



Essential Oil Components and Antitrichomonal Effects of *Piper nigrum* L.

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Abstract

Background: Trichomoniasis, caused by *Trichomonas vaginalis* protozoan, is the most common nonviral sexually transmitted infection (STI) worldwide. Although metronidazole and tinidazole are the only approved drugs for treatment, drug-resistant cases of infection are on the rise. The aim of this study was the evaluation of antitrichomonal potential of *Piper nigrum* and limonene. The phytochemical profile of *P. nigrum* oil was also investigated.

Methods: The parasites were treated *in vitro* with essential oil and different extracts of *P. nigrum* seed and limonene using microtiter plate method. The oil of *P. nigrum* was also analyzed by gas chromatography-mass spectrometry and gas chromatography-flame ionization detector. Furthermore, the cytotoxicity assay of *P. nigrum* oil and limonene were screened on Vero cell line by MTT method.

Results: The tested *P. nigrum* fractions were able to kill 100% of *Trichomonas* trophozoites at minimum lethal concentration (MLC) and reduce the trophozoite viability at sub-MLC and lower concentrations. After 48 hours exposure, the most potent fraction was the n-hexane extract with MLC of 78 µg/mL followed by the essential oil and methanol extract with MLC of 156 µg/mL, limonene (MLC = 1250 µg/mL), and then, aqueous extract with MLC value of 25 mg/mL. Moreover, according to cytotoxicity assay, *P. nigrum* oil was less toxic to Vero cell than limonene, with a selectivity index (SI) of 13.2 and 2.04, respectively.

Conclusion: This study clearly demonstrated the trichomonacidal potential of *P. nigrum*. Thus, *P. nigrum* fractions can be considered promising antiprotozoal agents and the basis for further development to discover new phytochemicals compounds.

Keywords: Essential oil, Extract, Limonene, *Piper nigrum*, *Trichomonas vaginalis*

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Introduction

Trichomonas vaginalis is a protozoan parasite that causes human urogenital trichomoniasis. The infection is considered as the most common nonviral sexually transmitted infection (STI) worldwide, with a global prevalence of 5.3% in women and 0.6% in men, in 2016. The estimated curable STIs incidence for 2016 was 376.4 million cases, of which 156.0 million were related to trichomoniasis, higher than chlamydia (127.2 million), gonorrhoea (86.9 million) and syphilis (6.3 million). Asymptomatic trichomoniasis occurs in at least 50% of women and 70 to 80% of men. Vaginal or urethral discharge, dysuria, pelvic pain, and itching appear in symptomatic infection. Increased risk of HIV transmission, infertility, and adverse pregnancy outcomes are among the complications of trichomoniasis (1-3). Metronidazole is still the drug of choice for the treatment of trichomoniasis in most parts of the world.

Metronidazole-resistant strains of *T. vaginalis* have been demonstrated. In the United States, the prevalence of resistant strains is estimated to be 2% to 5% among clinical isolates (4,5). Due to the high prevalence of trichomoniasis and the emergence of drug-resistant strains, research on new drugs is needed to effectively control the infection.

Medicinal plants are an important source of the active natural compounds from which many medicines are made. Therefore, herbs and phytochemical compounds are of special importance for pharmaceutical research. *Piper nigrum* L. (Piperaceae), known as black pepper, is grown in the tropics and is native to India. In addition to be used as a spice, *P. nigrum* have been widely used in traditional medicines. Among the applications of black pepper in traditional medicine are: treatment of abdominal tumors, abdominal fullness, cholera, cold, colic, asthma and headache, in Thailand, and treatment



of epilepsy, and respiratory or gastric cancers, in China. It is also used in traditional Ayurvedic medicine as an antipyretic, and for improving pulmonary and gastrointestinal disorders (6). *P. nigrum* is considered as an antihypertensive, antispasmodic, anti-inflammatory, anti-diarrheal, hepato-protective, and anti-thyroid agent. Its antimicrobial activity has also been demonstrated. In addition to antibacterial and antifungal activity, *P. nigrum* have an inhibitory effect on *Trypanosoma* and *Leishmania* species (7-9). Due to the antiprotozoal activity of *P. nigrum*, this study was performed to evaluate the antitrichomonal property of *P. nigrum* and limonene, as one of the main essential oil compounds of *P. nigrum*.

