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Evaluation of inhibitory effect of Salvia officinalis extracts and volatile oils on the life of Leishmania donovani parasite

Hala Talib Abd Ali Al-Taei

Department of Biology, College of Education for pure Sciences, University of Kerbala, Karbala, Iraq
Corresponding author email: hala.t@s.uokerbala.edu.iq

Yarub Modhar Al-Qazwini

Department of Biology, College of Education for pure Sciences, University of Kerbala, Karbala, Iraq
Email: yaarob.m@uokerbala.edu.iq

Hanan Zwair

Department of Biology, College of Education for pure Sciences, University of Kerbala, Karbala, Iraq
Email: hanan.mikhlif@uokerbala.edu.iq

Abstract---Visceral leishmaniasis (VL) is considered one of the endemic diseases in Iraq, and due to the distinction of the anti-leishmaniasis treatments currently used by their many side effects in addition to their toxicity to the human body, as well as the length of treatment period, and the problems they cause to the digestive system, *Salvia officinalis* (Saga) was used as a natural preventive treatment against leishmaniasis. Because of its therapeutic properties for many health symptoms, including heart attacks, ulcers, and cancerous tumors, in addition to its antibacterial and antimicrobial nature, the current study aimed to know the inhibitory activity of plant extracts and the volatile oil of *Salvia officinalis* on the life of *Leishmania donovani* parasite, using the colorimetric test (MTT assay, to measure toxicity) promastigote parasitoid cells, six concentrations of the aqueous extract and plant emulsion of the mentioned plant were prepared (6.25, 12.5, 25, 50, 100, 200) g/ml, the concentrations of the volatile oil were (31.25, 62.5, 125, 250, 500, 1000) g/ml. The results indicated that under the influence of treatment with the three drug spectra of *Salvia officinalis*, there were noticeable differences in the inhibitory percentage for each drug spectrum. The deterioration of its concentrations, as it gave the two highest concentrations of the aqueous extract, namely (100 and 200

µg/ml), a percentage of 92%, which is the highest percentage of cellular growth inhibition under the influence of treatment with the aqueous extract of the plant, while the second concentration, which is (100 µg/ml), gave a percentage value is 89%, which represents the highest percentage of cell killing under the influence of the plant emulsion of the same plant, while the highest concentration was effective in inhibiting the growth of promastigote under the influence of treatment with volatile oil of *Salvia officinalis* plant was the fifth concentration (62.5 µg/ml), the highest killing rate was 86%.

Keywords---visceral leishmaniasis, *Salvia officinalis*, anti-leishmaniasis, MTT assay, plant extracts, volatile oil, inhibitory percentage.

Introduction

Leishmania donovani belongs to the protozoa, which belonging to the class of kinetoplastid flagellates of the family Trypanosomatida (Millán et al., 2014). This parasite is the main cause of visceral leishmaniasis, which is considered one of the most deadly forms of the disease in the human race if untreated. As a result of infection with the *Leishmania* complex *L. donovani* complex, which consists of two sexes of *Leishmania*, namely (*L. donovani* and *L. infantum*), In addition to *L. chagasi*, these species cannot be distinguished morphologically, but molecular techniques are used to identify their types (Lukeš et al., 2007). *Leishmania* parasites have a genetic life cycle consisting of two main stages, so they need two hosts to complete their life cycle, as the two stages include two stages: the promastigote, inhabiting the alimentary canal of the invertebrate host represented by the sand fly of the genus *Phlebotomus*, and Amastigote is an oval flagellum that inhabits the macrophages of the vertebrate host, represented by humans (Debrabant et al., 2004; Hojjat, 2015).

Leishmaniasis visceral is spread in 101 countries with approximately 350 million susceptible people, and these areas are characterized as active sites of parasitic transmission, There are an estimated 1.3 million cases that occur annually worldwide, and it is a serious systemic parasitic disease caused by parasites of the visceral spectrum of the disease (Savoia, 2015) (Sundar and Singh, 2016). There are more than 20 known types of leishmaniasis that can infect humans and can cause different clinical symptoms, Leishmaniasis has three known clinical forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL) and visceral leishmaniasis (VL), (Hakkour et al., 2020).

