Indian Journal of Experimental Biology
Vol. 58, October 2020, pp. 699-705

Antileishmanial apoptotic activity of *Nigella sativa* L. essential oil and thymoquinone triggers on *Leishmania tropica*

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Received 22 June 2019; *revised 15 June 2020*

Nigella sativa L., commonly called Black cumin, is well-known in folk medicine and numerous studies have shown its various pharmacological activities. In this study, we estimated the cytotoxic effects of *N. sativa* essential oil (NEO) and its major bioactive component Thymoquinone (TQ) on *Leishmania tropica* promastigotes that cause cutaneous leishmanias, and also observed the programmed cell death features. The extraction of NEO was done by hydro-distillation and analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). Antileishmanial activity of NEO and TQ was determined using 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) assay and the obtained results are expressed as 50% inhibitory concentration (IC_{50}). The leishmanicidal activity of NEO and TQ was mediated via apoptosis as evidenced by *in situ* labelling of DNA fragments using terminal deoxyribonucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) and cell cycle arrest at sub G_0/G_1 phase. The IC_{50} values were 5 µg/mL and 1.3 µg/mL, respectively. We noted a significant increase in DNA fragmentation in treated parasites with IC_{50} of both NEO and TQ as well as a cell cycle arrest. These results revealed that NEO and TQ possess potential antileishmanial activity that mediated high possibility by programmed cell death.

Keyword: Black Cumin, Cell cycle arrest, Cutaneous leishmaniasis, Programmed cell death, Promastigotes

Leishmaniasis is one of the most neglected diseases in the world, with poor people being the highest affected population, mainly in Developing Countries; 350 million people are considered at risk of contracting leishmaniasis, and nearly 0.7 to 1.2 million cases of CL occur yearly[1]. Leishmaniasis, caused by the protozoan parasite from more than 20 *Leishmania* spp., is regarded as a major public health problem in tropical and sub-tropical areas, where infection is transmitted by the bite of a female sandfly[2,3]. More than 90 species of sandfly are known to transmit this parasite[3].

Cutaneous leishmaniasis (CL) caused by *Leishmania tropica* is the most prevalent clinical form of leishmaniasis worldwide. The 10 countries with the highest number of cases reported in 2018 are Afghanistan, Algeria, Bolivia, Brazil, Colombia, Iran, Iraq, Pakistan, Peru, Syria and Tunisia, which together account for 85% of global reported CL incidence[4].

In Syria, CL is an old endemic disease, *Leishmania tropica* is still an endemic in Aleppo, also in Edlib, Lattakia, Tortous, Hama, and Damascus. It represents actually about 90% of all the cases[5]. The population density in urban areas of Syria has increased, due to the population movement across the country, which might explain the rise in the number of cases. A recent survey estimated that some of the cases are not reported or treated, a considerable human reservoir exists[5,6].

At present, there is no effective vaccine against different forms of leishmaniasis; therefore, chemotherapy is the only choice in hand. Pentavalent antimonials have been in use for more than 70 years[7]. Moreover, Sodium stibogluconate and meglumine antimoniate are widely used. Unfortunately, both of these drugs are very toxic and can have serious side effects that include cardiac arrhythmia and pancreatitis, and could lead to life-threatening situations. Additionally, there is a widespread emergence of drug resistance due to non-standard intake and misuse. Amphotericin B, Miltefosine, Paromomycin, and Sitamaquine are gradually replacing antimonials, either given alone or in combination with them[8,9].

Plants are clearly a potential source of new antiprotozoal drugs[10]. Plant extracts or plant-derived compounds provide a valuable source of new medicines[10,11-15]. Furthermore, the leads obtained from the search for natural products with antileishmanial

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activity arises new impetus for obtaining valuable synthetic compounds[16]. Therefore, plant medicines are extremely needed for developing new effective treatment alternatives[11-15,17].