Material and Methods

Plant and chemicals

Black pepper fruits were purchased from the market of medicinal plants in Hamadan, western Iran, and botanically authenticated. D-limonene (8.18407.0100) was bought from Merck Chemicals Co. (Darmstadt, Germany) and Metronidazole (M3761) and Dimethyl sulfoxide (D2650, BioReagent) were bought from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.).

Extracts and essential oil preparation

The fruits of black pepper were crashed and extraction was performed by maceration method (10). In brief, the powdered black pepper (100 g) was sequentially soaked in three solvents of increasing polarity: *n*-hexane, methanol and distilled water (3×1 L, rt for 72 hours). The mixtures were filtered, by Whatman filter paper (No. 1), and concentrated by a rotary evaporator under vacuum at below 40 °C. Essential oil was extracted by hydrodistillation of 100 g of black pepper powder in 250 mL of distilled water for three hours' extraction time, using a Clevenger-type apparatus (11). Then, the oil was dehydrated by anhydrous sodium sulfate and stored in dark airtight container at 4 °C until use.

Isolates and solutions

Five clinical *T. vaginalis* isolates were used for drug susceptibility testing. The isolates were axenically cultured in TYI-S-33 medium and used in logarithmic growth phase (12). Metronidazole was dissolved in distilled water and its concentration was reduced from 200 µg/mL to 0.1 µg/mL by two-fold serial dilution method in Diamond's medium. The natural products were dissolved in distilled water or DMSO according to the degree of their polarity. Then, the natural solutions were diluted in culture medium to achieve concentrations between 2500 µg/mL and 19.5 µg/mL.

MLC, GI% and susceptibility testing

The minimum lethal concentration (MLC) is defined as the lowest concentration of an antimicrobial agent that immobilized and killed all of *T. vaginalis* trophozoites (13).

The growth inhibitory percentage (GI%) is considered as sublethal (sub-MLC) and less concentrations that relatively inhibit the growth of *T. vaginalis* trophozoites (14). A 96-well microtiter plate method was used for antitrichomonal susceptibility testing as described by the Centers for Disease Control and Prevention (13). Aerobic susceptibility testing was conducted in duplicate against control, under sterile condition. The tests were repeated independently at least two times. Concisely, 100 µL of the parasite culture (2×10^5 cells/mL) was added to the wells of microtiter plate, containing 100 µL of the antitrichomonal agents, and incubated at 35.5 °C for 24 and 48 hours. Then, the microtiter plates were investigated by inverted microscope for determination of MLCs. To achieve GI% of the agents, the number of active trophozoites was counted in the control and sub-MLC wells and compared, according to the following formula:

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

Where, a = Mean number of trophozoites in the negative control wells and b = Mean number of trophozoites in the test wells at sub-MLC concentration (14). Finally, MLC of the tested agents was confirmed by achieving negative culture of the exposed trichomonads (12).

Identification of essential oil compositions

The essential oil compositions were identified by gas chromatography-mass spectrometry (GC-MS). GC analysis was conducted using a Thermoquest gas chromatograph with a flame ionization detector on DB-5 column (60 m×0.25 mm; film thickness 0.25 µm). Also, GC-MS analysis was done similarly by GC column using gas chromatograph coupled to a TRACE mass spectrometer (10).

Cytotoxicity assay

Cytotoxicity effect of *P. nigrum* essential oil and limonene was tested on African green monkey kidney (Vero) Cell Line. Vero cells were obtained from national cell bank of Iran (NCBI, Pasteur Institute of Iran) and cultured in DMEM medium supplemented with 10% fasting blood sugar (FBS), 100 u/mL penicillin and 100 µg/mL streptomycin, at 37 °C and 5% CO₂. Vero cells were trypsinized, followed by washing with PBS. The cells were seeded in 96-well plates (5×10^3 cells/well) for 24 hours of incubation. Then, the cells were treated with different concentrations of the natural products for 24, 48, and 72 hours of incubation time. Cell viability was assessed by MTT assay. Briefly, 10 µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added into each well and after 4 hours, the wells were substituted with 100 µL of DMSO to terminate the assay. The optical density was measured at 570 nm using an ELISA plate reader. The viability (%) was calculated according to the following equation:

$$\% \text{Viability} = (B/A) \times 100$$

Where, A and B are the optical density of untreated and treated cells, respectively. The half maximal cytotoxic concentration (CC_{50}) was considered as the natural products concentration, which reduced cell viability by 50% compared to untreated cells. CC_{50} values were calculated from the plot of percentages of cell viability versus concentrations of the natural products (15). The half-maximum inhibitory concentration (IC_{50}) and the selectivity index (SI) were obtained from the linear regression curve and the CC_{50}/IC_{50} ratio, respectively.

