MSc. (Department of Biotechnology)

"INVITRO ANTILEISHMANIAL ACTIVITY OF METHANOLIC BLACK SEED EXTRACT."

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

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JUNE, 2021

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Title of paper: Invitro antileishmanial activity of *Nigella sativa* methanolic black seed extract.

Authors name: Syed Tawqeer Ali, Prakash Chandra

Name of Journal: Research Journal of Biotechnology

Conference date with Venue (if applicable): NA
Have you registered for the conference (yes/no)?: NA
Status of paper: Communicated

Date of paper communicated: 29th May, 2021

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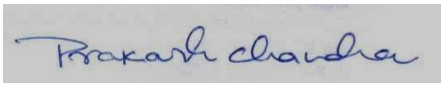

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ABSTRACT

Leishmaniasis is a disease that is caused by obligate intramacrophage protozoa belonging to the genera Leishmania. The major Leishmania species responsible for the condition are L.major, L.infantum, L.chagasi, and L.donovani, including 20 other Leishmania species. The disease is endemic in 60 countries worldwide. This study aims to evaluate the antileishmanial activity of black seed extract of Nigella sativa collected from Kashmir. The extract was prepared by maceration along with the methanol. The antileishmanial activity was evaluated against Leishmania major using MTT (3-(4.5-dimethylthiazol-2yl)-2.5-diphenyltetrazolium bromide) assay. The black seed extract shows a reduction in cell promastigotes viability with different concentrations of doses (0- 2000 ug/ml). The extract showed the highest antileishmanial activity at 2000 ug /ml concentration. The methanolic extract of Nigella sativa showed potential antileishmanial activity at an inhibition% value of 80.29% ± 0.65%. IC 50 was calculated after 48hrs to be 964.3 µg/ml. Considering these results, these medicinal plants from Kashmir could serve as potential drug sources for antileishmanial compounds.

Keywords—MTT assay, Antileishmanial, Cell viability, *Nigella sativa*

ACKNOWLEDGEMENT

It is my privilege to express my profound sense of gratitude and indebtedness to my mentor **Dr. Prakash Chandra**, Assistant Professor in the Department of Biotechnology, Delhi Technological University for his valuable guidance and consistent encouragement during the progress of the project work. The dissertation wouldn't be completed within a short period without his insightful suggestions and support. I am equally grateful and wish to express my wholehearted thanks to respected research scholars of our lab, Mr. Rahat Ali, Shamas Tabrez for their kind support and help in the course of my research work. On a personal note, I wish to express my gratitude and affection to my family for their constant love and support. I would like to thank all my friends for their boundless love and trust which motivated me to complete the project work in the given time.

Syed Tawqeer Ali

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ABBREVIATIONS

L. Major : Leishmania major

MTT: 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide

N.sativa: Nigella sativa

CHAPTER 1

INTRODUCTION

Leishmania, a single-celled parasite that causes an infectious disease known as Leishmaniasis [1]. The World Health Organization classified this disease as a neglected tropical disease, affecting predominantly the individuals from developing countries with low socioeconomic levels [2]. Several reports suggest that the Leishmania species affects mammals and reptiles, out of which 20 species infect humans. Moreover, 88 countries have reported this disease as endemic affecting 14 million people globally [3]. The vertebrate host receives these parasites through the bites of infected sand flies. There are various mammalian hosts for these parasites, such as rats, dogs, hamsters, and humans. Moreover, more than 70 animal species act as natural reservoir hosts of *Leishmania* parasites [4]. The life cycle of Leishmania consists of two developmental stages, the promastigote, and amastigotes form, which are found in the alimentary canal of sandfly and mammalian host, respectively. These parasites infect other cells by attacking the host cells, such as phagocytes, neutrophils, or macrophages. Clinically this diverse spectrum disease with several manifestations shows two distinct forms: the self curable cutaneous leishmaniasis and fatal visceral leishmaniasis [5].