Many researchers have revealed the wide spectrum of *Nigella sativa* L. (Fam. Ranunculaceae; commonly known as Black Cumin) pharmacological potential[18-20]. *N. sativa* is native to Southern Europe, North Africa, and Southwest Asia and it is cultivated in many countries like Middle Eastern Mediterranean region, South Europe, India, Pakistan, Saudi Arabia, Turkey and Syria[20]. Seeds and oil have a long history of folklore usage in various systems of medicines and food. *N. sativa* seeds have been widely used in the treatment of different diseases and ailments[18-2018-21]. It has been widely used as an antihypertensive[22], liver tonics[17,20], diuretics[23], digestive[24], antidiarrheal[25], appetite stimulant[20], analgesics[26], apoptotic[17], antibacterial and in skin disorders[27,28]. Extensive studies on *N. sativa* have been carried out by various researchers and a wide spectrum of its pharmacological actions have been explored which may include anticancer[29], anti-inflammatory[30], antimicrobial and antioxidant properties[31,32].

In this study, we investigated the antileishmanial activity of the essential oil of local *Nigella sativa* (NEO) and its major component thymoquinone (TQ) against *Leishmania tropica* promastigotes that causes cutaneous leishmaniasis (CL). Further, we explored its apoptotic potential using flow cytometry test to consider *N. sativa* as a possible alternative to chemotherapy in parasitic disease control.

Materials and Methods

Parasite culture

Promastigotes of local strain of *Leishmania tropica*, previously identified by molecular genotyping at the Animal Biology Department, Damascus University, Syria, were maintained by weekly transferring in RPMI-1640 medium supplemented with L-glutamine (100 U/mL), 10% fetal calf serum (FCS) and penicillin-streptomycin (100 U/mL) at 26°C (all products from Sigma, USA).

Extraction and analysis of essential oil

Nigella sativa seeds were purchased from the local market and powdered. Two hundred grams of powdered seeds was submitted to hydrodistillation using a Clevenger-type apparatus according to the European Pharmacopoeia and extracted with 1.0 L water for 2.5 h. The essential oil was collected, dried over anhydrous sodium sulphate and stored at 4°C until used.

Essential oil analysis was run on an Agilent 7890A GC-Ms system coupled to a quadruple mass spectrometer (model 5975C) with a capillary column of HP-5MS 5% Phenyl Methyl Silox; 30 m × 250 µm × 0.25 µm. GC-MS interface, ion source, selective mass detector and injector temperatures were maintained at 280, 230, 150 and 260°C, respectively. Carrier gas used was helium with a flow rate of 1.0 mL/min. The oven temperature was programmed at 60°C then increased from 60 to 200°C at the rate of 4°C/min and held at the rate of 8°C/min and held at 260°C for 7.5 min[33]. Diluted oil in n-hexane (0.5/100, v/v) of 1.0 µL was injected in the split ratio 1:10.

Identification of compounds

The compounds were identified on the basis of comparison of their retention indices and mass spectra with published data[34], also computer matching was done with the NIST Mass spectral version 2.0 f (2008) and the National Institute of Standards Technology libraries provided with the computer controlling GC-MS systems. The retention indices were calculated using a homologous series of n-alkanes C_8-C_{20} (Sigma, USA).

Viability assay

NEO and Thymoquinone (TQ; Sigma) were reconstituted aseptically to 100 mg/mL in dimethyl-sulfoxide (DMSO; Sigma) and diluted further in culture medium to achieve a final DMSO concentration of not more than 0.1%. The viability of *L. tropica* promastigotes was evaluated by colorimetric cell viability MTT assay (Sigma, USA) according to the manufacturer's instructions. In order to get IC_{50}, serial dilutions of NEO and TQ were performed separately in 96-well microtiter plate then 100 µL of culture medium containing 5×10^5 promastigotes was added to each well and incubated at 26ºC for 24 h. Untreated promastigotes were used as a positive control. After incubation, 10 µL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution (5 mg/mL) was added to each well and incubated at 26ºC for 3 h[35]. After that, the resulting formazan crystals were dissolved by adding MTT Solubilization Solution. Finally, the absorbance was measured by an ELISA reader (Huma Reader HS, Germany) at 540 nm. The percentage of viability was calculated as follows:

$$\text{Viability \%} = 100 \times \frac{\text{absorbance of Sample}}{\text{absorbance of Control}}$$

Each concentration was tested in duplicate together with the control and repeated three times in separate experiments.