The WHO estimated that 80% of people around the world depend on medicine herbal as an essential part of their health (Dekanski et al., 2009). *Salvia officinalis* has been used in traditional folk medicine since ancient times to treat various human diseases (Craft et al., 2017). Through studies, it was found that *Salvia officinalis* contains high levels of terpenoids and flavonoids, which are bioactive chemicals (Ghorbani and Esmaeilizadeh, 2017), these compounds are characterized by their powerful antioxidant effects, and this plant is a broad-

spectrum remedy for many different health disorders (Pedro et al., 2016), In addition to its lethal toxicity to single-celled organisms (Patel et al., 2022).

Materials and Methods

Parasite samples were collected and grown in artificially prohibited culture media, composed of The RPMI-1640 culture medium was prepared by adding RPMI-1640 solution and a certain amount of bovine fetal serum as well as the antibiotic Penicillium., and then six different concentrations of sage were prepared for each drug spectrum, which were as follows: For the aqueous extract and the plant emulsion, the concentrations were prepared (200, 100, 50, 25, 12.5, 6.25 µg/ml), The volatile oil concentrations were (1000, 500, 250, 125, 62.5, 31.25 µg/ml), The inhibitory effect of these drug spectra on the growth and vitality of the parasitic flagella was measured by conducting a cytotoxicity test (MTT assay) on the parasitic cells of *Leishmania donovani* after treating them with different concentrations of plant extracts and volatile oil of *Salvia officinalis*. Where 3 replications were made in the microtiter plate for each drug spectrum and for each concentration of it, leaving holes for the negative control group, which were filled with the culture medium containing the parasitic cells only, while the remaining holes were filled with the cell suspension consisting of (promastigote + plant extract or volatile oil), The plate was incubated in the refrigerated incubator for a certain period of time, after which Dimethyl sulfoxide (DMSO) was added to each hole, and then dyed it with tetrazolium dye (MTT). After the experiment was completed, the microtiter plate etching parameters of the MTT assay were read using the ELISA reader.

Preparation of the aqueous extract of *Salvia officinalis*

Dry sage leaves were purchased from the local markets in the holy city of Karbala, Iraq, and then ground by the electric mill to obtain a fine powder used for extraction, Where the aqueous extract was prepared by 50 g of *Salvia officinalis* powder in 500 ml of distilled water in a glass beaker and inserted into the vibrating incubator for 24 hours Then the liquid was filtered by filter paper to get rid of plankton, then poured into glass containers and placed in the oven at a temperature of 40 ° C, then scraped off the dry matter to obtain the plant extract of brown color that was placed in the oven. A glass container and kept in the refrigerator, then six concentrations of the plant extract were made by making a series of dilutions based on a standard solution of 200µg/ml, which represented the highest concentration of the aqueous extract down to the lowest concentration, which was 6.25µg/ml, These concentrations were diluted using distilled water (Afonso et al., 2019).

Preparation of Essential oils extraction for *Salvia officinalis*

A total 100 g of dry *Salvia officinalis* leaves were weighed and then crushed with an electric mill to obtain a fine powder used in the volatile oil extraction process of the plan, Then it was placed in a glass boat for the clavinger volatile oil extraction device with the addition of 500 ml of distilled water and mixed well, then the mixture was heated at a temperature of 60 ° C for 3 hours at the end of the device. The essential oil was collected after cooling and condensing. Then, six

concentrations of the Essential oil were made by making a series of dilutions, depending on the standard solution prepared by adding 100 microliters of the volatile oil to 10 ml of Dimethyl sulfoxide (DMSO) to form a standard solution with a value of 1000, which represents the highest concentration for the volatile oil treatment, and then making the six required concentrations of it (Badiee et al., 2012).

Preparation of vegetable emulsion of *Salvia officinalis*

The plant emulsion was extracted during the process of extracting the volatile essential oil, as it was the result of condensation of water containing oil droplets, and after emptying the oil from the Clevenger extraction device, we will notice the formation of misty water containing oil droplets, which is withdrawn by the medical syringe and kept in a sterile glass tube away From light and at a relatively low temperature (in the refrigerator), then six concentrations of the vegetable emulsion were made by making a series of dilutions based on a standard solution of 200 µg/ml, which represented the highest concentration of the emulsion down to the lowest concentration, which was 6.25 µg/ml, These concentrations were diluted using distilled water and less of (DMSO) (Cassiday, 2014).