The current approaches for treating this disease are primarily based on chemotherapeutic drugs such as miltefosine, paromomycin, amphotericin B, and pentavalent antimonials; however, these drugs lead to some side effects like high toxicity and makes parasites resistible. Since there are currently no vaccines, we have to find new strategies and approaches to treat this disease.

The indigenous systems of medicine or folk medicines include the usage of medicinal plants for many centuries for curing diseases.

In addition, such medicinal plants find their use in herbal medicines that are relatively better than allopathic medicines. Many researchers focus their interest in such medicinal plants because of their less exploration and investigation in their properties, viability, and toxicities. Nigella sativa, owing to its historical and religious importance, is greatly revered in many parts of the world. The seeds of Nigella sativa are commonly known as Black seeds. Across the globe, the seeds, along with the oil extracted, find their extensive uses, particularly in resisting diseases. The drugs obtained from N.sativa are proven effective, increasing their value in traditional medicine systems, including Unani and Ayurveda. Among many biological and therapeutic properties, N. Sativa is also known for its analgesics, anti-inflammatory, anti-oxidant properties etc. In addition, it is also used as an appetite stimulant, liver tonic and helps increase milk production in nursing mothers to aid in the immune system [6]. The oil's therapeutic effect can be attributed to one of its key components, i.e., thymoquinone (TQ). This study suggests that black seed extract could serve as a potent drug against leishmaniasis.

CHAPTER 2

REVIEW LITERATURE

Leishmania donovani

Leishmania donovani is a single-celled organism that causes a lethal disease in humans called ― visceral leishmaniasis.‖ The whole-genome sequencing reveals that L.donovani has 36 chromosomes, 32444968 nucleotides, 8032 protein genes and 112 RNA genes.[7] L.donovani is a digenetic parasite, completes its life cycle in two hosts. The primary host is the man. In contrast, the secondary host is sandfly. The parasite occurs in two distinct morphological forms:

1. Promastigote: The flagellated form is found in sandflies, where the parasite survives and proliferates extracellularly in the alimentary tract.

2. Amastigote: It is a non-flagellated form that replicates in macrophage phagosomes in the mammalian host, where it adopts an obligatory intracellular form that thrives inside the phagolysosome.

Leishmania donovani causes kala-azar with the following characteristics- pyrexia, splenic enlargement, liver enlargement, hyperplasia, neutropenia and in some cases, cutaneous lesions.