Statistical analysis

Data were presented as means \pm standard deviation of experiments. Data were analyzed by Friedman's test using SPSS 16 software and a probability value of $P < 0.05$.

Results

The extracts and essential oil of *P. nigrum* and limonene were able to kill all of *T. vaginalis* trophozoites by causing cell lysis at MLC values. They were also able to reduce the trophozoite viability at sub-MLC and lower concentrations of the tested agents. The effect of plant products was dependent on the concentration and exposure time (Figures 1 and 2). Figure 1 shows the antitrichomonal activity of plant products after 24 hours of incubation. The oil and n-hexane extract of *P. nigrum* were the strongest antitrichomonal agents with MLC of 156 $\mu\text{g}/\text{mL}$ and the MLC of methanol extract and limonene were 312 and 1250 $\mu\text{g}/\text{mL}$, respectively. The aqueous extract was the weakest agent with MLC of 25 mg/mL , among the tested agents ($P < 0.001$). Also, the growth inhibitory property of plant products at sub-MLC concentration was ranged from 70.07% to 92.4%. According to the data in Figure 2, the growth inhibition potential of plant products increased after 48 hours' incubation and consequently, the lethal concentration of n-hexane (MLC = 78 $\mu\text{g}/\text{mL}$) and methanolic extract

(MLC = 156 $\mu\text{g}/\text{mL}$) decreased by one fold ($P < 0.001$).

Drug susceptibility testing demonstrated the susceptibility of *Trichomonas* isolates to metronidazole, as a standard antibiotic and antiprotozoal medication. The sensitivity of parasites was 3.1 ($\mu\text{g}/\text{mL}$) and 12.5 ($\mu\text{g}/\text{mL}$) at 24 and 48 hours of incubation, respectively (Table 1). The maximum essential oil was obtained with a yield of 2% (w/w %) by hydrodistillation. GC/MS analysis was detected 27 constituents for 98.06% of the entire essential oil of *P. nigrum* (Table 2). β -caryophyllene (40.62%), limonene (12.64%) and β -pinene (11.20%) were the major compounds in the essential oil (Figure 3). The *P. nigrum* oil and limonene were tested for their toxicity against Vero cell line. CC_{50} and SI of the oil and limonene have been presented in Table 3.

Discussion

Successful determination of phytochemical compounds largely depends on the type of solvent used in the plant extraction process. Therefore, we used different solvents, both polar and non-polar, for extraction, which led to relatively different results. Overall, the results of this study indicate the antitrichomonal potential of black pepper. The tested agents were able to kill all of the *Trichomonas* trophozoites at MLC concentrations. Among the tested plant products, the oil and n-hexane extract of black pepper exhibited strong activity against the parasites. The experiments also showed that the antitrichomonal activity of plant agents depends on dose and time exposure. Therefore, after 48 hours' exposure, the growth inhibitory potency of the agents was increased.

Pungent and volatile oil compounds are main components of black pepper. Piperine, the main black pepper alkaloid, is responsible for the pungency of black pepper. The amount of piperine in black pepper is high and varies between 2% to 9%, depending on the environmental conditions (16). The antimicrobial potential of piperine was previously demonstrated. Aldaly investigated the antimicrobial activity of piperine against

