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RESEARCH ARTICLE

ANTILEISHMANIAL ACTIVITY EVALUATION OF BLACK CUMIN EXTRACTS AGAINST *LEISHMANIA TROPICA*

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Abstract

Objectives: In addition to the high cost of antileishmanial drugs, the resistance that develops against these drugs and their side effects has led to the investigation of leishmanicidal active ingredients from natural sources that may be cheaper and have no side effects. The purpose of this study is to use *in vitro* assays to examine the antileishmanial effect of various extracts made from black cumin seeds.
Methods: By using the shaking maceration technique, extracts of black cumin seeds were prepared by water, 60% aqueous ethanol, ethanol, methanol, chloroform, and n-hexane. The extracts' ability to inhibit *Leishmania tropica* isolates with the code MHOM/TR 2012/CBCL-LT was examined.
Results: The IC_{50} values for methanol, *n*-hexane, chloroform, water, ethanol, and 60% aqueous ethanol extracts were determined as 172.4, 355.5, 400.3, 673.3, 818.4, and 1148 µg/ml, respectively.
Conclusion: The extracts of black cumin seeds have demonstrated antileishmanial activity against *Leishmania tropica* promastigotes. To verify black cumin seeds' potential as an antileishmanial source, *in vivo* antileishmanial research is required.
Keywords: Antileishmanial, *Leishmania tropica*, *Nigella sativa*, promastigotes.

INTRODUCTION

People have been using *Nigella sativa* L. (*Ranunculaceae* family) as a medicinal herb and food flavoring for hundreds of years. Traditionally, the seeds and the fixed oil have been utilized as medicinal. In addition to its antimicrobial, anti-inflammatory, antioxidant, anticancer, antidiabetic, gastroprotective, and hepatoprotective qualities, its volatile constituents, minerals, and vital fatty acids are known to boost the immune system[1,2]. Because of its aromatic, ornamental, and nutritional qualities, it is commonly used as a spice in international cuisines. Furthermore, because of its appetite-stimulating, milk-increasing, menstrual-regulating, anti-jaundice, gas-relieving, and diuretic activities, it is a plant that is commonly chosen as a treatment for inflammatory diseases, and illnesses including colds, headaches, asthma, and rheumatism[3].
In western culture, the whole or ground seeds of *Nigella sativa* known as "black cumin or black seed" have a three-thousand-year history of use as a food component in Middle and Far Eastern cultures. Black cumin seeds contain thymoquinone, which is thought to be a successful medicinal agent and is responsible for most of the bioactive properties[4-7]. The use of *N. sativa* as a food ingredient has been approved by the FDA in the United States. *N. sativa* has been categorized as Generally Recognized As Safe (GRAS) by the Flavor and Extract Manufacturers Association (FEMA)[8]. Due to the presence of certain alkaloids, the essential oil with anti-inflammatory, antibacterial, antifungal, antiviral, anticancer, and antiparasitic activities has been categorized as a "chemical of concern" by the European Food Safety Authority (EFSA)[9-11].
The parasitic disease leishmaniasis is spread by the blood-feeding of female sandflies carrying the *Leishmania* species. There are three primary clinical manifestations of the disease: mucocutaneous (MCL), cutaneous (CL), and visceral (VL). Leishmaniasis is a serious public health issue, especially in developing countries. According to research by the World Health Organization (WHO), more than 1 billion people in 92 countries and 83 regions worldwide lived in areas endemic to leishmaniasis and were at risk of infection, with an additional 30,000 new VL cases and over 1 million new CL cases added each year[12]. Globally, 0.5 million VL cases and 1.5–2 million CL cases are

reported each year. Each type of the illness is expected to spread quickly to the majority of non-endemic locations due to wars, migration, climate change and global warming. *Leishmania infantum*, *L. donovani*, *L. aethiopica*, *L.* major, and *L. tropica* are the species that cause CL in the Old World. In Turkey, *L. tropica* is the most frequent causal agent of CL. It has been reported that in addition to the *CL*, *L. tropica* parasite has also caused VL in recent years[13,14].

Since there is no safe and reliable vaccine against leishmaniasis, chemotherapy is used to treat leishmaniasis. However, existing antileishmanial drugs have various disadvantages, such as toxicity, long-term application, resistance, and high costs that limit their use in clinical applications. To fight the illness, it is crucial to create novel antileishmanial formulations[15]. Hence, interest in plant-based compounds is increasing, *in vitro* and *in vivo* studies are being conducted to investigate plants and their extracts as new candidates for antileishmanial agents. In a study conducted by Mahmoudvand *et al.*, the *in vitro* antileishmanial effects of thymoquinone, the methanol extract and the essential oil of *Nigella sativa* were investigated against *L. tropica* and *L. infantum*[16]. TiAgNps-N sativa oil combination was previously investigated on *in vitro* and *in vivo*, *L. tropica* promastigotes and amastigotes[17]. There is limited study on the antileishmanial activity of this valuable plant. The comparative antileishmanial activity of black cumin seed extracts prepared with solvents having different polarities has been *in vitro* investigated against *L. tropica* for the first time in this study.

