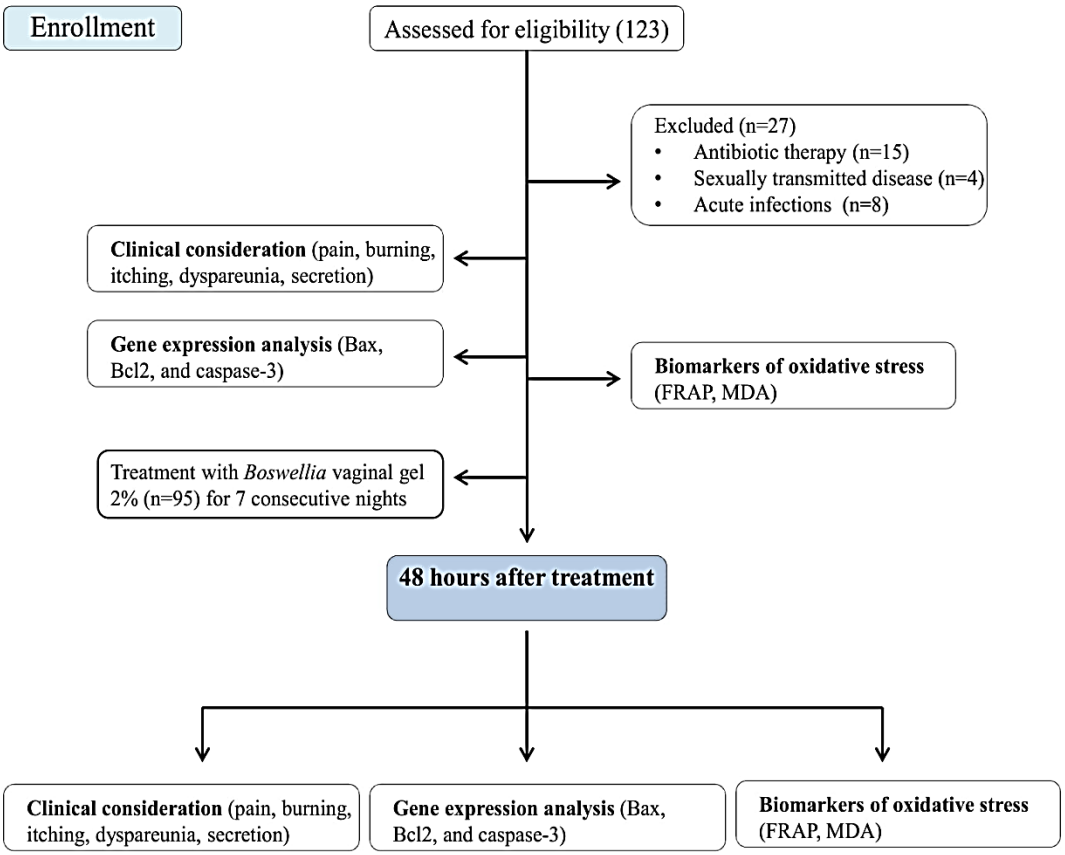

Figure 1. Diagram of the process of sample selection and study method

Results and Discussion

A total of 95 cases with vaginal candidiasis were enrolled in the study. The mean age of individuals was 35.89 ± 9.07 years. The clinical outcomes of *Boswellia* vaginal gel therapy on pain, burning, itching, dyspareunia, and secretion are summarized in Table 2. A significant different was found in the frequency of these clinical outcome before and after therapy. *Boswellia* vaginal gel treatment significantly decreased the frequency of pain, burning, itching, dyspareunia and secretion compared to before study ($p<0.001$; Table 2).

Comparison of FRAP value before and after showed that the treatment significantly enhanced the mean values of FRAP from 1.27 ± 0.41 µmol/mL before the study to 4.69 ± 0.53 µmol/mL after the study ($p<0.001$) (Figure 3).

Boswellia vaginal gel treatments significantly decreased MDA values from 31.47±6.69 nmol/L before the study to 13.85 ± 3.72 nmol/L after the study ($p<0.001$) (Figure 3).

A significant difference was found in expression pattern of Bax, Bcl2, Bax/Bcl2 ratio and Caspase 3 between before and after the therapy (Figure 2). The vaginal discharge of women showed significantly downregulation of Bcl2, but overexpression of Bax, Caspase 3 and Bax/Bcl2

ratio before the study. However, *Boswellia* vaginal gel treatment improved the expression Bcl2 and downregulated Bax, Bax/Bcl2 ratio and Caspase 3 expression.

Compared to the baseline, the gel resulted in a significant decrease in Bax and Caspase-3 expression, and Bax/Bcl2 ratio by 2.34-fold ($p=0.018$; Figure 2), 2.86-fold ($p=0.002$; Figure 2), and 12.72-fold ($p<0.001$; Figure 2), respectively. In contrast, the treatment significantly enhanced the expression of Bcl2 by 5.42-fold ($p<0.001$; Figure 2) compared to the before study.