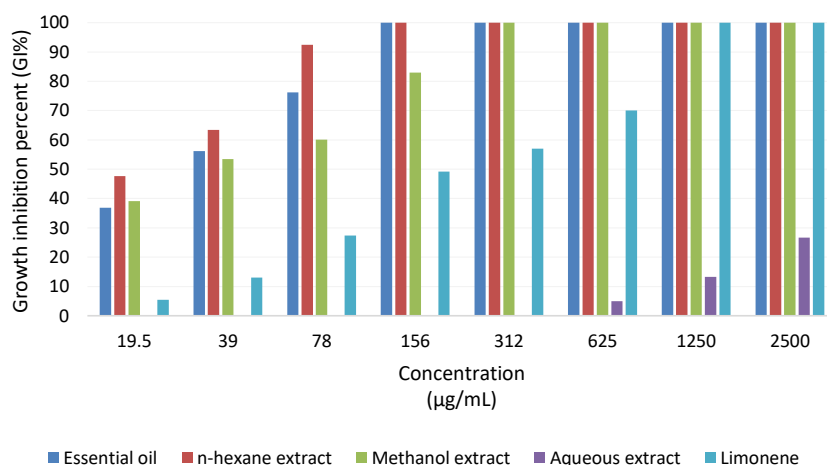


Figure 1. The efficacy of essential oil and extracts of *Piper nigrum* L. and limonene on *Trichomonas vaginalis* after 24 hours' incubation time

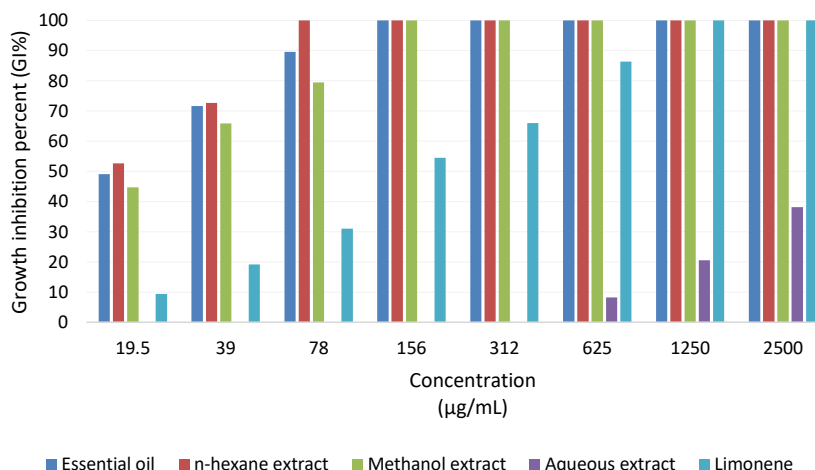


Figure 2. The efficacy of essential oil and extracts of *Piper nigrum* L. and limonene on *Trichomonas vaginalis* after 48 hours' incubation time

Table 1. The results of metronidazole susceptibility testing of *Trichomonas vaginalis* isolates

| Incubation time | Mean & standard deviation of growth inhibition percent (GI%) 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 h | 55.8±6.4 | 60.1±5.6 | 75.8±5.6 | 85.6±2.7 | 98.4±0.7 ^b | 100.0±0.0 ^a | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
| 48 h | 51.0±3.9 | 72.4±2.8 | 83.5±4.3 ^b | 100.0±0.0 ^a | 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 Minimum lethal concentration (MLC) is related to the lowest concentration of the antitrichomonal agents that kill all trichomonads.

^b Sub-MLC concentration is related to inhibition of trichomonads growth.

gram positive and negative bacteria and *Candida albicans*. In this study, piperine was able to inhibit the growth of microorganisms in the range between 3.125 and 100 mg/mL, with the greatest effect on *C. albicans* (17). Piperine and its derivatives also possess anti-trypanosomatid activity. They have an inhibitory effect on promastigotes of *Leishmania* species and epimastigotes of *Trypanosoma cruzi* (16). Antimalarial Activity of piperine was also studied by Thiengsusuk et al and IC₅₀ values of piperine against chloroquine-resistant (K1) and chloroquine-sensitive (3D7) *plasmodium falciparum* strains were estimated to be 111.5 and 59 µM, respectively (18). In the present study, the extracts of black pepper also exhibited considerable effect on *T. vaginalis* which is expected to be due to piperine or other compounds in the extracts.

In addition, we investigated the composition of essential oils by the GC and GC/MS techniques. The main compounds identified in the essential oil were β-caryophyllene, limonene and β-pinene. Limonene is the second ingredient in black pepper oil. Limonene, classified as a cyclic monoterpene, is one of the most common terpenes found in nature and aromatic plants. Limonene and its metabolites have a variety of biological properties, including antiproliferative activity. The anticancer properties of limonene have been demonstrated by its ability to induce apoptosis. In addition, limonene has good antimicrobial activity against microbial pathogens (19,20). These properties of limonene have been confirmed by research. In the study of Han et al, the antibacterial activity of limonene has been demonstrated.

