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Original Article

In vitro antitrichomonal activity of Satureja khuzestanica and main essential oil components carvacrol, thymol, and eugenol

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Abstract

Introduction: Human trichomoniasis is a widespread sexually transmitted disease and the concern of drug resistance in the parasite is growing. Hence, this study was performed to evaluate *in vitro* antitrichomonal activity of *Satureja khuzestanica*, carvacrol, thymol, eugenol, and phytochemical evaluation of the *S. khuzestanica* oil.

Methodology: Extracts and essential oil of *S. khuzestanica*, and the components were prepared. Then, susceptibility testing was performed using the microtiter plate method and *Trichomonas vaginalis* isolates. The minimum lethal concentration (MLC) of the agents was determined in comparison with metronidazole. Also, the essential oil was investigated by gas chromatography-mass spectrometry and gas chromatography-flame ionization detector.

Results: After 48 hours of incubation, carvacrol and thymol were the most effective antitrichomonal agents with MLC of 100 µg/mL, followed by the essential oil and hexanic extract (MLC = 200 µg/mL), then eugenol and methanolic extract (MLC = 400 µg/mL), in comparison with the metronidazole MLC of 6.8 µg/mL. Overall, 33 identified compounds accounted for 98.72% of the total essential oil composition with carvacrol, thymol, and p-cymene being the major constituents.

Conclusions: The results suggested the potency of *S. khuzestanica* and its bioactive ingredients against *T. vaginalis*. Thus, further *in vivo* studies are required to evaluate the efficacies of the agents.

Key words: Carvacrol; eugenol; *Satureja khuzestanica*; thymol; *Trichomonas vaginalis*.

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Introduction

Human trichomoniasis, caused by a protozoan parasite, is one of the most prevalent sexually transmitted infections (STIs) in the world, with a global prevalence of 276.4 million cases per year. Risk of HIV transmission, adverse consequences of pregnancy, infertility, and cervical neoplasia are important consequences of *Trichomonas vaginalis* infection transmission [1-3]. Metronidazole has been the drug of choice for the management of human trichomoniasis since 1961 [4]. The failure of Metronidazole in the treatment of trichomoniasis was first reported in 1962, and its incidence has been growing [5-7]. Consequently, the evidence suggests the need for research on new antitrichomonal agents for the treatment of the infection.

Medicinal plants are essentially considered the main natural sources of drugs; hence, various bioactive properties of plant compounds are of particular interest in medicine. *Satureja khuzestanica* (Lamiaceae), with the common Persian name "marzeh khuzestani", is an endemic plant of southern Iran. It has been traditionally considered as antiseptic and analgesic agent among the nomadic inhabitants of the area. Notably, its antimicrobial properties have also been proven in research. Carvacrol, known as 2-methyl-5-1-methylethyl-phenol and thymol, chemically named 2-isopropyl-5-methylphenol, two monoterpenoid phenols, are the main constituents of *S. khuzestanica* oil. In addition to their use as safe food additives, carvacrol and thymol are particularly known for their antimicrobial and antioxidant properties [8-11]. Eugenol (1-allyl-4-hydroxy-3-methoxybenzene), a phenylpropanoid compound, is another component of *S. khuzestanica* oil showing a wide range of antimicrobial activity against bacteria and fungi [12]. To examine the antitrichomonal properties of the plant and the natural compounds, the present study was specifically designed

to appraise the effect of *S. khuzestanica*, carvacrol, thymol, and eugenol on *T. vaginalis* parasite.

Methodology

Plant and Chemicals

The cultivated *S. khuzestanica* aerial part was prepared by the Khorraman Pharmaceutical Company (Lorestan, Iran). Carvacrol, thymol, and eugenol were purchased from Merck Chemicals Company. Metronidazole and Dimethyl sulfoxide (BioReagent) were purchased from Sigma-Aldrich Chemical Company.