TUNEL assay

In situ detection of DNA fragments in promastigote was analyzed using an in-situ cell death detection kit (*In situ* cell death detection kit, Roche, Germany) according to the manufacturer's instructions. Briefly, promastigotes (2×10^6 cell/mL) were treated separately with an IC_{50} concentration of NEO and TQ at 26°C. After 24 h, the cells were washed, fixed with 4% para formaldehyde (v/v) for 1 h at 25°C, washed with PBS then permeabilized with freshly prepared 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. The cells were washed twice with PBS, after which 50 µL of the reaction mixture TUNEL containing TdT and labeled dUTP was added for 1.0 h at 37°C[36], washed and finally resuspended in PBS for run in a FACS Calibur (Becton–Dickinson, Rockville, MD, USA), and analyzed using ProCellQuest Software (BD Biosciences).

FACS analysis of cell cycle

Parasites (10^6 cell/mL) were treated separately with an IC_{50} concentration of NEO and TQ at 26°C for 24 h. Then cells were fixed in chilled methanol (90%) at least 30 min and kept at 4°C until used. Cells were washed with PBS, centrifuged and the resultant pellet was resuspended in 500 µL of cell suspension solution with RNase (200 µg/mL), 0.2% Triton X-100 (Promega) and propidium iodide PI (50 µg/mL) (Sigma, USA) Suspension mixed well and kept for 1 h at 25°C in the dark[37]. The DNA content was analyzed using FACS Calibur (Becton–Dickinson, Rockville, MD, USA), where 10,000 cells were counted in each assay. In each experiment, the distribution of sub G_0/G_1, G_0/G_1, S and G_2/M phases was calculated from each histogram using the CellQuest software (BD Biosciences).

Results

Isolation of *N. sativa* essential oil and GC-MS analysis

The essential oil isolation by hydro-distillation gave a yield of 0.2 %. Results of GC-MS analysis and compounds identification are shown in Table 1. The major component was p-cymene (39.9%) followed by α-thujene (18.2%), thymoquinone (17.2%), β-pinene (4.5%), and α-Pinene (4.4%).

Evolution of growth inhibitory activity of NEO and TQ against *L. tropica* promastigotes

Leishmania tropica promastigotes treated with graded concentration showed a reduction in cell viability in a concentration- dependent manner. IC_{50} of NEO and TQ was ≥5 µg/mL and ≥1.3 µg/mL, respectively. Details of the inhibitory effect of NEO and TQ against *L. tropica* promastigotes are presented in Fig. 1. ANOVA analysis showed a significant difference (P <0.01) between IC_{50} of NEO and TQ.

Table 1 — Major chemical composition of *Nigella sativa* essential oil analyzed by GC-MS

Compounds	Area%	RT	RI
α-Thujene	18.2	4.848	925
α-Pinene	4.4	4.997	932
Sabinene	2.2	5.869	973
β-Pinene	4.5	5.965	978
p-Cymene	39.9	7.198	1027
D-Limonene	2.6	7.275	1030
γ-Terpinen	1.6	8.088	1059
Thymoquinone	17.2	14.136	1254
Total identified	90.6		

[Compounds listed in order to their elution on the HP-5MS column, RT Retention Time, RI Retention Indices on the HP-5MS column relative to C_8–C_{22} n-alkanes]