MATERIALS AND METHODS

Preparation of plant extracts

Black cumin seeds were obtained from the Arifoğlu spice company. The seeds were ground into powder and weighed in 5 gm portions, after which the extracts were prepared using the shaking maceration technique with water, 60% aqueous ethanol, ethanol, methanol, chloroform, and n-hexane at room temperature. The liquid portions were filtered and evaporated to dryness under low pressure in a rotary evaporator. The extracts have been stored in a deep freezer at -20ºC until analysis[18].

Production of *Leishmania tropica* Isolates

The *Leishmania tropica* isolate coded as MHOM/TR/ 2012/CBCL-LT was obtained from the Parasite Bank of Manisa Celal Bayar University Faculty of Medicine. The isolate was removed from the liquid nitrogen tank, thawed in a water bath, transferred into 5 ml of RPMI-1640 medium, mixed, and then centrifuged at 4000 rpm for 5 minutes. At the end of the centrifugation, the upper part was discarded, and 5 ml of medium was added to the pellet, and the centrifugation process was repeated. This washing procedure was repeated three times. After the final washing step, 500 µl of culture medium was added to the pellet, making it homogeneous, and 100 µl was taken from it and inoculated into Now-Mc Neal-Nicolle (NNN) culture media. NNN media were placed in an incubator at 26ºC. NNN media were checked for growth on consecutive days, and when the growth of parasites was observed, the parasites were taken from the media and inoculated into 5 ml of RPMI-1640 medium containing 10% Fetal Bovine Serum in a cell culture flask. The flask was incubated in an oven at 26˚C. In the following days, the culture medium was checked for growth, and the parasite's reproductive density was observed. After the control, the proliferated *L. topica* promastigotes (10^6 promastigotes /ml) were used for the study[18].

In vitro antileishmanial activity of plant extracts

The antileishmanial activity of plant extracts was evaluated using the Cell Titer-Glo®Cell Viability kit with *L. tropica* promastigotes that entered the logarithmic phase and reached the required reproductive density for the study. Concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.62, and 7.81 µg/ml of the plant extracts were prepared. Amphotericin B was used as the active substance for control purposes. The study was conducted on 96-well flat-bottom cell culture plates. The plates were marked as control, drug-free parasite control, Amphotericin B (positive control), methanol extract, ethanol extract, chloroform extract, water extract, *n*-hexane extract, and 60% aqueous ethanol extract. After 100 µl of RPMI-1640 medium containing 10% Fetal Bovine Serum was added to each well of the plate, 100 µl of the drug and extracts were then added to the first well of each of the three sections designated for Amphotericin B and plant extracts. Using an automatic dispenser pipette, the drug and plant extracts in the first well were mixed homogeneously with the medium, and a serial dilution was performed downward in the plate. Then, 100 µl of the medium with a parasite density of 10^6 promastigotes/ml was added to all wells except the ones reserved for the control. The plates were placed in an incubator at 26ºC. At 24 and 48 hours, the plates were removed and subjected to the CellTiter-Glo®Cell viability kit according to the manufacturer's instructions to evaluate their antileishmanial activities. These procedures were repeated three times[18,19].

Statistical analysis

The statistical analysis of the results was evaluated using the SPSS 23.0 statistical softwarpackage program. Student's *t*-test was performed. *P* value less than 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

L. tropica promastigotes were exposed to different concentrations of black seed extracts. The decrease in cell viability of the parasites showed a concentration dependent activity (Figure 1). At the lowest concentration (19.53 µg/ml) all off the extracts had 100 % parasite viability, whereas at the highest concentration (2500 µg/ml), the parasite viability percentages for methanol, water, chloroform, ethanol, n-hexane and 60 % ethanol extracts were determined as 0.0±0.0, 3.33±1.25, 7.0±0.82, 31.0±1.63, 36.0±2.45 and 38.0±1.63 %, respectively. With the obtained results, the IC_{50} values of the extracts prepared from black cumin seeds against *L. tropica* were calculated (Table 1). With an IC_{50} value of 172.4 µg/ml, the black

cumin seed methanol extract was found to have the strongest antiparasitic activity. In contrast, the n-hexane, chloroform, water, ethanol, and 60% aqueous ethanol extracts had IC_{50} values of 355.5, 400.3, 673.3, 818.4, and 1148 µg/ml, respectively.