Extracts and essential oil preparation

The aerial parts of the dried plant were powdered and extraction was done by maceration technique [13]. The powdered plant sample (100g) was immersed in 1-liter *n*-hexane, methanol, and distilled water for 72 hours. Evaporation of the plant extracts was done by a rotary evaporator at temperatures less than 40 °C. Essential oil of the plant powder (200 g) was obtained by hydro-distillation method using a Clevenger device for 3 hours [14]. Then, the obtained essential oil was dehydrated by anhydrous sodium sulfate.

Parasite isolates and solutions

Four distinct clinical isolates of *T. vaginalis* were grown in Diamond's medium and used for susceptibility testing in the log phase of growth [15]. Distilled water was applied to dissolve metronidazole powder and the aqueous extract, and dimethyl sulfoxide (DMSO) was employed to prepare the other plant solutions.

In vitro sensitivity testing, MLC and GI%

The minimum lethal concentration (MLC) indicates the least amount of an agent which immobilizes and kills all of the parasites after exposure [16]. The growth inhibitory percentage (GI %) refers to sublethal concentration (sub-MLC) causing some percentage of growth inhibition of parasites [17]. Susceptibility testing of the isolates was carried out on 96-well microtiter plates, according to the method provided by the Centers for Disease Control and Prevention [16]. Briefly, the serial concentrations of the plant products (50, 100, 200, 400, 800, 1600, and 3200 µg/mL) were prepared by serial twofold dilutions in Diamond's medium. Then, the parasite culture (1×10^5 trophozoites per mL) was transferred to the wells of the plate test and followed by incubation, for 24 and 48 hours, under aerobic conditions at 35.5 ºC. Susceptibility assay was performed in comparison with metronidazole at concentrations of 0.1 to 200 µg/mL. The MLCs of the agents was detected by inverted microscopic examination of the test wells [15]. The GI% was calculated according to the equation:

$$\mathrm{GI\%} = \frac{a-b}{a} \times 100$$

Where: a = the average number of cells in negative control wells, and b = the average number of cells in test wells [17].

Each susceptibility test was conducted in duplicate and repeated three times, in comparison to control. Finally, the MLCs of the agents were checked by no growth of the exposed trophozoites in fresh medium [15].

Analysis of the essential oil composition

Analysis of the essential oil components of *S. khuzestanica* was conducted by retention indices (RI) obtained by gas chromatography/mass spectroscopy. Thermoquest gas chromatograph with a flame ionization detector was used for GC investigation. Components separation of the essential oil in GC-FID and GC/MS was done by the DB-5 column (60 m × 0.25 mm; film thickness 0.25 µm). GC-MS analysis was carried out using gas chromatograph paired with a TRACE mass spectrometer equipped by ADAMS library [13].

Table 1. Efficacy of essential oil and extracts of *Satureja khuzestanica* and natural compounds against *Trichomonas vaginalis*, after 24 hours' exposure.

Agents	Growth inhibition *percent* (Mean[a] ± SD) at different concentrations of plant products						
	50 (µg/mL)	100 (µg/mL)	200 (µg/mL)	400 (µg/mL)	800 (µg/mL)	1600 (µg/mL)	3200 (µg/mL)
Essential oil	14.8 ± 5.7	48.6 ± 4.6	[c]93.6 ± 4.9	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Hexane extract	74.6 ± 5.1	[c]95.4 ± 4.9	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Methanol extract	32.6 ± 5.3	56.5 ± 3.4	[c]93.4 ± 8.4	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Aqueous extract	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Carvacrol	[c]92.4 ± 2.2	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Thymol	55.3 ± 3.1	[c]81.6 ± 2.2	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Eugenol	35.3 ± 5.7	68.2 ± 3.5	[c]90.1 ± 0.0	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

[a]Mean of results; [b]MLC is related to the lowest concentration of the agent that kill all parasites ; [c]Sub-MLC is related to the concentration of agent that inhibit growth of parasite.