In the present research we evaluated the effect of a formulated *Boswellia* vaginal gel on clinical symptoms, oxidative stress biomarkers and gene expression of apoptotic biomarkers in vaginal discharges of women with vaginal candidiasis. Our findings revealed that *Boswellia* gel treatment for a period of 7 days significantly improved clinical symptoms of vaginal candidiasis. The frequency of pain, burning, itching, dyspareunia, and vaginal secretion were significantly decreased after the therapy.

We found that vaginal candidiasis is associated with a significant depletion of total antioxidants and increased levels of MDA in vaginal discharges. Vaginal candidiasis was significantly associated with overexpression of Bax and Caspase 3 genes and down-regulation of Bcl2 gene in the vaginal discharges. Our findings support the idea that clinical symptoms of vaginal candidiasis are likely mediated through the induction of oxidative stress apoptosis of vaginal cells. Several documents have reported apoptosis and oxidative stress in the vaginal epithelial cells of women with vaginitis. For instance, based on the study by Chen et al. [6], high levels of MDA, H_2O_2, antioxidants depletion, and DNA breaks were reported in vaginal epithelial cells of 100 women with vaginitis. More importantly, high levels of apoptotic biomarkers such as Bax, caspase 3 and cytochrome C were reported in the same cases. These data are in agreement with our findings. Desdicioglu et al. [17] compared the content of disulphide and native/total thiol ratio in cases with vaginitis and healthy females; unlike the disulphide level, levels of native/total thiol ratio, were surprisingly lower in women with vaginitis. They concluded that in the absence of native thiol groups as the pivotal elements of antioxidant enzymes activity, oxidative stress and inflammation can be considered as the chief causes of vaginitis. Roselletti et al. [18] evaluated the rate of apoptosis induced by oxidative stress in the vaginal epithelial cells collected from women with bacterial vaginosis. Based on their findings, inflammatory biomarkers and caspase-3 activity significantly increased in the separated cells.

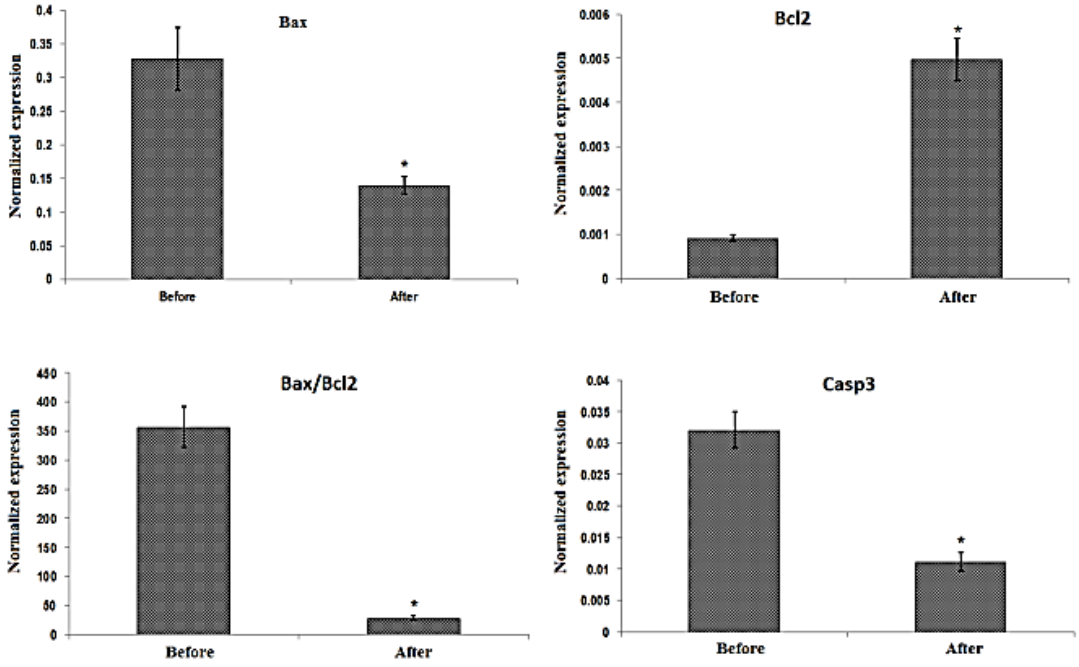

Figure 2. Comparison of the ratio of gene expression before and after treatment with *Boswellia* vaginal gel. Paired-t-test was applied to compare the gene expression before and after therapy. *$p<0.001$ compared to before study