Statistical analysis

The statistical analysis program SAS 2012. Statistical Analysis System was used to detect the effect of the different factors in the study parameters, as well as the LSD Analysis of Variation-ANOVA test (the value of the least significant difference) was used for the important comparison between the methods used, the results were calculated by the ELISA reader device. reader of the two microtiters used under study, where the data were expressed on the basis of an equation represented by the mean + standard deviation (SD), at a probability level of 0.05 (Mohammad et al., 2022). In addition, the statistical analysis program SPSS and Excel 2010 were used to calculate the value of the concentration that inhibits 50% of the growth of parasitic cells, which is called IC₅₀., where the X-axis represents the logarithm of concentration, while the Y-axis represents the Inhibition percentage (Abe et al., 2012; Gharban and Al-Shaeli, 2021).

Result and Discussion

The results revealed the inhibitory effects of the aqueous extract, the crude essential oil, and the emulsion of *S. officinalis* on the growth of promastigots of *L. donovani* parasite. The cytotoxicity assay which was conducted by the (MTT assay) on the microtiter plate showed a difference in the chromatic absorption, depending on the six concentrations treated with it, which differed according to the type of drug substance, The concentrations of the aqueous extract were as follows: (200, 100, 50, 25, 12.5, 6.25 µg/ml), As for the concentrations of the essential oil, it was (1000, 500, 250, 125, 62.5, 31.25 µg/ml). Then, the emulsion concentrations were (200, 100, 50, 25, 12.5, 6.25 µg/ml). The figure (1) shows the plate after planting it in the cell suspension and incubating it for 24 hours and then treating it with the drug substance in its three spectrums according to the

concentrations mentioned above for each substance, and after four hours of incubation in the refrigerated incubator.

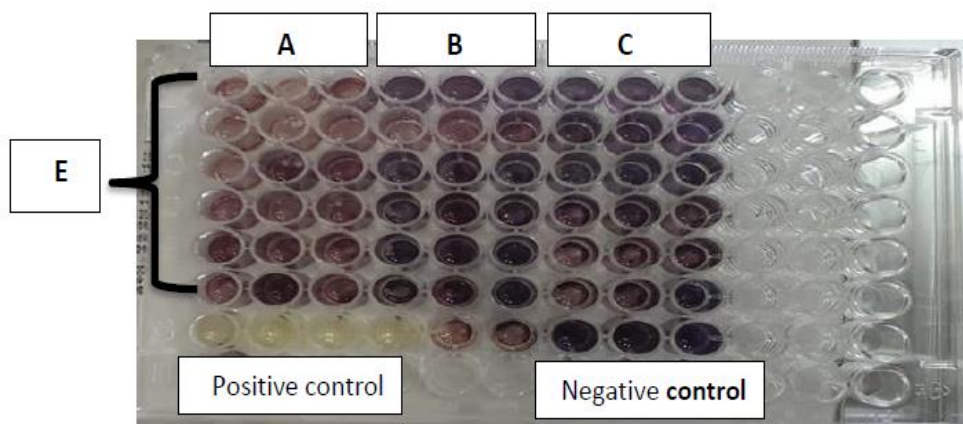


Figure 1. Microtiter plate after culturing in cell suspension with addition of drug concentrations of *saliva officinalis* with the addition of dye to perform the cytotoxicity test MTT assay

Where (A) repeats of the aqueous extract, (B) repeats of vegetable emulsion, (C) repeats of volatile oil, while (E) the six concentrations of the three drug spectra gradual from the highest concentration to the lowest concentration, the negative control consists of (Promastigotes + media), Positive control consists of (Cell suspension + Media + Pentostam drug).

The results indicated in the table (1) The two highest concentrations (200, 100 $\mu\text{g/ml}$) showed the highest anti-parasite inhibitory effect after treatment, the value of the least significant difference LSD at the probability level ($P \leq 0.05$) with a percentage of 92% at the probability level ($P \leq 0.01$). Moreover, the concentration (50) $\mu\text{g/ml}$ showed an inhibitory effect on cell growth, reaching a percentage of 83%, while the two concentrations (25, 12.5 $\mu\text{g/ml}$) were equal in their anti-parasitic effect with an inhibitory percentage of 84% with a slight decimal difference between them, As for the minimum concentration, which was (6.25) $\mu\text{g/ml}$, it gave a percentage inhibition of 72%, which triangle of the lowest anti-parasitic growth rate, which is due to the lowest concentration used for the aqueous extract of *S. officinalis*, As shown in the table below:

Table 1. Inhibition percentage for concentrations of aqueous extract

Inhibition percentage (%)	Mean \pm SD	Concentrations of aqueous extract $\mu\text{g/ml}$
92%	0.0269 \pm 0.0549 b	200
92.2%	0.0280 \pm 0.0373 b	100
83%	0.063 \pm 0.128 b	50
84%	0.059 \pm 0.0146 b	25
84.3%	0.0513 \pm 0.0109 b	12.5