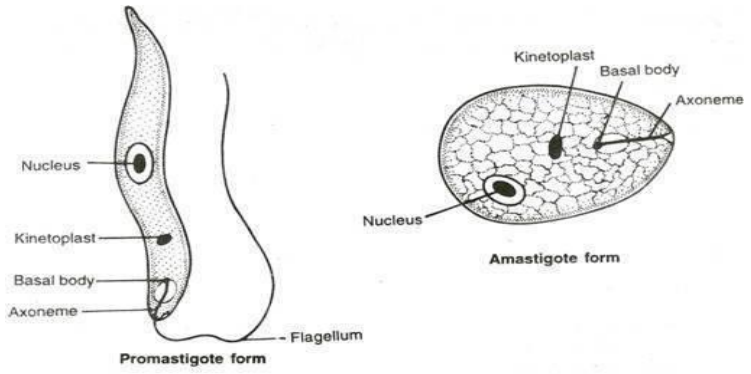


Fig. 178. Morphological forms of *Leishmania donovani*

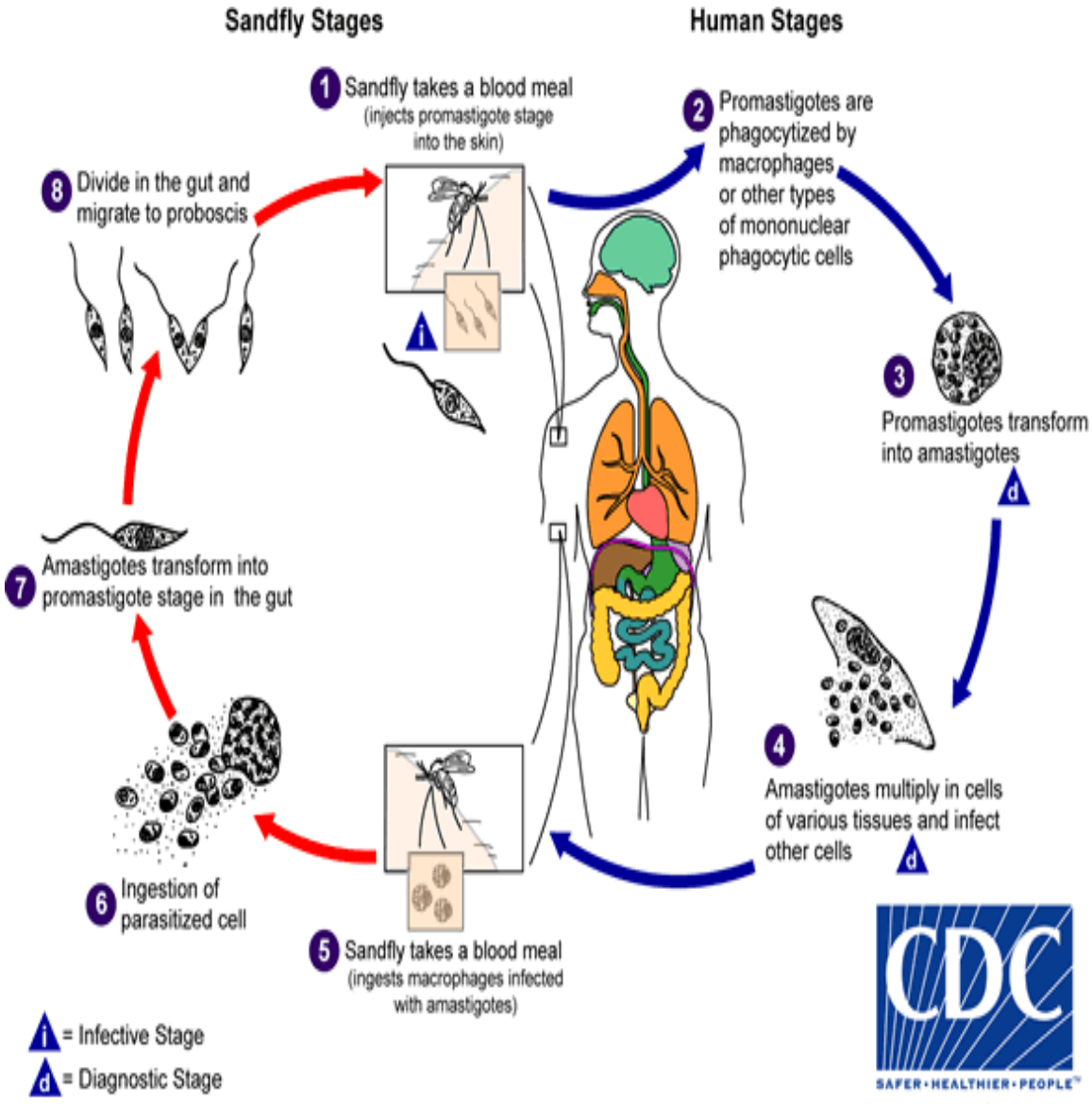


Fig 1 B :Life Cycle of Leishmania donavani

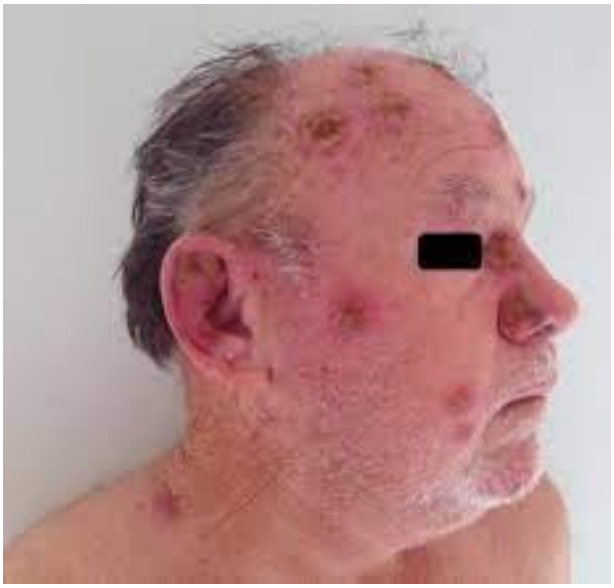


Fig 1 C: Cutaneous Leishmaniasis

Fig 1D : Visceral Leishmaniasis

Fig 1 E : Muocutaneous Leishmaniasis

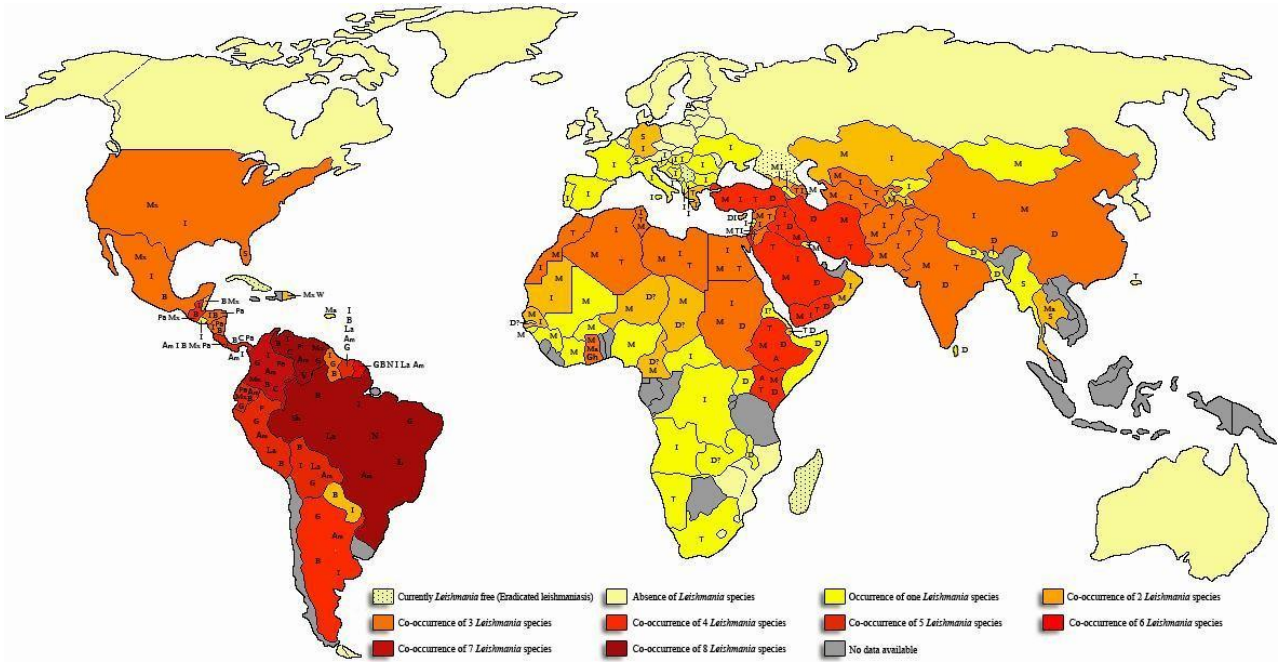


Fig 1 F : Worldwide distribution of Leishmaniasis

CHAPTER 3

Nigella sativa

Plant Morphology:

This plant grows up to a height of 22-90 cm. It consists of delicate flowers that are present within 6-10 petals with various colors such as yellow, pink, pale blue and white. The plant bears large-sized fruits, the swollen capsule containing a plethora of seeds [8].