Table 1: IC_{50} values of black cumin seeds extracts against *L. tropica* promastigotes

Extracts	IC_{50}±SD (µg/ml)
Methanol	172.4±1.63
n-hexane	355.5±2.00
Chloroform	400.3±2.05
Water	673.3±2.00
Ethanol	818.4±1.63
Aq. ethanol (60%)	1148± 1.63
Amphotericin B	0.500±0.00

In a previous study conducted by Özge Yılmazlı in 2013, as part of the Master's program in Microbiology at the Institute of Health Sciences, Istanbul University, it was observed that *N. sativa* did not inhibit the proliferation of *L. infantum* promastigotes at concentrations of 4, 8, 16, 32, 64, and 125 µg/ml, but inhibited proliferation by 94% at a concentration of 1000 µg/ml[20]. It was also observed that *Zingiber officinale* did not inhibit the proliferation of promastigotes at concentrations of 4, 8, 16, 32, 64, 125, and 250 µg/ml, but inhibited proliferation by 77% at a concentration of 1000 µg/ml. For *N. sativa* and *Z. officinale* essential oils, the *in vitro* IC_{50} values against *L. infantum* promastigotes were 265.957 µg/ml and 961.538 µg/ml, respectively[20]. Mahmoudvand *et al.*, demonstrated that the vitality of promastigotes of *L. tropica* was greatly decreased by the ethanolic extract of *Berberis vulgaris* (IC_{50} 4.83 µg/ml) and the chloroform extract of *N. sativa* (IC_{50} 7.83 µg/ml)[21]. Mahmoudvand *et al.*, searched for antileishmanial actrivities of essential oil and methanolic extract of *N. sativa*, and thymoquinone on promastigotes of *L. tropica* and *L. infantum*[21]. The essential oil of *N. sativa* (*L. tropica* IC_{50} 9.3 µg/mL and *L. infantum* IC_{50} 11.7 µg/ml) and methanolic extract of *N. sativa* (*L. tropica* IC_{50} 14.8 µg/ml and *L. infantum* IC_{50} 15.7 µg/ml) and thymoquinone (*L. tropica* IC_{50} 1.16 µg/ml and *L. infantum* IC_{50} 1.47 µg/ml), was shown to have potent antileishmanial activity on promastigotes of both species[16]. Moreover nanaoparticles of *N. sativa* oil prepared by polycaprolactone and silver doped titanium dioxide were demonstrated to have strong antileishmanial activities on *L. infantum* and *L. tropica*, respectively[17,22]. The results of antileishmanial activity and IC_{50} values obtained with *N. sativa* vary depending on the experimental procedure and the duration of parasite observation.

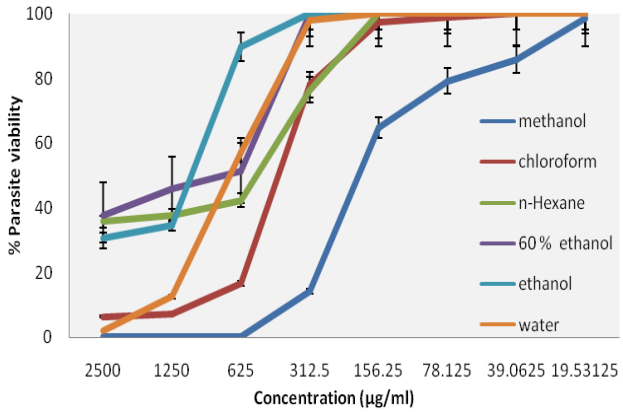

Figure 1: The cell viability percentages of *L. tropica* promastigotes obtained with various concentrations of black cumin seeds extracts (µg/ml).

Additionally, it is likely that the commercially obtained black cumin seeds might have different compositions of phytoconstituents. Based on the findings of our study, in addition to the methanol extract of the seeds, which contains components with a more polar character, the n-hexane and chloroform extracts, rich in nonpolar chemicals, are also worth further in vivo and clinical research.

Limitation of the study

The study of plant material obtained from one supplier and the lack of *in vivo* study constitute the limitations of the study.

CONCLUSION

The relative effectiveness of extracts made from black cumin seeds using solvents with varying polarity against *L. tropica* has been investigated for the first time. Future *in vivo* antileishmanial investigations could support the use of black cumin seed methanol extract as a natural resource with a high potential for treatment.

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AUTHOR'S CONTRIBUTION

Sayar S, **Cavus I**, **Kayalar H**: investigation, data curation, supervision. **Ozbilgin A**: methodology, supervision. Final manuscript was read and approved by all authors.

DATA AVAILABILITY

The data will be available from the corresponding author.

CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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