72%	0.111 ± 0.226 b	6.25
0 %	0.322 ± 0.657 a	Control
Chi-Square (χ^2) =9.216**	0.8391 *	LSD value
where*represents the probability ratio ($p \leq 0.05$),where** ($p \leq 0.01$)		

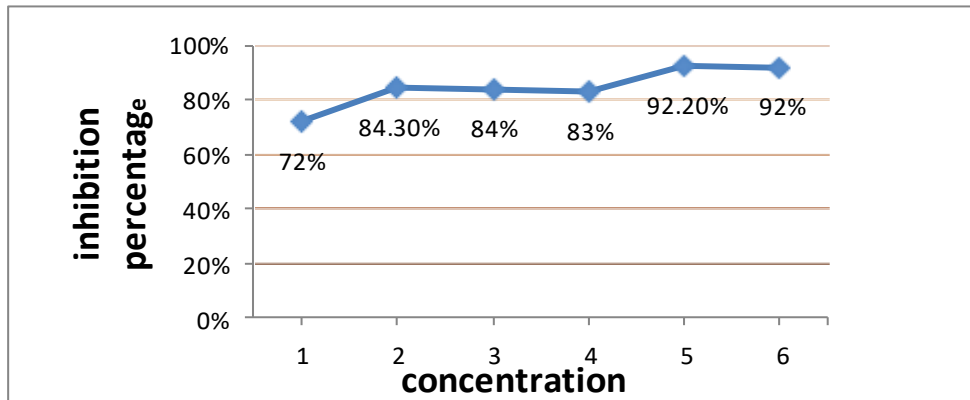


Figure 1. Inhibitory percentages under an influence of aqueous extract of *Saliva officinalis*

In view of the results presented in the table above, we note that the aqueous extract of *Saliva officinalis* possesses a high efficacy to inhibit the cellular growth of protozoa, and this is consistent with a close study on the inhibitory effects of the aqueous extract in addition to its promising activity in cytotoxicity (Afonso et al., 2019) and with study (Martins et al., 2015), While the results of the current study differed with a study (Serakta et al., 2013) conducted to measure the effectiveness of the extract on inhibiting the cellular growth of *Leishmania* parasite, where the researcher confirmed the ability of the aqueous extract on the total inhibition of the parasite cells.

The results of the cytotoxicity assay (MTT assay) for *Leishmania* visceral parasite *L. donovani* under the influence of the plant emulsion of *S. officinalis* showed that, different percentages of cellular growth inhibition efficacy and according to the concentrations of the drug spectrum. At the probability level of $P \leq 0.01$, the value of the least significant difference LSD at the probability level of $P \leq 0.05$. The second concentration (100 $\mu\text{g/ml}$) showed the highest percentage of inhibiting the cellular growth of the promastigots with a percentage of 89%, while the fourth concentration (25 $\mu\text{g/ml}$) ranked second in terms of its ability to kill cells with a percentage of 71%, while the first concentration gave (200 $\mu\text{g/ml}$) a percentage of 64%, while the concentrations (50, 12.5, 6.25 $\mu\text{g/ml}$) recorded percentages of (11%, 31%, 59%), respectively, as shown in the table below:

Table 2. Inhibition percentage for concentrations of the plant emulsion of *S. officinalis*

Inhibition percentage	Mean ± SD	Concentrations of emulsion ($\mu\text{g/ml}$)
64%	0.126 ± 0.259	200
89%	0.033 ± 0.068	100

11%	0.227 ± 0.464	50
71%	0.113 ± 0.230	25
31%	0.224 ± 0.457	12.5
59%	0.081 ± 0.165	6.25
0%	0.322 ± 0.657	Control
Chi-Square (χ^2)=6.635 **	*0.9956	LSD value
where*represents the probability ratio ($p \leq 0.05$), where** ($p \leq 0.01$)		

