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An Epidemiological, Diagnostic, and Therapeutic Study of the *Leishmania tropica* Parasite in Iraq's Anbar Province

Suad Shallal Shahatha* ***Thaer Abdelqader Saleh***

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Abstract:

This paper involved the registration of 1,936 cases of infection of the *Leishmania tropica* parasite observed at hospitals and health centers in Ramadi, Fallujah, Baghdadi, and Hit during 2017. The results revealed that the highest rates of infection were found in Ramadi and Fallujah. The 1-10 years age group recorded the highest rate at 35.5%. There was no significant difference ($p \geq 0.05$) between the sexes. December and January saw the highest rate of infection, where the rate in rural townships was found to be 65.5%, higher than in urban regions which saw a rate of 34.4%. Facial lesions were the most prominent area of infection, recorded at a rate of 41.3%. The study also included an examination of 180 rodents (94 mice and 86 black rats) - the investigation demonstrated the presence of the amastigote stage at a rate of 43.6% among mice and 53.4% among rats. The study also involved an analysis of the impact of the use of a water extract from the *Rhanterium epapposum* plant, also locally known as the Arfaj plant, on *Leishmania tropica* parasite growth. As part of this study, a concentration of between 0.05-5 mg/ml was used. The application of these concentrations led to an inhibitory effect on parasite growth - an application of relatively higher concentrations caused greater effects in times of growth between 1-5 days.

Keywords: epidemiology, therapeutic, *Leishmania tropica*, Arfaj plant

Introduction:

The *Leishmania* parasite is a protozoan parasite belonging to the flagellated eukaryotic monocyte. It lives in pharyngeal cells, embedded in the retinal lining of the vertebrate host and is the cause of a disease called leishmaniasis, which is transmitted through the bites of female mosquitoes (sand flies) of the *Phlebotomus* genus (Fig.1). Dogs, foxes, and rodents are the main reservoir hosts of the parasite (1). The *Leishmania* parasite exists in two forms known as the amastigote and the promastigote. The first form is found in vertebral hosts, while the latter is located in vector hosts as well as in culture media (2).

Leishmania tropica causes cutaneous leishmaniasis, also known as the Baghdad boil, the Delhi boil, and oriental sore. It is identified through the presence of a skin lesion ranging from a small pimple to a large sore typically located on the face, hands, and legs (3). Leishmaniasis is endemic in more than 98 countries across the world, there are 12 million cases of *Leishmania* and 350 million are exposed to the infection. Moreover, the annual incidence is 2–2.5 million (4).

Department of Biology, College of Science, University of Anbar, Anbar, Iraq.
*Corresponding author: alhuda7730@yahoo.com

Several studies have been conducted to investigate the spread of cutaneous leishmaniasis in Iraq; Al-Samarai and Al-Obaidi recorded a 57% rate of infection of cutaneous leishmaniasis (5). Elsewhere, Daham and Al-Husseini found 468 cases in Salah Al-Din (6). In addition to the aforementioned investigations, a number of studies have been carried out in response to the *Leishmania* epidemic in the city of Kirkuk, Iraq (7).

Many plants and medicinal herbs have been used in the treatment of diseasses including parasitic diseases such as leishmaniasis. They have been found to contain many effective compounds including alkaloids, glycosides, fats and flavones, resins, carbohydrates, titans, volatile oils, and others (8). The Arfaj plant, or the *Rhanterium epapposum* plant، belongs to the Asteraceae family, which is characterised by clusters of small, spherical flowers with an approximate height of 30-55cm (Fig.2). It blooms between mid-May and late June, and the plant grows in sandy and calcareous soils. It has antibacterial and fungal properties and is used as a dermatologic. Moreover, this plant aids digestion and can be used to help with renal colic. It contains 17 chemical compounds ranging from aromatics, flavonoids, citrolates, and volatile oils such as Linalool, Camphere, Camphene, Terpinene,