Table 2. Efficacy of essential oil and extracts of *Satureja khuzestanica* and natural compounds against *Trichomonas vaginalis*, after 48 hours' exposure.

Agents	Growth inhibition *percent* (Mean[a] ± SD) at different concentrations of plant products						
	50 (µg/mL)	100 (µg/mL)	200 (µg/mL)	400 (µg/mL)	800 (µg/mL)	1600 (µg/mL)	3200 (µg/mL)
Essential oil	35.8 ± 4.8	[c]74.3 ± 3.9	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Hexane extract	76.3 ± 5.3	[c]98.5 ± 4.5	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Methanol extract	52.4 ± 4.3	76.5 ± 3.5	[c]98.4 ± 3.4	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Aqueous extract	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Carvacrol	[c]99.4 ± 2.1	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Thymol	[c]70.3 ± 2.6	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Eugenol	55.4 ± 7.2	78.6 ± 3.2	[c]95.7 ± 0.0	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

[a]Mean of results; [b]MLC is related to the lowest concentration of the agent that kill all parasites; [c]Sub-MLC is related to the concentration of agent that inhibit growth of parasite.

Statistical analysis

SPSS software, version 16, was used for data analysis. The mean of MLC values were compared by Friedman's test and the *p value* < 0.05 was applied to be detected as significant.

Results

The essential oil and extracts of *S. khuzestanica* and its oil compounds had antitrichomonal activity and caused death of the parasites. The obtained MLCs were confirmed by no growth of the parasites in a fresh medium. After 24 hours, the most potent antitrichomonal compound was carvacrol with an MLC of 100 µg/mL. Also, among the oil and extracts, the hexanic extract was the most potent antitrichomonal agent with MLC of 200 µg/mL ($p < 0.001$) (Table 1). After 48 hours, the efficacy of most antitrichomonal agents increased and carvacrol and thymol were found as the most potent antitrichomonal agents with MLC of 100 µg/mL ($p < 0.001$) (Table 2). At the sublethal concentration (sub-MLC), GI% of the agents ranged from 81.6 ± 2.2 to 95.4 ± 4.9 and 70.3 ± 2.6 to 99.4 ± 2.1 for 24 and 48 hours of exposure, respectively (Table 1 and 2). Metronidazole susceptibility testing of the isolates revealed sensitivity of the parasite with MLCs of 12.5 and 6.2 µg/mL after 24- and 48-hours' incubation (Table 3).

Hydro-distillation of *S. khuzestanica* afforded an essential oil with 1.4% yield (w/w on the plant dry weight basis). The compounds of the essential oil were detected and they are listed in Table 3 with retention indices and presenting quantitative results. The compounds indicate 98.72% of the whole essential oil profile. Monoterpenes such as carvacrol, thymol, and p-cymene constituted the major class of compounds (91.67%) in the essential oil, as presented in Table 4 and Figure 1.

Discussion

The findings suggested that the studied agents had a potent antitrichomonal activity. The antimicrobial property of *S. khuzestanica* may be due to the bioactive phytochemical components such as p-cymene, myrcene, γ-terpinene, terpinene-4-ol, and especially thymol and carvacrol [8,9]. Growth inhibitory activities of the agents were increased on the second day of exposure. After 48 hours, carvacrol and thymol were the most potent antitrichomonal agents (MLC = 100 µg/mL). They were followed by the oil and hexanic

Figure 1. GC-Mass chromatogram of Satureja khuzestanica essential oil.