In the mentioned study, limonene inhibited the growth of *Staphylococcus aureus* at a concentration of 20 mL/L (19). The antimicrobial properties of black pepper oil may be due to the presence of bioactive phytochemical compounds alone or synergistically with other components. The mechanism of cell lysis of plant essential oil and its compounds has been partially elucidated by research. Cell membrane disruption is the most well-known cell lysis pathway for these compounds. Limonene, as one of the main components of black pepper oil, can affect the permeability of cell membranes and disrupt it. In addition, limonene can interfere with cellular energy metabolism and inhibit ATP synthesis by inhibiting ATPase and respiratory chain enzymes (19,20). In the present study, as expected, the antitrichomonal activities of black pepper oil and limonene were also remarkable. The results of our study show that the antitrichomonal activity of black pepper oil (MLC = 150 µg/mL) is higher than limonene (MLC = 1250 µg/mL). This fact may be due to the synergy of the compounds or the presence of more effective compounds in the oil than in limonene. Also, the results of cytotoxic test showed that limonene was more toxic to Vero cells than the oil and has a lower SI.

As far as we know, information about the antiparasitic properties of black pepper is limited. Chouhan and colleagues studied the antileishmanial efficacy and metabolic profiles of black pepper extracts. In Chouhan and colleagues' study, hexane and ethanolic extracts of black pepper were able to inhibit the growth of *L. donovani* promastigotes at IC₅₀ of 31.6 and 37.8 µg/mL,

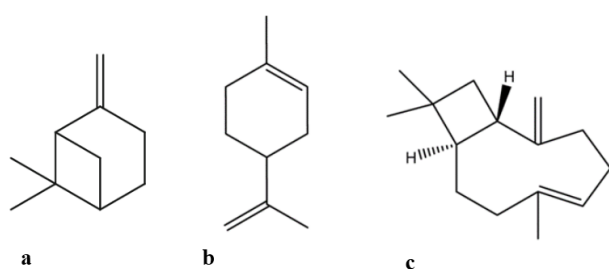


Figure 3. Chemical structures of β -pinene (a), limonene (b) and β -caryophyllene (c)

Table 2. Composition of the essential oil of *Piper nigrum*

| RT | Compound | RI ^a | Percentage |
|-------|---------------------------|-----------------|------------|
| 5.62 | α -Thujene | 923 | 2.3 |
| 6.26 | Camphene ^b | 945 | 0.2 |
| 6.91 | Sabinene | 967 | 2.95 |
| 7.04 | β -Pinene | 974 | 11.20 |
| 7.85 | α -Phellandrene | 1002 | 0.30 |
| 8.10 | δ -3-Carene | 1007 | 9.6 |
| 8.59 | O-Cymene | 1021 | 0.73 |
| 8.69 | Limonene | 1022 | 12.64 |
| 8.70 | β -Phellandrene | 1025 | 0.74 |
| 9.78 | γ -Terpinene | 1053 | 0.21 |
| 10.98 | Terpinolene | 1086 | 0.20 |
| 11.32 | Linalool ^b | 1094 | 0.35 |
| 14.66 | Terpinen-4-ol | 1172 | 2.1 |
| 15.21 | α -Terpineol | 1185 | 0.15 |
| 23.49 | α -Copaene | 1374 | 0.50 |
| 24.15 | β -Elemene | 1387 | 2.65 |
| 25.06 | α -Cedrene | 1408 | 0.71 |
| 25.10 | α -cis-Bergamotene | 1410 | 0.82 |
| 25.36 | β -Caryophyllene | 1416 | 40.62 |
| 28.37 | β -Selinene | 1486 | 2.1 |
| 29.04 | β -Bisabolene | 1502 | 1.77 |
| 29.42 | β -Curcumene | 1511 | 0.31 |
| 29.72 | δ -Cadinene | 1520 | 0.31 |
| 31.96 | Spathulenol | 1575 | 0.43 |
| 32.16 | Caryophyllene oxide | 1580 | 1.45 |
| 34.63 | Cubenol | 1642 | 2.4 |
| 36.17 | α -Bisabolol | 1682 | 0.32 |
| | Total | | 98.06 |