Limonen, and anthraguinones (9). In 2015, Ezatpour and his colleagues studied the impact of *Pistacia khinjuk* on *Leishmania tropica* promastigotes (10). In the same year, Mahmoudv and his colleagues conducted another study on the impact of extracts of essential oils and methanolic extracts of *Myrtus communis L.* on the *Leishmania tropica* parasite (11). On the other hand, studies have been carried out analysing the impact of *Zataria multiflora* on *Leishmania tropica* (12). However, due to the lack of studies on the *Leishmania tropica* parasite specifically in the province of Anbar and the spread of the ailment epidemically, particularly in light of the province's political conditions over the past two years and considering the deterioration of public health across the province and indeed the country, this paper has chosen the Anbar province as its focus. As part of this study, the impact of the water extract from the Arfaj plant on the growth of parasite in culture will also be looked upon. This study is the first in the country where the use of the Arfaj plant to treat this parasite in order to reduce infections and its spread has been included.

Figure 1. Samples of sand flies

Figure 2. The Arfaj plant *Rhanterium epapposum*

Materials and Methods:

Examination and diagnosis of parasites

Information on the 1,936 cases of cutaneous leishmaniasis that were reviewed as part of this study included the identification of patients' age, sex, area of residence, date of infection, and its location in the body. The data was collated from patients attending hospitals and their respective health centers in Ramadi, Fallujah, Hit, and Baghdad during the year 2017.

The parasite was diagnosed in two ways:

1-**The direct examination method**, where thin blood smears or biopsies from the active edge of the lesion were taken from patients. Following this, the blood smears were installed and dyed with a Giemsa stain according to method used by de Vries *et al.* (13), and then examined at 40X for parasite viewing.

2-**The method of cultural media**, whereby a tissue biopsy of the active edge of the lesion was crushed with a mount of solution or a lock sterilizer. Alternatively, liquid was collected from the active lesion. The samples were then injected into the semi-solid culture media and prepared according to the method used by de Vries *et al.* (13). The next step was incubation of the samples at 27°C. After a five-day incubation period, the samples were examined by taking a drop from the center and placing it on a slide before being observed at 40X to examine the parasite.

Preparation of the culture media

Toby's media is used to develop *Leishmania* parasites. This media consists of two phases, the solid phase and the liquid phase. Both were prepared according to the method mentioned by Al-Hamairy (14), and using Lock's solution.

Complete blood preparation

O and B blood groups were specifically selected to fortify the *Leishmania* strain. These two groups are more susceptible to the growth of *Leishmania*. 15 ml of human blood was added to 85 ml of the solid media. Then, the media was distributed in sterile glass bottles of 35 ml and 5 ml for each vial in a slanted manner to be hardened. Following this, they were inserted into the incubator for 24 hours and stored at a temperature of 26°C to ensure that they were free from contaminants. Finally, 2 ml of Lock's solution was added to each vial (15).

Development and count of the number of parasites

The parasites were cultured by adding 0.1 ml of Lock's solution containing live amastigote parasites after 4 days to the vials containing 1.9 ml of Lock's

solution and ($10^5 \times 2$) cell/cm^2 after which they were stored at 26°C for 5 days. The number of parasites in each culture was measured using a haemocytometer and was obtained by taking 0.9 ml of the culture and adding 0.1 ml of diluted formalin solution by 10%. Then, the parasite was positioned under the light microscope to be observed at 40X and thus the number of parasites was obtained in each 1 cm^3 sample of the culture (11).

Preparation of the water extract of the Arfaj plant

The water extract was prepared from the Arfaj plant. Firstly, it was collected from an island area in Ramadi، which was diagnosed in Anbar University Herbarium. The plants were cleaned and dried in an incubator set at 30-35°C. The plant was extracted according to method Ezatpour *et al.* (10) by crushing 40 grams of the plant with 160 ml of distilled water in a ration of 4:1 and using a blender. The mixture was placed inside a snow bath to ensure that the active compounds were not harmed by the high temperature. The substance was then mixed for 60 minutes using an electric magnetic motor stirrer and left to sit for 24 hours at 4°C. After that, the mixture was nominated using filtration papers to dispose of non-plant fiber parts and then the plant extract was dried using a lyophilizer. The powder was stored after the drying process in glass bottles at -10°C until use. The standard concentration of the extract was prepared by taking 2 grams of raw extract and 20 ml of distilled water. Based on the standard concentration (100 mg/ml), the required concentrations were prepared.