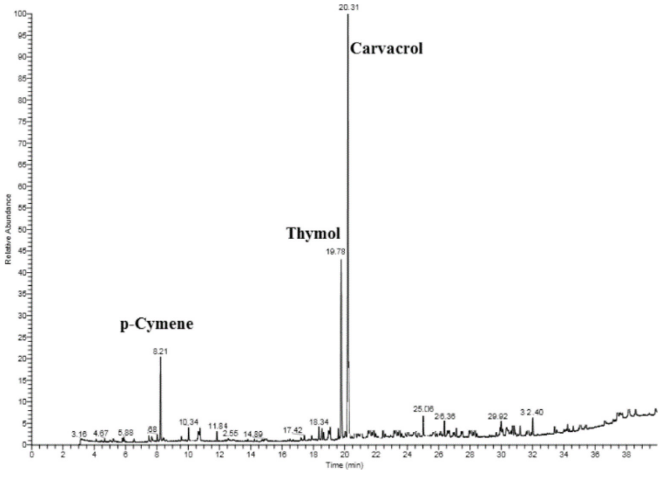

Table 3. Efficacy of metronidazole against *Trichomonas vaginalis*.

Incubation time	Growth inhibition *percent* (Mean[a] ± SD) at different concentrations of metronidazole									
	0.4 (µg/mL)	0.8 (µg/mL)	1.6 (µg/mL)	3.1 (µg/mL)	6.2 (µg/mL)	12.5 (µg/mL)	25 (µg/mL)	50 (µg/mL)	100 (µg/mL)	200 (µg/mL)
24 hours	52.8 ± 2.6	55.0 ± 4.6	72.6 ± 2.3	84.6 ± 2.7	[c]93.4 ± 0.6	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
48 hours	64.0 ± 2.2	83.4 ± 2.5	92.5 ± 2.3	[c]98.2 ± 0.4	[b]100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

[a]Mean of results; [b]MLC is related to the lowest concentration of the agent that kill all parasites; [c]Sub-MLC is related to the concentration of agent that inhibit growth of parasite.

extract of *S. khuzestanica* (MLC = 200 µg/mL). Although the results are remarkable but the antitrichomonal activity of the tested agents significantly lower than that of metronidazole. The *Trichomonas* isolates were sensitive to metronidazole. The MLCs of metronidazole were 12.5 and 6.2 µg/mL, after 24 and 48 incubations, respectively.

The cytoplasmic membrane is a cellular target for essential oil components. The antimicrobial activity of essential oils is mainly due to their phenolic compounds. However, there is evidence that minor components can play a synergistic role with other components. Carvacrol and thymol are synthesized from γ-terpinene via p-cymene as precursors. The role of the hydroxyl group in the properties of phenolic compounds such as carvacrol and thymol has been demonstrated. Carvacrol and thymol, as phenolic monoterpenes, are structurally similar but differ in the hydroxyl position in the phenolic ring. Monoterpenes

Table 4. Essential oil compounds of leave and flower of *Satureja khuzestanica*.

No	Compound	RI[a]	Percentage
1	α-Thujene	924	0.23
2	α-Pinene[b]	932	0.47
3	Camphene	946	0.56
4	β-Pinene	974	0.49
5	cis-Pinane	982	0.21
6	Myrcene	988	0.45
7	α-Phellandrene	1002	0.21
8	α-Terpinene	1014	0.9
9	ρ-Cymene	1020	7.31
10	o-Cymene	1022	0.65
11	1,8-Cineole	1026	1.62
12	γ-Terpinene	1054	2.05
13	cis-Sabinene hydrate	1065	0.22
14	Terpinolene	1086	0.9
15	o-Guaiacol	1087	0.12
16	Linalool[b]	1095	0.25
17	Borneol	1165	1.32
18	Pinocampheol	1166	0.78
9	Cryptone	1183	0.4
20	α-Terpineol	1186	0.66
21	Thymol	1289	14.74
22	Carvacrol	1298	54.81
23	Eugenol	1356	0.29
24	Z-Caryophyllene	1408	2.03
25	Aromadendrene	1439	1.16
26	Seychellene	1444	0.13
27	α-Humulene	1452	0.6
28	Viridiflorene	1496	0.35
29	β-Bisabolene	1505	0.62
30	Germacrene A	1508	0.14
31	Z-γ-Bisabolene	1514	1.67
32	Spathulenol	1577	0.69
33	Caryophyllene oxide	1582	1.69
Classification of the constituents			
	Monoterpenes		91.67
	Sesquiterpenes		7.05
	Total		98.72