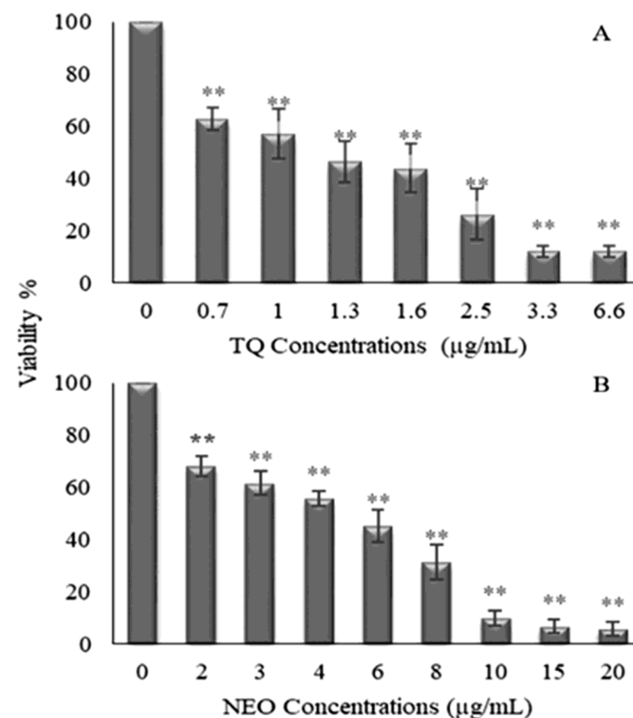

Fig. 1 — Antileishmanial activity of serially diluted concentrations of (A) TQ; and (B) NEO against *Leishmania tropica* promastigotes monitored by MTT assay at 24 h incubation. [Values are expressed as means ± SD in triplicate, and in three independent assays, Statistical significance was measured by comparing treated groups to control groups (**P <0.01)]

TUNEL assay

Degradation of nuclear DNA into oligonucleosomal units is one of the hallmarks of apoptotic cell death. The DNA fragmentation was documented through TUNEL assay, wherein the 3′OH groups are generated in abundance and serve as a substrate for TdT mediated fluorescence conjugated-dUTP binding. *In situ* TUNEL labeling was performed to evaluate the endonuclease activity, where the proportion of DNA nicks was equivalent to the fluorescence obtained from dUTP. Treatment with IC_{50} concentrations of both NEO and TQ for 24 h induced a significant increase in DNA fragmentation in parasites which were 65.04 and 68.66 %, respectively in comparison with the control. The TUNEL analyses demonstrated that apoptosis was the main mechanism of cell death induced by NEO and TQ (Fig. 2).

Cell cycle

To determine the amount of sub G_0/G_1 cells percent, promastigotes were treated separately with IC_{50} concentrations of NEO and TQ for 24 h. The treated cells were permeabilized, stained with PI and analyzed by flow cytometry. In a given cell, the amount of bound dye correlates with the DNA content and thus DNA fragmentation in apoptotic cells translates into fluorescence intensity lower than that of G_0/G_1 cells, i.e. a sub G_0/G_1 peak. NEO and TQ triggered apoptosis in *L. tropica* promastigotes with a significant increase (P <0.05) in sub G_0/G_1 population to 14.9 and 36 %, respectively in comparison with untreated control parasite (Fig. 3). This data suggests that NEO and TQ arrest cell cycle of parasites at sub G_0/G_1 phase which leads to apoptosis.