Figure 3.1 N. sativa (whole plant, flower and seeds) adopted from internet

Taxonomic classification:

KINGDOM	**Plantae**
SUPERDIVISION	**Spermatophyta**
PHYLUM	**Magnoliophyta**
CLASS	**Magnoliopsida**
ORDER	**Ranunculales**
FAMILY	**Ranunculaceae**
GENUS	**Nigella**
SPECIES	**Sativa**

The seeds and powder Characteristics

This plant contains tiny dicotyledonous, angular and trigonous seeds that are externally black and are white inside. N. sativa seeds transversely show uni-layered epidermis that consists of thick-walled cells followed by 3-4 layers of elongated parenchymatous cells. This layer is again followed by a reddish-brown pigmented layer that contains rectangular cells. Inner to this layer, there is a elongated columnar cell that are thickly walled. Endosperm of this plant consists of polygonal cells that are filled with oil globules [9].

Chemical Composition of black seeds

Many compounds have been identified, isolated and reported from different varieties of N. sativa black seeds. Some of the active compounds are thymoquinone (30%-48%), dithymoquinone, p-cymene (7%-15%), thymohydroquinone, 4-terpineol (2%-7%), t-anethol (1%-4) etc [10]. There are many other compounds also present in trace amounts. These seeds consist of two types of alkaloids; isoquinoline alkaloid, and pyrazol.

Certain other substances are also found in rare amounts such as limonene, citronellol etc. The prominent component of quinine i.e TQ confers the wide pharmacological properties to N. sativa. The composition distribution of various biochemical compounds of seeds of N.sativa remains; protein(26.7%), fat(28.5%), carbohydrates(24.9%), crude fibre(8.4%), and ash(4.8%).Its seeds also contain appreciable amounts of vitamins and minerals such as Cu, P, Fe etc.carotene present in the seeds gets converted into vitaminA inside liver.Vanilic acid is also reported to be found in its roots as well as shoots [11].

Scientific research on *Nigella sativa*

Researchers employed novel scientific methods in order to explore its curative properties against certain genetic anomalies and other such defects.. There are many pharmacological actions of N.sativa that have been researched from couple of decades.

CHAPTER 4

METHODOLOGY

Plant Material

This plant part was collected from the botanical garden of Kashmir, and dust was cleaned from its surface. The collected part was wholly dried in a ventilated room and was grounded to a fine powder.

Solvents and Chemicals

Dimethyl sulfoxide (DMSO, R & M), MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide), Formaldehyde, RPMI cell culture media, M1-99 parasite media and PBS.

Preparation of plant extract

The dried powered part of the plant (50 g) of *Nigella sativa* was extracted with methanol followed by reduced pressure at 500C with the help of a rotary evaporator. The adhesive material was obtained and, thus, subjected to drying vacuum desiccators (oil pump). The extract was kept at 40 C inside the dark bottles. The filtration method was used to sterilize the extracted material with the help of a membrane filter (0.22um). Now, freshly extract was used and prepared with different concentrations to be used for an antileishmanial activity.

Macrophage cell line (THP-1) and parasite cultures

The THP-1 human macrophage was purchased from the NCCS Pune. RPMI media was used for cell line culture. The parasites (L. major) were cultured at 250 C in M1-99 media.

Evaluation of the antileishmanial activity

The black seed methanolic extract was dissolved in DMSO followed by centrifugation at 2700rpm, 250 C for 5 min. The supernatant was discarded, and the remaining material was stored in a freezer at 40 C. By using colorimetric MTT assay, the antileishmanial activities of this extract were analyzed on THP-1 human macrophage. RPMI cell culture medium was used with 10% inactivated FBS. The cells were added to 6 well-plate followed by counting of parasites on a hemocytometer. The average was taken by the formula:

No. of parasites x dilution factor x 104 x vol = Avg no. of parasites.