[a] RI, Retention indices relative to C7 – C24 n-alkanes on the DB-5 column; [b] The identification was also confirmed by co-injection with an authentic samples.

substances probably cause cell membrane permeability changes or cell wall lysis [18,19]. They can cause apoptosis in eukaryotic cells by altering the permeability of mitochondrial membranes. Monoterpenes also interfere with protozoa metabolism by inhibiting the DiHydroFolate Reductase enzyme and lead to cell death [20]. Antiprotozoal Activity of carvacrol, and thymol was investigated by Tasdemir *et al*. [21]. In this study carvacrol was effective on *Trypanosoma brucei rhodesiense* (IC_{50} = 0.15 ± 0.04 µg/mL), *Trypanosoma cruzi* (IC_{50} > 90 µg/mL), *Leishmania donovani* (IC_{50} = 13.1 ± 3.9 µg/mL), and *Plasmodium falciparum* (IC_{50} = 6.4 ± 0.9 µg/mL). Thymol, as an isomer of carvacrol, was one of the phytochemicals tested by Tasdemir *et al.* that had a potent antiprotozoal effect on *T. b. rhodesiense* (IC_{50} = 0.11 ± 0.01 µg/mL), *T. cruzi* (IC_{50} > 90 µg/mL), *L. donovani* (IC_{50} = 17.3 ± 4.1 µg/mL), and *P. falciparum* (IC_{50} = 5.7 ± 0.03 µg/mL). The nematocidal activity of the two monoterpenoids has been demonstrated against parasitic and free-living nematodes.

The substances have a toxic effect on *Bursaphelenchus xylophilus*, a plant pathogenic nematode, with LC_{50} of 1.23 mg/mL for carvacrol and LC_{50} of 1.08 mg/mL for thymol [22]. The free-living nematode *Caenorhabditis elegans* and the pig roundworm *Ascaris suum* were used for other investigations. In this research, mortality rates of carvacrol and thymol were 99 ± 1% and 100 ± 0% at 0.67 mM concentration, respectively [23]. Carvacrol and thymol interact with nematode tyramine receptor (TyrR) in desensitizing for tyramine activation and in translocating *C. elegans* TyrR (SER-2) receptor from membrane to cytoplasm [19].

The present study showed the antitrichomonal properties of eugenol, although its effect was less than that of carvacrol and thymol. previously, antileishmanial activity of eugenol-rich essential oil and eugenol derivatives was demonstrated. The eugenol-rich essential oil of *Ocimum gratissimum* inhibited the growth of *L. amazonensis* at a concentration ranging from 100 to 1000 µg/mL [24]. Some compounds of 1,2,3-triazole eugenol derivatives had antileishmanial activity. The most potent compound was 4-(3-(4-allyl-2-methoxyphenoxy)propyl)-1-(4-methylbenzyl)-1H-1,2,3-triazole with IC_{50} of 7.4 ± 0.8 µmol/L [25].

According to our knowledge, there is no comprehensive information on the antiprotozoal activity of *S. khuzestanica*. The antileishmanial property of *S. khuzestanica* was investigated by Sadeghi-Nejad *et al.* In this study, ethanolic and methanolic extracts of *S. khuzestanica* inhibited the

growth of *L. major* promastigotes after 48 hours of incubation, at concentrations of 2.4 and 4.8 mg/mL, respectively [26]. This result suggested that the methanolic extract of *S. khuzestanica* is more effective on *T. vaginalis* (MLC = 400 µg/mL) than on *L. major* promastigotes. *In vitro* antigiardial activity of *S. khuzestanica* was reported by Fallahi *et al.* This research indicated the efficacy of *S. khuzestanica* oil on *Giardia* cysts with a fatality rate of 38.05% at a concentration of 5 mg/mL after 4 hours of exposure [27].