Effect of different concentrations on the growth of parasites

The concentrations 0.05, 0.5, 1.5, 2.5, and 5 mg/ml of the water extract of the Arfaj plant were added separately to 35 ml glass bottles. The parasite grew on Toby's media and after 4 days of inoculation, 0.1 ml of the culture was added to the glass bottles and incubated for 5 days at 26°C. The parasites were then counted at different intervals, after 1, 2, 3, 4, and 5 days of growth.

Collecting and testing rodents

180 rodents (94 mice and 86 black rats) were caught using metal traps and ready-made adhesive papers. The rodents were killed using chloroform and the anatomical dissection was performed by opening the abdomen below the chest area and sterilized with ethanol (Fig.3). Swabs of the blood samples were taken, and a biopsy was taken from the liver and the spleen, where light pressure swabs were carried out to detect the parasite (16). It was placed on a filter paper and thinly sprayed on a slide and covered with methyl alcohol for one minute to dry. Finally, it was washed with water and dyed with Giemsa stain for 30 minutes, before being washed with water and left to dry.

Chemical detection of active compounds in plants (17**)**

Detection of flavonoids: A 10 ml solution of 50% ethanol was added to 10 ml of 50% potassium hydroxide and mixed in an equal amount; the presence of a yellow color indicated the existence of flavonoids.

Detection of phenols: 1 ml of extract was added to 1 ml of 1% ferric chloride solution; the surfacing of a green or green-like shade indicated the presence of phenols.

Detection of alkaloids: 10 grams of plant extract was boiled with 50 ml of distilled water containing 4% hydrochloric acid (HCL). The solution was cooled and filtered. Following this, 0.5 ml of leachate was tested in a watch glass with 0.5 ml of Meyer reagent; the emergence of a white deposition confirmed the presence of alkaloids.

Detection of Terpenes: a mixture of the following chemical materials was prepared: 1 gram of plant extract was dissolved in 2 ml of chloroform, followed by a drop of anhydrous acetic acid and a drop of concentrated sulfuric acid. The presence of brown sediment indicated the existence of terpenes.

Detection of the saponins: 3 ml of mercuric chloride solution (1%) was added to 5 ml of the extract; the appearance of a white sediment indicated the presence of the saponins.

Detection of tannins: A few drops of lead acetate solution (1%) were added to 5 ml of the plant extract; the emergence of white gelatin deposits confirmed the presence of tannins.

Detection of resins: 50 ml of ethyl alcohol (95%) was added to 5 grams of plant extract and heated in a water bath. The solution was then filtered and combined with 100 ml of distilled water containing 4% of HCL. The appearance of turbidity indicated the presence of resins.

Statistical analysis

The results were analyzed using the one-way analysis of variance (ANOVA) table. The results were compared using least significant difference (LSD) and the SAS (18).

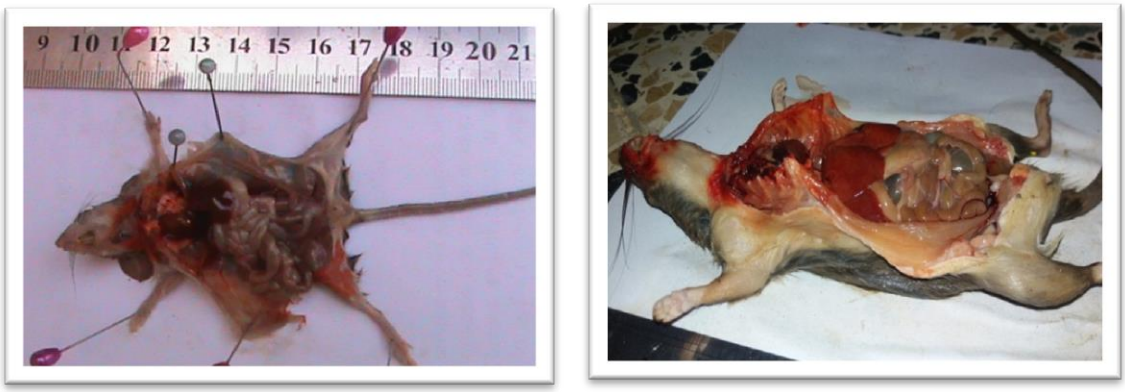


Figure 3. Photos of a mouse and a rat after autopsy

Results:

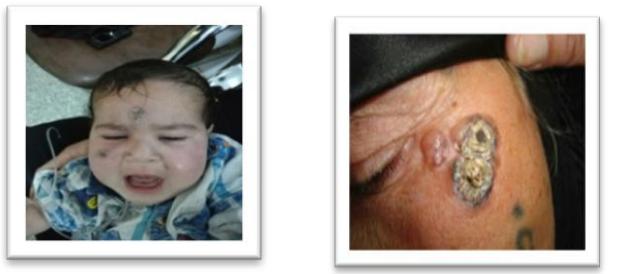
The study showed clear cases of infection of leishmaniasis in Anbar. The city of Ramadi recorded the highest percentage of infection with a rate of 32.2%, followed by Fallujah with a rate of 30.9% (Table 1). The 1-10 years age group recorded the highest infection rate (35.5%) as depicted in Table 2. There are no significant variation between the rates amongst males (50.4%) and females (49.5%) as Table 3 presents. The incidence rate in rural areas was 65.6%, higher than that found across urban inhabitants who had an infection rate of 34.4% (Table 4). Table 5 portrays the relationship between infection rates and months of the year, indicating that there is a high incidence when low temperatures are recorded. December and January recorded the highest infection rates, 26.2% and 27.8% respectively; in contrast, the infection was not reported during the summer months. Table 6 shows that the incidence of facial infections was 41.3% and that of abdominal infection was 3%, significantly higher than other areas of the body (Fig. 4).

The proportion of rodent infection was 48.3% (in the domestic mouse it was at a rate of 43.6% and 53.4% for the black rat) as depicted in Table 7. and Fig. 5 illustrates the existence of amastigote *Leishmania* in one of the blood swabs of a rodent. The 0.05, 0.5, 1.5, 2.5, and 5 mg/ml concentrations were used to determine the impact on the development of cutaneous leishmaniasis parasites in the culture media and to compare them with non-treated parasites (control). The 5 mg/ml concentration resulted in the extermination of the parasites during the first day of treatment, while the 2.5 mg/ml concentration completed the inhibition of it within 2 days of treatment. The 0.05, 0.5, and 1.5 mg/ml concentrations reduced the growth rate to 50.0%, 61.4%, and 71.3% respectively within 1 day of treatment; after 2 days, the rates had fallen to 37.0%, 55.0%, and 66.2 %; after 3 days the growth rate decreased again to 22.8%, 48.4%, and 56.6%; after 4 days, growth was reduced to 15.8%, 40.5%, and 49.3%. After 5 days, all concentrations resulted in the complete inhibition in the culture media compared with the control group (Table 8). Table 9 shows the active substances found in the plant extract of Arfaj, namely flavonoids, phenols, alkaloids, saponins, terpenes, and tannins.

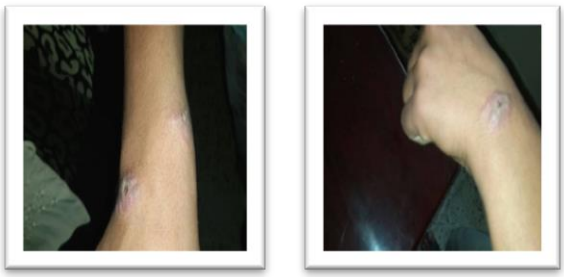
Table 1. Number and Percentage of Infection of *Leishmania tropica* Patients in Anbar province.

