

## Isolation and Molecular Diagnosis of *Leishmania major* and Study Activity of Aqueous Extract of Plant *Nigella sativa* Against the Parasite *In Vitro*

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### ABSTRACT

The current study included isolating and diagnosing the cutaneous leishmaniasis parasite from patients with Baghdad boil and the reviewers of Al Hussein Teaching Hospital in Thi-Qar province for the period from November to December 2016 and then studying the effect of aqueous extract of the plant *Nigella sativa* in the vitality of isolated parasite in vitro. Through the results, the isolated parasite was diagnosed depend on lesions appearance and characterization then confirm the diagnosis by KDNA- PCR which revealed that *leishmania* spp. was *Leishmania major*. In this study three different concentrations from aqueous extract of the *Nigella sativa* plant were used (50, 100 and 150 mg / ml), the parasite growing in NNN medium (3 replicates used) It was observed that the percentage of parasite destruction increased with prolong exposure time of the extract and increased concentration of the extract, results also exhibited that the percentage of the destruction was 100% after four hours of exposure to the concentration of 150 mg / ml which was the highly killed percentage compared to control, which was 5.98%.

**Keywords:** *leishmania major*, parasite, *nigella sativa*

### INTRODUCTION

Leishmaniasis is a parasitic disease caused by parasite *leishmania* that is transmitted by a bite of sand fly of a genus *Phlebotomus* its an endemic disease in Iraq (AL-Hamash ,2012 ;Saporito *et al.*,2013). The World Health Organization (WHO) has reported that 350 million people are at risk of developing leishmaniasis, especially people living in developing countries (WHO,2010) .This disease has become endemic in 88 countries worldwide (Youssef, 2002) .The disease is known by several local names, including the Baghdad boil , oriental sore and Aleppo boil It is spread around the world with an annual rate of injuries 600,000 and the spread of more than 12 million from around the world and there are at least 20 species of *leishmania* are pathogenic to humans (Banuls *et al.* , 2008) Cutaneous leishmaniasis is characterized by two types: the first zoonotic, that causes it *Leishmania major* (wet type) or Rural type and the second type anthroponotic that causes it *L. tropica*(dry type ) or Urban type (AL-Samarai and AL-Obaidi, 2009).

Therefore, there is a need to reach safe parasitic antibiotics for human use from natural sources, both animal and plant, and from these sources black seed plant (*Nigella sativa* ) Which is characterized by seeds a small black oval Rough texture and cooled surface It has a distinctive smell and a stinging taste belongs to the Ranunculaceae family .The composition contains many quantities of active substances

such as Glycosides ,Ratings ,phenols and others ,this plant is used to kill worms and treat headaches, scabies, diuretics and menstrual cycles in women (AL- Al-Dujawi ,1996 ) It also has a counter-effect in the growth of many germs (Bilal *et al.* , 1998) .

The aimed of study to know the effect of the aqueous extract of the plant *Nigella sativa* in the vitality of promastigote of *Leishmania major in vitro*

### MATERIAL AND METHOD

#### Source of parasite

The leishmania parasites were isolated from patients with Baghdad boil (50 samples )who visited Al Hussein Teaching Hospital in the city of Nasiriyah, the center of Thi-Qar province for the period from November until December 2016 and was isolated on the semi solid medium which was prepared according to method Adler and Theodor (1930).

the parasite was diagnosed depend on clinical appearance of lesions after consultation with a specialist and confirm the diagnosis of leishmania by KDNA –PCR then parasites were growing on NNN medium which was prepared according to method Kang and Norman (1970) and Meredith *et al.* (1995) .

#### DNA Extraction

DNA extraction from blood samples from patients using the Wizard Genomic DNA Purification Kit (Qiagen,

Table 1: detection of *Leishmania* PCR (kDNA).

kDNA PCR	Positive No.	%	Negative No.	%	Total
<i>Leishmania major</i>	48	96 %	2	4%	50
<i>Leishmania tropica</i>	0	0%	50	100%	50