Until now, a significant number of reports have been published about the effect of medicinal plants against *T. vaginalis*. Three families of plants including *Asteracea*, *Lamiaceae,* and *Myrtaceae* cover most plant species with antitrichomonal properties, with some of their species displaying considerable anti-parasitic activity [28]. According to Jiménez-Arellanes *et al.*, extracts of avocado seeds (*Persea Americana*) are one of the most potent antitrichomonal agents. The chloroformic and ethanolic extracts of *P. americana* inhibited growth of *T. vaginalis* by 50% at concentrations of 0.524 and 0.533 µg/mL, respectively, compared to metronidazole with an IC_{50} at 0.037 µg/mL [29]. The effect of these extracts has been far stronger than that of the extracts used in the present study, although their effects were evaluated after 72 hours of incubation. Ezz Eldin and his colleagues demonstrated the efficacy of *Ocimum basilicum* oil on *T. vaginalis* at MLCs of 30 µg/mL, after 24 hours, 20 µg/mL after 48 hours, and 10 µg/mL after 96 hours [30]. Since the major ingredient of the essential oil of *O. basilicum* is linalool and that of *S. khuzestanica* is carvacrol, consequently, linalool should be more effective than carvacrol on the parasite. Another encouraging result has been reported by Taran and colleagues. In this study, they investigated the antitrichomonal efficacy of *Allium hirtifolium* (Persian Shallot). The results suggested hydroalcoholic and dichloromethane extracts of *A. hirtifolium* were highly effective on *T. vaginalis* at concentrations of 10 and 5 µg/mL, respectively after 48 hours [31]. The antimicrobial properties of *Allium* species are attributed to organosulfur compounds such as allicin, alliin, ajoene, and diallyl sulfides [32]. Spermicidal and antitrichomonal activities of *Sapindus saponaria* were also demonstrated by Damke *et al.* Water-ethanol and butanolic extracts of *S. saponaria* were able to immobilize spermatozoa at the concentration of 2.5 mg/mL, with antitrichomonal activity of the extracts being as follows: an MLC of 0.156 mg/mL (related to the two extracts) for a clinical strain and an MLC of 0.312 and 0.156 mg/mL (related to water-ethanol and butanolic extract, respectively) for ATCC strain [33]. These results almost reflect the similarity of the antitrichomonal activity of *S. saponaria* and *S. khuzestanica*. In addition, the susceptibility variation in this study has been in accordance with our results. Other potent antimicrobial plants are *Verbascum thapsus*, *Lobophora variegate*, *Xanthium brasilicum*, *Zataria multiflora*, *Eucalyptus camaldulensis*, *Artemisia aucheri*, and *Myrtus communis*, which have shown evidence of remarkable *in vitro* efficacy on *T. vaginalis* [28]. Some phytochemicals, such as terpenoids, saponins, alkaloids, and glycosides, have been described to possess *in vitro* activity against *T. vaginalis* [27]. Hederagenin, a triterpenoid saponin derived from *Cussonia holstii* plant, has proven to be a strong antitrichomonal natural compound causing 50% growth inhibition of *T. vaginalis* with an IC_{50} 2.8 mM after 72 hours (He *et al.,* 2003). In another study, saponins derived from *S. saponaria* were investigated against *T. vaginalis* by Damke *et al.* The results indicated significant antitrichomonal activity of saponins with an MLC of 0.078 mg/mL after 24 hours [33]. The antitrichomonal activity of saponins was similar to that of carvacrol obtained in our study.

Conclusions

Although the antitrichomonal potency of *S. khuzestanica* and its bioactive components were demonstrated in this study, but, further researches are required to evaluate the effects of the agents under in-vivo conditions.

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