Region	Number of Infections	%
Ramadi	624	32.2*
Falluja	600	30.9
Baghdadi	510	26.3
Hit	202	10.4
Total	1936	100
Significant variations		0.001

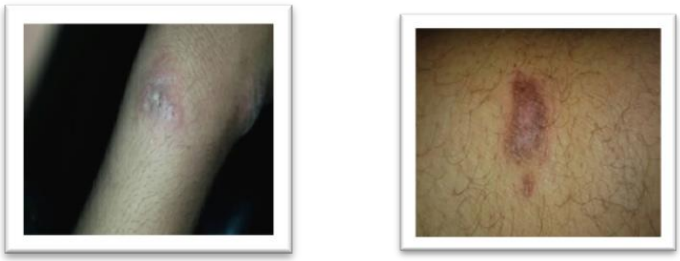
* = significant



A. Images of facial infection



B. Images of hand and arm infection



C. Images of leg infection

Figure 4. Photos of various body areas infected with *Leishmania*

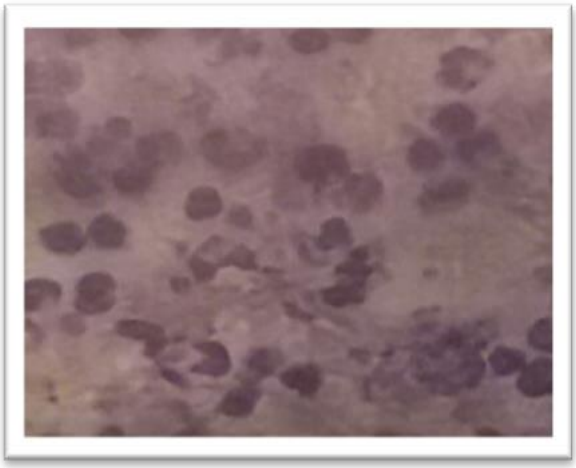


Figure 5. The *Leishmania* amastigote Phase dyed with a Giemsa stain in a blood smear of one of the examined rodent(100x)

Table 2. Number and percentage of *Leishmania tropica* infection by age.

age	Number of Infections	%
1-10	688	35.5**
11-20	620	32.0
21-30	282	14.5
31-40	204	10.5
41-50	98	5.0
51-60	40	2.0
61-70	4	0.2
Total	1936	100
Significant variations		Less than 0.001

** = high significant

Table 3. Number and percentage of *Leishmania tropica* by sex.

sex	Number of infections	%
male	976	50.4*
female	960	49.5
Total	1936	100
Significant variations		Less than 0.001

* = no significant

Table 4. Number and percentage of *Leishmania tropica* infection by Accommodation area.

Accommodation area	Number of infections	%
rural	1270	65.5**
urban	666	34.4
Total	1936	100
Significant variations		Less than 0.001

** = high significant

Table 5. Number and percentage of *Leishmania tropica* infections by month

Month	Number of infections	%
January	540	27.8**
February	162	8.3
March	44	2.2
April	236	12.1
May	0	0
June	0	0
July	0	0
August	0	0
September	198	10.2
October	116	5.9
November	132	6.8
December	508	26.2*
Total	1936	100
Significant variations		Less than 0.001

* = significant ** = high significant

Table 6. Number and percentage of *Leishmania tropica* infections by bodily location

Location of infection	Number of infections	%
Face	800	41.3**
Neck	82	4.2
Back	72	3.7
Abdomen	60	3.0**
Hands	500	25.8
Legs	422	21.7
Total	1936	100
Significant variations		Less than 0.001

** = high significant

Table 7. Number of rodents examined and infection percentage of *Leishmania tropica*

Type of Rodent	Number examined	Number of infections	%
Mouse	94	41	43.6
Black rat	86	46	53.4*
Total	180	87	48.3
Significant variations			0.05

* = significan

Table 8. Effect of water extract of the Arfaj plant on the growth of *Leishmania tropica* parasites in culture media