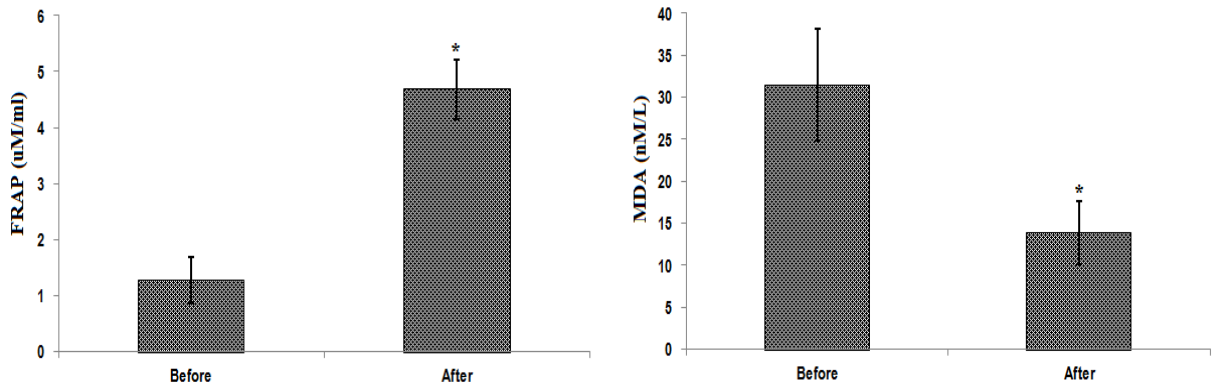

Figure 3. Comparison of the mean of FRAP and MDA value before and after treatment with *Boswellia* vaginal gel. Paired-t-test was applied to compare mean value of FRAP and MDA between before and after therapy. *p<0.001 compared to before study.

In another research, Guo et al. [10] analyzed cervico-vaginal fluid of women with vaginitis for oxidative stress biomarkers. Their results showed a remarkable increase in the levels of MDA and H_2O_2 while the catalase (CAT) activity was dropped. Similar to these findings we realized that antioxidant capacity is peculiarly decreased in females with vaginitis. Furthermore, our data confirmed the previous findings that vaginal mucosa epithelial cells in patients with vaginitis undergo oxidative stress induced apoptosis. Hence, all these data support the idea of involvement of oxidative stress in vaginitis and that antioxidant therapy can be adopted as a viable solution for vaginitis.

According to these findings and the concepts of vaginitis pathogenesis, it would be logical to use antioxidants that protect vaginal mucosal cells against oxidative stress and apoptosis. Here, we considered the effect of a formulated *Boswellia* vaginal gel to mitigate oxidative stress and vaginal mucosal cells apoptosis caused by vaginitis. In our study, the treatment significantly decreased vaginitis symptoms. This effect was associated with a significant increase in total antioxidant capacity and a significant decrease in MDA content in vaginal discharge. Interestingly, we found that *Boswellia* vaginal gel treatment not only improved the total antioxidants capacity, but also attenuated oxidative stress, expression of apoptotic (Bax, Caspase 3 and Caspase 8) and increases anti-apoptotic mediators (Bcl2). These data indicate that *Boswellia* vaginal gel can be helpful in mitigating oxidative stress and vaginal mucosal cells apoptosis in individuals who chronically suffer from vaginitis. To support these findings, some studies revealed that antioxidants therapy attenuate inflammation and oxidative stress by diminishing ROS production and apoptosis, as well as down-regulation of inflammatory cytokines and increasing of anti-inflammatory mediators and antioxidants contents. For instance, in a one week study by Chen et al. [6], patients with vaginitis were daily treated with vitamin C vaginal tablets (250 mg). They reported that vitamin C can significantly decrease oxidative stress biomarkes and cell death in mucosa epithelial cells. Angelucci et al. [19amin A, C and E on vaginal dryness in young females. According to them, vaginal health index was improved in 87% of patients after antioxidant therapy which is possibly due to inhibition of oxidative stress and inflammation.

Plants have always been in the core of attention for their antioxidant and anti-inflammatory effects and thus considered for the treatment of vaginitis [20]. For example, Ma et al. [21] surveyed the anti-apoptotic effects of berberine, in 180 women with bacterial vaginitis. They reported that berberine therapy can not only decrease the rate of cell program death apoptosis and oxidative stress biomarkers, but also can surge the activity of catalase and superoxide dismutase in the cells removed from the vagina.

Conclusion

In summary, oxidative stress, antioxidant depletion and vaginal mucosal cells apoptosis are the main reasons for the development and

pathogenesis of vaginitis. *Boswellia* vaginal gel treatment has positive effect on vaginitis symptoms through protecting vaginal mucosal cells against oxidative damages and apoptosis. Thus, it may have successful effects against vaginitis; however, the open label design was the main limitation of our study.

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Author contributions

Fazele Heydarian Moghadam was responsible for data collection and writing the primary draft of the manuscript; Mojgan Tansaz supervised the study and revised the final draft; Soheila Aminimoghaddam contributed to the data analysis; Homa Hajimehdipoor prepared the gel.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

STD: sexually transmitted disease; PID: pelvic inflammatory disease; OS: oxidative stress; ROS: reactive oxygen species; BVG*: Boswellia* vaginal Gel*;* KOH: potassium hydroxide; UTI: urinary tract infection; TAC: total antioxidant capacity; FRAP: ferric reducing antioxidant power; MDA: malondialdehyde; TBA: thiobarbituric acid