Discussion

Current treatment of infections caused by *Leishmania* relies solely on chemotherapy. Typical

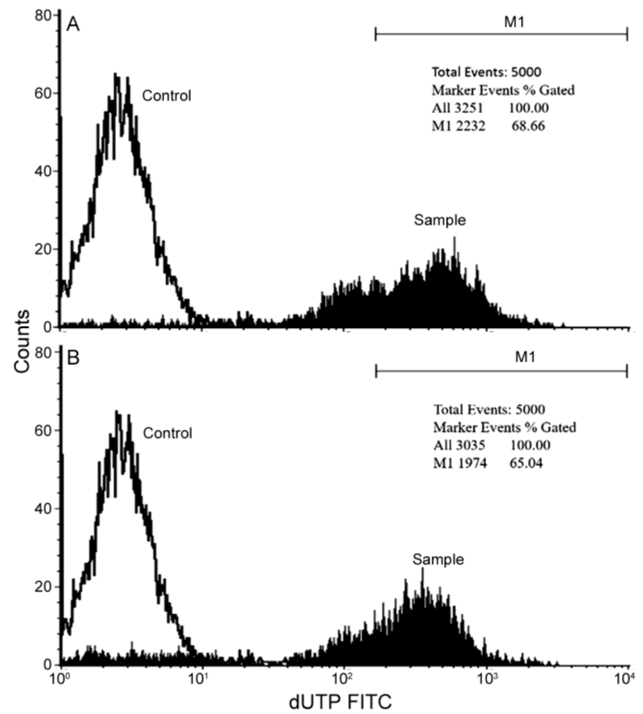

Fig. 2 — Analysis of DNA fragmentation in *L. tropica* promastigotes. Cells were incubated or not (as a control) with IC_{50} of (A) TQ; and (B) NEO for 24 h and stained using TUNEL assay. [Treatment parasites with IC_{50} of both NEO and TQ induced a significant increase in DNA fragmentation]

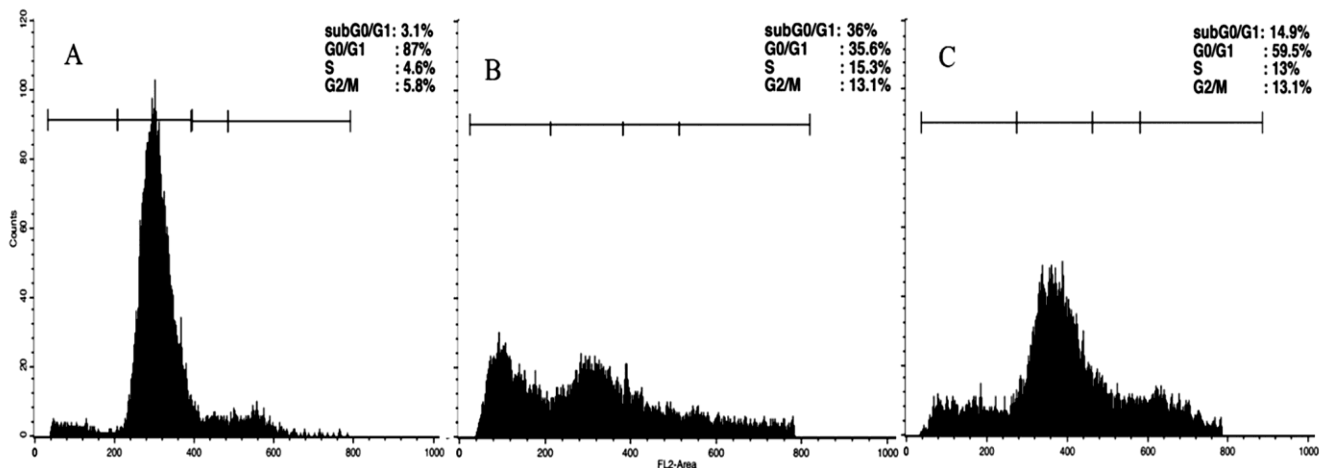

Fig. 3 — Effect of NEO and TQ on the *L. tropica* promastigotes cell cycle. The figure illustrates the cell cycle analysis of promastigotes after treatment and staining with propidium iodide. (A) Untreated parasites (Control); (B) Parasites treated with IC_{50} of TQ; and (C) Parasites treated with IC_{50} of NEO. [Both NEO and TQ induce cell cycle arrest in promastigotes at sub G_0/G_1 phase as evaluated after 24 h treatment]