Exposure period / Concentration(mg / ml)	Growth (%)				
	1 day	2 days	3 days	4 days	5 days
0.05	71.3*	66.2*	56.6*	49.3*	0
0.5	61.4	55.0	48.4	40.5	0
1.5	50.0	37.0	22.8	15.8	0
2.5	21.5	0	0	0	0
5	0	0	0	0	0
Control	100	100	100	100	100
Significant variations	0.004	0.013	0.007	0.005	0.019

* = significant

Table 9. Chemical reagents on active compounds in extract of the Arfaj plant.

active compounds	Type of Reagents	Reagents Guide	Result Reagents
Flavonoids	Potassium hydroxide 50%	Yellow precipitate	+
Phenols	Ferric chloride 1%	Greenish green sediment	+
Alkaloids	Meyer Reagent	White deposit	+
Terpenes	Sulfuric acid concentrates with chloroform	Brown deposit	+
The Saponins	Mercuric chloride 1%	White precipitate	+
Tannins	Lead acetate 1%	White gelatin deposits	+
Resins	Acid 4% HCL	The turbidity	-

(+) the presence of an effective compound
(-) Lack of effective compound

Discussion:

The study confirmed that the 1,936 patients who participated in this research had been infected with cutaneous leishmaniasis from areas of the province of Anbar. This result is in agreement with a study Abdulsadah (19), attributed to the deterioration of public health in Anbar, especially over the past two years as a result of the destabilization and displacement endured by the province and the migration of local populations. Where the population density has increased, the incidence of *Leishmania* also increased and spread epidemically due to a lack of interest in and observation of hygiene standards. Additionally, the volume of insects and animal stockpiles fuelled the spread of the disease and resulted in higher rates of infection of cutaneous leishmaniasis. The 1-10 years age group recorded the highest rate of infection and this is in compliance with a study Al-Sayad (20). A possible explanation for this could be that children do not adhere to public health standards due to a lack of knowledge and experience, and that there is insufficient immunity against this parasite in children because there is no euglobulin in their bodily system, which is present in adults and leads to the annihilation of the parasites (21). Therefore, children are more likely to catch the infection in endemic areas.

There were no noteworthy contrasts amongst males and females as per the results of this study - this agree with the findings of Daham and Al-Husseini (6), but contradicts the results of Al-Mashhadany (22) and Khademvatan *et al.* (23). This is because the disease affects both females and males in equal measure. On the other hand,

environmental and social conditions play an important role in the spread of the disease, as well as factors such as people's sleeping habits, as sand flies fly low in search of prey. People in the province tend to sleep outside their houses or on the roof due to the regular power shortages experienced. Throughout the summer, high humidity and high temperatures create conditions which lead to a marked increase in the activity of the insect carriers and their contact with human skin. The incidence of infection in rural areas is higher than in urban ones, this corresponds to the findings of Al-Jaf (24).This is due to the undeveloped nature of cultural and social norms and lifestyles in rural areas, which are presumably responsible for the lack of education around hygiene, as well as the presence of animals residing close to or within people's living quarters. The distribution of insects, tankers, bulk dogs, and rodents across rural areas of the province facilitate the spread of the infection and play a notable role as the reservoir hosts of the disease. Moreover, the large ranches and plantations in the villages are perfect breeding grounds for the insects, including sand flies, which is also responsible for allowing the spread of the infection.

The increase in the infection rate was observed in the months of December and January, which is similar to what was found in the study of Fakhar *et al*. (25). This could be due to the fact that the insects multiply and increase in population during the months of March and April, and the process of chewing also occurs during these months followed by an incubation period of 4-6 months. The infection then appears in the winter months and disappears in the summer months because the proliferation of vector insects requires moist and mild conditions. Furthermore, temperatures play a significant role in the spread of leishmaniasis.

The highest incidence of infection was recorded in the face, hands, and legs of the body which corresponds with a study Gomes *et al.* (26). The reason for this is that these areas of the body are easily accessible and therefore most prone to bites of insects due to their exposure. Sleeping habits as previously mentioned and play time outside the house facilitate the spread of the infection.