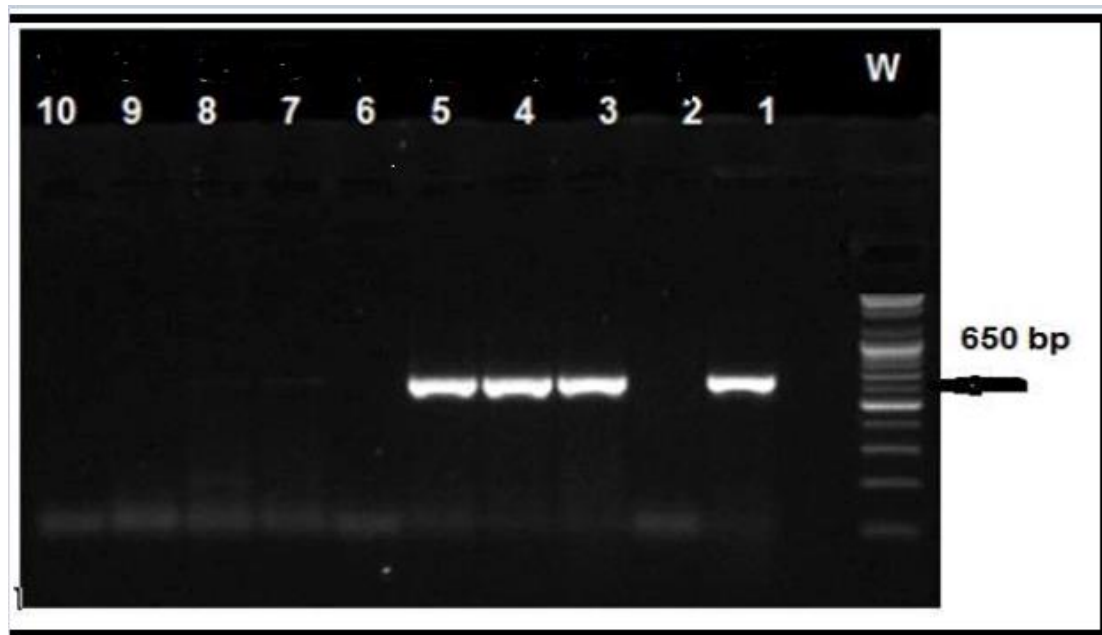


Figure 1: Gel electrophoresis of kDNA-PCR products, patients samples, MW 100 bp ladder (the first five) *L. major*, (the last five) *L. tropica*

Hilden, Germany) following the manufacturer's protocol. The quantification and quality control of the DNA extraction procedures were performed using a nano spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific). All reactions were performed in appropriated places, following the good practice of laboratories to avoid sample contamination, then the extracted DNA were stored at  $-20^{\circ}\text{C}$  for further process DNA Amplification kDNA – PCR.

#### kDNA-PCR

The technique was performed based on the protocol described previously (Nada *et al.*, 2017) using the following primers to confirm and differentiate between two types these primers amplify special gene No bands was shown for *L. tropica* and 650 bp as product for *L. major* kDNA forward Primers, *L. tropica* F(5-AGGTGTTTTTGGGCTTGAC-3), Reverse(5 ACTCAAGTCCCGTCCATCAAC-3) (*L. major* F (5-TCGCGTGTCTGACTTTTGC-3), Reverse 5 ACTCAAGTCCCGTCCATCAAC-3) were carried out as described by [ Nada *et al.*, 2017 ] for amplifying the variable region of the Leishmania species The reaction mixtures were prepared in a final volume of 20  $\mu\text{L}$  that containing Master mix (Bioneer, Korea), 10 p mol primers and DNA 2mL. The reaction mixtures were incubated in thermal cycler (Appendrof, USA) as follows: initial denaturation at 95oC for 5min by 30 cycles of 94oC for 30 sec, and at annealing temperature 60oC for 30 sec and 72oC for 40 sec. and final extenstion at 72oC for 5min. The PCR products were electrophoresis in 1.2% agarose gel

stained with ethidium promide, in the kDNA –PCR determined by the observation of expected bands.

#### Source of the Plant and preparation of aqueous extract

The seeds of the plant *Nigella sativa* were obtained from local markets in Nasiriyah city and the aqueous extract was obtained according to the method Harborne (1984) took 50 grams of plant seed powder and added 250 ml of distilled water and placed in a  $45^{\circ}\text{C}$  water bath for four hours, then filtered, dried and stored for use.

#### Studying the effect of the *Nigella sativa* plant on the parasite

Four tube containing 1 mL of sterile lock's solution containing 20 million parasite / ml each group consisting of three replicates and each Three different concentrations of plant extract were added to each tube as follows :

The first group used concentration 50 mg / ml

The second group used concentration 100 mg / ml

The third group the concentration used was 150 mg / ml

The Fourth group control (no treated with plant extract)

All the tubes were incubated at a temperature of  $26^{\circ}\text{C}$  and the parasites vitality was calculated after 1, 2, 3 and 4 hours of addition the plant extract. The vitality of the parasite was measured using a dye erythrocin –B according to method Hodgkinson *et al.* (1980).