Invitro antileishmanial- the activity of the black seed extract on promastigote IC50

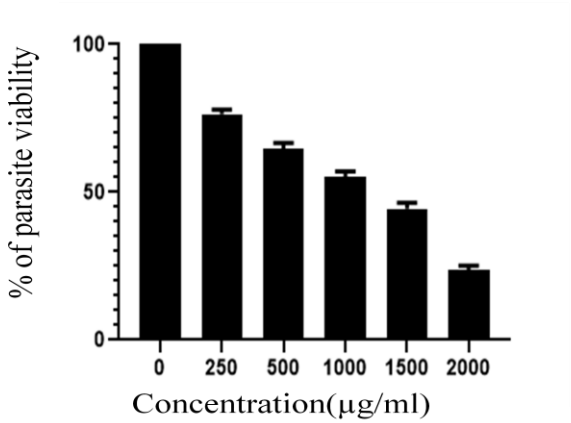
The effect of using an increased concentration of black seed extract (250-2000 ug/ml) on the Leishmania Major parasites (2 x 106 cells) was quantified for 48 h at 250 C. The result was determined by colorimetric assay, and concentration was calculated at which 50% promastigote were killed.

In-vitro cytotoxicity on macrophages (CC50)

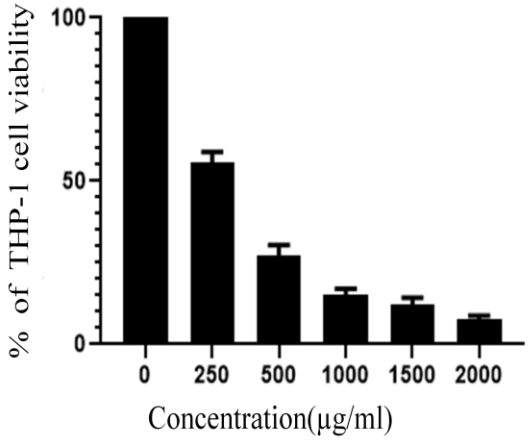
The human THP-1 macrophages (2 x 106 cells) were loaded along with different growing concentrations (250 – 2000 ug/ml) in a 96 – well culture plates. The CO2 concentration was 5% for 48 h at around 370C. The cell viability was determined through MTT assay. The absorbance values were quantified at O.D 570-nm. PBS and miltefosine were used as controls. The results were depicted as mean % reduction of human macrophages when compared with untreated samples x 100. At last, CC50 was calculated, i.e., the concentration at which 50% cytotoxicity (ug/ml) was observed. The software used to calculate CC50 was Graph Prism-pad.

CHAPTER 5

RESULTS



Dose dependent effect of methanolic extract *Nigella sativa* seed on *Leishmania* parasite.
IC 50 was calculated after 48 hrs to be 964.3 µg/ml.



Evaluation of cytotoxic studies of *Nigella sativa* methanolic seed extract on THP-1 derived human macrophages after 48 hrs with CC_{50} of 231.2 µg/ml.

CHAPTER 6

DISCUSSION

Leishmania, a single-celled parasite that causes an infectious disease known as Leishmaniasis. The major disease-causing species of Leishmania include, L.major, L. infantum etc. The disease is endemic in more than 60 countries worldwide. The black seed extract is reported to possess some medicinal properties. There is no published report about its anti-leishmanial property. In the present study, black seed extract was found to have various biological activities and broad traditional uses against infectious and non-infectious diseases. *Nigella sativa* black seed is used in folk medicine as an anticancer, antioxidant, antifungal, and antimicrobial. Hence also its antileishmanial activity was observed due to the presence of many phytochemicals inside its extract.

CHAPTER 7

CONCLUSIONS AND FUTURE CONSIDERATION

The results demonstrate that the medicinal plants can serve as good source of new antileishmanial drugs. Future studies will be carried out with a view of studying the different compositions of the extracts to identify the main phenolic components that are responsible for the antileishmanial and anticancer activities.

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