The results indicate the presence of parasites in rodents and this confirms that they play the role of reservoir hosts to the parasite in the province of Anbar, which is in agreement with the study of Foroutan *et al*. (27) The dog family is also considered to be an important reservoir for the parasite and plays a significant role in the spread of the disease as a result of the free reign of dogs in the province. In addition, dogs' bulk and spread contribute significantly to the spread of the infection. This happens because of the lack of health and municipal services as well as the deterioration of public health across the province. As a result of war, migration, and displacement, the spread of the disease has been made much easier as many families are now living in camps which are also home to dogs and insects. All these factors have prompted the spread of this endemic illness.

Also, the results of this study demonstrate the clear inhibitory impact of the water extract of the Arfaj plant on the growth of *Leishmania* parasites; the findings clearly show that it can kill these parasites and can therefore be employed as a possible treatment for the infection. After one day of treatment at high concentration levels, the infection in the culture media was significantly reduced. In addition, the growth rate was decreased by increasing concentration levels during different periods of treatment, which was also noted in a study Al- yasary (28) and a study Al- Shukur (29). The plant contains many compounds, which have the capacity to directly affect the centers of enzyme production and other vital components, thus causing the annihilation of the cells and, consequently, elimination. This is in agreement with the studies mentioned above, which confirmed the high inhibitive power of extracts from the plants and their impact on parasite growth in culture media. Moreover, the plant contains a quantity of tannins which are phenolic compounds and significantly affect the acetylcholinesterase enzyme. The fact that this enzyme controls all physiological events and the passage of ions to and from the cell through the membrane of the cell and thus controlling the mobility of the parasite, inhibition of this enzyme will lead to the death of the parasite.

Conclusion:

1-The study showed clear cases of infection of leishmaniasis in Anbar.
2- The 1-10 years age group recorded the highest infection rate.
3-. There were no noteworthy contrasts amongst males and females
4- The incidence of infection in rural areas is higher than in urban ones.
5- The increase in the infection rate was observed in the months of December and January.
6- The results of this study demonstrate the clear inhibitory impact of the water extract of the Arfaj plant on the growth of *Leishmania* parasites.

Conflicts of Interest: None.

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دراسة وبائية وتشخيصية وعلاجية لطفيلي *Leishmania tropica* في محافظة الانبار- العراق

سعاد شلال شحاذة **ثائرعبد القادر صالح**

قسم علوم الحياة، كلية العلوم، جامعة الانبار، الانبار، العراق.

الخلاصة:

تضمنت الدراسة الحالية تسجيل 1936 حالة أصابة بطفيلي *Leishmania tropica* للمراجعين لمستشفيات (الرمادي، الفلوجة، البغدادي، وهيت) والمراكز الصحية التابعة خلال عام 2017، أظهرت النتائج أعلى نسبة أصابة في مدينتي الرمادي والفلوجة وأحتلت الفئة العمرية (1-10) سنوات أعلى نسبة أصابة بلغت35.5% ولم يسجل فرق معنوي($P\geq0.05$) بين اصابة كلا الجنسين، وسجل شهري كانون الاول وكانون الثاني أعلى معدل للاصابة، وكانت نسبة الاصابة في الريف 65.5 % أعلى منها في المدينة 34.4% وبفرق معنوي، وسجلت آفات الوجه اعلى نسبة 41.3%عن باقي اجزاء الجسم. كما تضمنت الدراسة فحص 180 قارض (94 فأر منزلي و 86 جرذ أسود)وبينت الدراسة وجود الأطوار المسوطة وبنسة43.6% للفئران و53.4% للجرذان. شملت الدراسة ايضا تأثير المستخلص المائي لنبات العرفج *Rhanterium epapposum*على نمو الطفيلي، أذ أدى أستخدام التراكيز ما بين (0.05-5) ملغم/مل الى تأثير تثبيطي على نمو الطفيلي وكلما زاد التركيز زاد التأثير خلال فترات النمو من 1-5 يوم.

الكلمات المفتاحية : وبائية ، علاجية ، *Leishmania tropica* ،نبات العرفج .