^a RI, Retention indices relative to C7–C30 n-alkanes on the DB-5 column

^b The identification was also confirmed by co-injection with an authentic sample

Table 3. Cytotoxic activity of *Piper nigrum* essential oil and limonene against Vero cell line

| Compound | 24 hours' exposure <i>Trichomonas vaginalis</i> | | | 48 hours' exposure <i>Trichomonas vaginalis</i> | | |
|-------------------------------|---|--------------------------------|-------|---|--------------------------------|------|
| | IC ₅₀ (μ g/mL) | CC ₅₀ (μ g/mL) | SI | IC ₅₀ (μ g/mL) | CC ₅₀ (μ g/mL) | SI |
| <i>Piper nigrum</i> essential | 29.5 | 309 | 10.47 | 19.5 | 257.42 | 13.2 |
| Limonene | 158.48 | 446.68 | 2.82 | 138 | 281.83 | 2.04 |

Data presented are the mean of three experiments.

SI: selectivity index.

respectively. Furthermore, the ex vivo assessment showed that the extracts stopped the growth of intra-macrophagic *Leishmania* amastigotes as follow: the hexane extract with IC₅₀ of 14.6 μ g/mL and the ethanolic extract with IC₅₀ of 18.3 μ g/mL. Also in this study, the analysis of black pepper extracts revealed that trans- β -caryophyllene (22.28%) and piperine (70.36%) are the main components in the hexane and ethanolic extracts, respectively (21). In our study, the efficacy of hexanic and methanolic extract of black pepper on *Trichomonas* is consistent with the efficacy of hexanic and ethanolic extract of black pepper on *Leishmania* in the study of Chouhan et al.

In another study by Chouhan et al, the mechanism of cell death and the biological effects of the black pepper extracts were investigated. The results of this study indicate that the antileishmanial effect of black pepper extracts is exerted through apoptosis induction. Evidence of apoptosis included phosphatidylserine externalization, chromosomal DNA cleavage, induction of cell cycle arrest in the G0/G1 phase, and mitochondrial membrane disorders and production of reactive oxygen species. *In vivo* investigation of the biological effects of extracts also showed that they provide a strong protection against *L. donovani* infection in BALB/c mice. This protection was due to enhanced cell-mediated immunity response by increasing the secretion of Th1 cytokines (INF- γ , TNF- α and IL-2) and decreasing IL-4 and IL-10 (9).

The other study on antiprotozoal properties of black pepper has been done by Shaba *et al.* In this study, antitrypanosomal activity of black pepper extract was evaluated. The methanolic extract induced immobilization, reduction in the number, and death of *Trypanosoma evansi* according to the concentration. The methanolic extract of black pepper reduced the number of trophozoites at 750 μ g/mL and killed all of the trypanosomes in concentration of 1000 μ g/mL, at the end of 7 hours of incubation.

Finally, it should be noted that these studies were performed on drug susceptible strains of parasites and the main limitation of our study was also the lack of access to drug resistant strains. Therefore, no prediction can be made about the effect of black pepper fractions or its derivatives on the metronidazole resistant *T. vaginalis*.

Conclusion

In summary, the results of this study clearly demonstrated the trichomonacidal potential of black pepper, with no significant toxicity on African green monkey kidney

cells. Thus, black pepper fractions can be considered promising antiprotozoal agents and the basis for further development to discover new phytochemical compounds.

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Authors' Contribution

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Funding acquisition: Mohammad Matini.

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Methodology: Mohammad Matini, Mohammad Fallah.

Project administration: Mohammad Matini, Dara Dastan.

Resources: Mohammad Matini.

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Supervision: Mohammad Matini, Dara Dastan.

Validation: Mohammad Matini.

Visualization: Mohammad Matini, Dara Dastan, Mohammad Fallah.

Writing—original draft: Ali Jamshidi-Zad, Farid Azizi-Jalilian.

Writing—review & editing: Mohammad Matini, Dara Dastan, Mohammad Fallah.

Competing Interests

The authors declare that they have no conflict of interests to disclose.

Ethical Approval

This study, with ethics code IR.UMSHA.REC.1397.885, has been approved by the ethics committee of Hamadan University of Medical Sciences.

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