Where equal volumes of 100 microliters were mixed for each of the suspended parasites and diluted dye solution. The mixture was cooled to  $4^{\circ}\text{C}$  and then kept in ice at  $-4^{\circ}\text{C}$  for 5 min. A drop of the mixture was then examined and at least 100 cells were calculated / 1 ml to estimate the percentage of cell vitality where the stained parasite

Table 2: The percentage of destruction of *leishmania major* parasite by the effect of aqueous extract of the plant *Nigella sativa*.

Conce. Mg/ml	percentage of destruction				
	0	1	2	3	4
Time /hour					
50	25	27	35	40	50
100	30	45	60	70	90
150	45	70	80	97	100
Control	4.5	4.98	5.01	5.5	5.98

refers to dead and non-stained parasite cells indicating living cells.

## RESULTS

### Diagnosis of parasite

Through the diagnosis of cutaneous leishmaniasis parasite, which was isolated from patients infected with the Baghdad boil in Nasiriyah city, the center of Thi – Qar province in a way KDNA- PCR which showed the presence of *Leishmania major* only.

### Evaluation of the efficiency of the kDNA-PCR method and traditional diagnostic methods.

kDNA-PCR was performed on all collected samples, and amplified has shown the existence of a parasite *Leishmania major* in 48 / 50 from all patient And absence of parasite *Leishmania tropica* ( Fig.1 ), (Table 1).

### Effect of aqueous extract of *Nigella sativa* on the vitality of *L. major* in vitro.

Table (2) shows the percentage of destruction of *leishmania major* parasite by the effect of the plant extract of the *Nigella sativa*. It was observed that the effect of the plant extract was increased by increasing the exposure time of the extract and increasing the concentration of the extract, It was noted that the percentage of destruction was 100% after four hours of exposure to the extract and 150 mg / ml concentration compared to the control in which the percentage of destruction was 5.98 %. It is noted that the percentage of destruction after four hours also 50% and 90% of the concentrations 50 and 100 mg / ml respectively.

## DISCUSSION

The results of the electrophoresis of cutaneous leishmaniasis sample which isolated from the patients in Thi-Qar province by KDNA- PCR showed that the sample is the *Leishmania major* type was given a positive result at molecular weight 650pb and was given a negative result This result corresponds to a study Nada *et al.*, (2017) showed the PCR was more specific technique for diagnosis of cutaneous leishmaniasis with (95%) specificity and out of 44 suspected Cutaneous Leishmaniasis patients, cases were positive PCR 95% (42\44).

In another study Marcela *et al.*, (2013) found that kDNA-PCR was performed on all 128 collected samples, and amplified *Leishmania* DNA was observed in 112/128 (87.5%) clinically suspected ATL samples The 16 remaining samples (12.5%) were considered negative

after confirmation of DNA integrity by human  $\beta$ -globin PCR analysis. In addition, these samples were also negative for ATL according to the traditional methods, excluding one sample.

this in agreement with other Iraqi study Rahi *et al.*, (2013) and other study in nearby countries such as Iran Azizi *et al.*, (2012), hence the high incidence of *L. major* may be due to the presence of reservoir animals in large numbers, especially rodents and dogs. Obviously, dense populations of natural hosts of *L. major*, together with abundant vector sand flies, are the key elements responsible for the high rate of human infection.

Use water as a solvent in the extraction process because it is neutral liquid that does not negatively or positively effect on the effectiveness of compounds derived from plants extracted for non-interference with these compounds, compared to organic solvents that may have a negative or positive interference with the compounds extracted (Leven *et al.*, 1979; AL-Hilli, 2000).

It was observed that the percentage of destruction *leishmania major* parasite was increased by increasing the concentration of the aqueous extract of the plant *Nigella sativa* and increasing the exposure time. The inhibitory effect shown by the plant extract against the parasite may be due to its containment of active substances such as Flavonoid, Tannins, Alkaloids and Thymoquinone. The inhibition of alkaloids compounds of the parasite may be due to the overlap of these compounds in the series of protein metabolism reactions necessary for the continued vitality of the microorganism or its ability to break down the cellular wall and its propellant of proteins and fats and thus parasite destruction (Anthony, 1979). There are many studies that confirm the inhibitory effect of the extract of this plant on the parasites and bacteria where Ali and Farzanen (2015) confirmed the inhibitory role of this plant on *Leishmania major* parasites they said it could be useful in treating leishmaniasis Mohamed Jawad (2011) also noted the inhibitory effect of black bean seed extracts on the Gram positive bacteria, particularly bacteria *Staphylococcus aureus* and *Staphylococcus epidermis*

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