drugs for leishmaniasis treatment are pentavalent antimonials such as meglumine antimoniate[7]. The use of these chemical compounds is limited due to their high price, toxicity, long-term treatment, and emergence of novel drug resistance[8], and until now, a small fracture of Leishmania treatment and prevention could be regarded as a promising approach[38]. All of which shed light on the importance of developing alternative treatments. Various traditional medicinal plants have been referred to possess antileishmanial activities proving their use in folk medicine[39]. Essential oils from plant and active components can be used as alternatives or additions to current antiparasitic therapy[40]. *N. sativa* is popular in folk medicine; numerous studies have shown various pharmacological activities[41]. Few studies have shown the cytotoxic activities of *N. sativa* and TQ on different forms of *L. tropica* and *L. infantum* but not the potential role of NEO and TQ on apoptosis[42,43]. Recently, Uysal *et al.*[18], have reported apoptotic effect of *N. sativa* seed oil on CCL_4 induced hepatotoxicity. Although *N. sativa* is widely used in the treatment of various diseases in traditional medicine, this is possibly the first report on the effect of NEO and TQ with an antileishmanial activity via apoptosis.

In this study, NEO and TQ showed growth inhibition of *L. tropica* promastigotes where IC_{50} were 5 µg/mL and 1.3 µg/mL, respectively. TQ IC_{50} was 4-fold lower when compared with NEO IC_{50} suggesting that TQ is the major bioactive components in essential oil[44]. Whereas another study reported that IC_{50} of NEO and TQ were 53.3 and 9.1 µg/mL after 24 h of treatment and 9.30 and 1.16 µg/mL after 72 h, respectively[45], The contrast with the previous study may be due to the difference between *L. tropica* strains where a local strain was used in this study[46], in addition to the difference in concentration of active components such as α-Pinene, β-Pinene, and *p*-Cymene and the interaction between them[40,47].

Leishmania species are known to display apoptosis like changes in response to a diverse range of stimuli including plant extracts[48,49]. *N. sativa* and TQ are known to stimulate apoptosis against a large variety of cancers[50,51]. Treatment with NEO and TQ also triggered apoptosis like change in *L. tropica* promastigotes characterized by induction of DNA fragmentation and cell cycle arrest at sub G_0/G_1 phase which is an indication of the cells entering into the apoptotic mode of death. *In situ* TUNEL of nicked DNA was studied to confirm apoptosis in *L. tropica* promastigotes. Apoptotic cells display active endonucleases translating into an increased cell population located before the G_0/G_1 peak on a DNA frequency histogram. Regarding promastigotes that were treated with different antileishmanial natural components such as withanolides[52], lapachol[53], zerumbone[54] and *Caryocar coriaceum* extracts[55], a considerable proportion of cells have been identified in sub G_0/G_1 region. Our study is in agreement with the previous reports and shows that both NEO and TQ treated *L. tropica* promastigotes exhibit a hypodiploid peak at sub G_0/G_1, containing parasites with reduced DNA content, substantiating DNA fragmentation and induction of apoptosis. This refers to the importance of NEO and TQ in leishmaniasis treatment generally.

Conclusion

In the absence of effective treatments and vaccines against *Leishmania tropica,* our study suggests a significant apoptotic effect and activity of the essential oil of *Nigella sativa* and its bioactive compound TQ, which were previously proven to have an efficient antileishmanial activity. However, further research needs to be conducted in order to highlight and investigate the mode of action of such compounds and to implicate the use of NEO and TQ to produce an efficient alternative leishmanicidal drug.

Acknowledgement

The authors would like to thank the Research Affairs team of Damascus University for the financial support and National Commission for Biotechnology Department of Molecular Biology and Biotechnology for help with flow cytometry.

Conflict of Interest

Authors declare no conflict of interests.

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