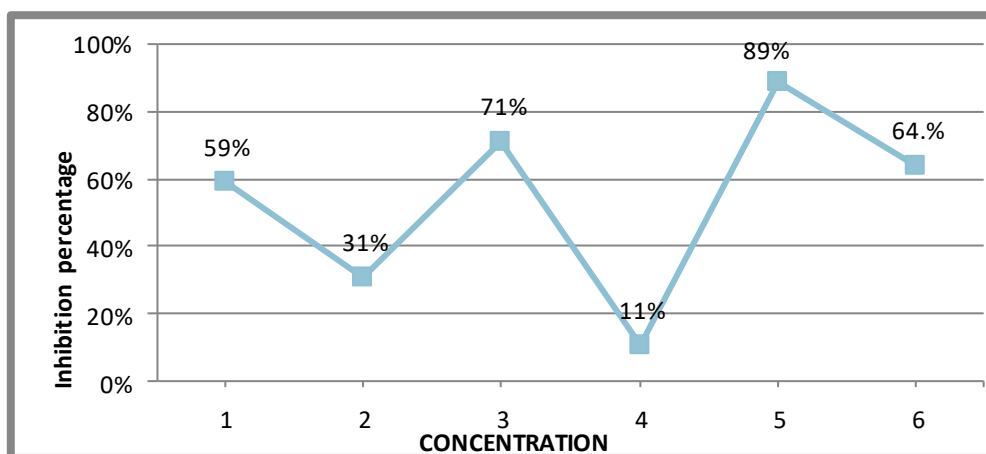


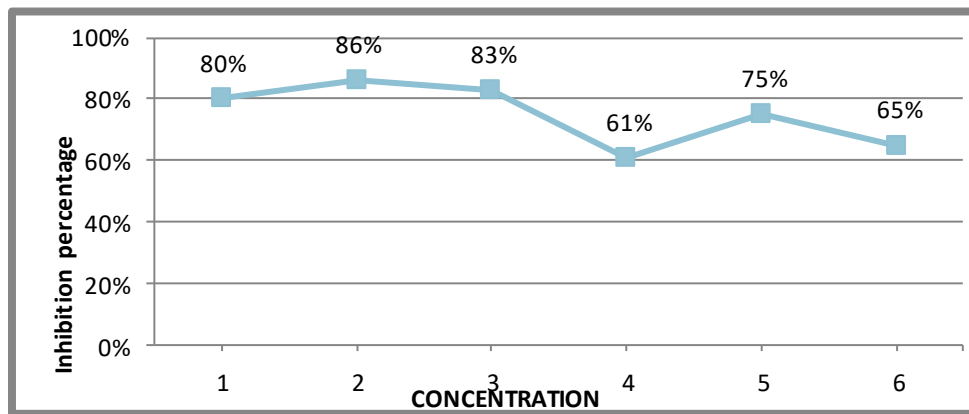
Figure 2. Inhibitory percentages under the influence of emulsion of plant *Saliva officinalis*

In comparison with the results presented in the table (2), it becomes clear to us the varying ability of *Saliva officinalis* emulsion to inhibit the cellular growth of the parasite, depending on the concentrations with which the parasitic cells were treated. Note that the use of plant emulsifier compounds represents a new drug technique to treat diseases caused by protozoa, including *Leishmania* parasites (Pund and Joshi, 2017).

While the experiments of treatment with the six concentrations of the CEO crude essential oil of the plant *S. officinalis* showed different results from the aqueous extract and the emulsion of the same plant alike, as the low concentrations of the Volatile Oil showed a greater ability to inhibit the growth of cells of the visceral parasite *L. donovani*. At the probability level $P \leq 0.01$, the value of the least significant difference LSD at the probability level $P \leq 0.05$. If the penultimate fifth concentration is given, which is (62.5 $\mu\text{g/ml}$) The highest cellular death rate was 86%. The fourth concentration (125 $\mu\text{g/ml}$) ranked second in terms of its ability to inhibit cellular growth with a percentage of 83%, As for the third place, it was occupied by the sixth concentration (31.25 $\mu\text{g/ml}$), as it showed a percentage inhibition rate of 80%, While the remaining concentrations were graded, giving varying percentages whose values reached (61%, 65%, 75%) for the concentrations (250 $\mu\text{g/ml}$, 1000 $\mu\text{g/ml}$, 500 $\mu\text{g/ml}$) respectively.

Table 3. Inhibition percentage for concentrations of volatile oil of *S. officinalis*

Inhibition percentages (%)	Mean \pm SD	Concentrations of volatile oil ($\mu\text{g/ml}$)
65%	0.123 \pm 0.251	1000
75%	0.082 \pm 0.167	500
61%	0.135 \pm 0.276	250
83%	0.054 \pm 0.110	125
86%	0,045 \pm 0,093	62.5
80%	0.073 \pm 0.149	31.25
0%	0,322 \pm 0,657	Control
Chi-Square (χ^2)=9.210**	0.7814*	LSD value
where*represents the probability ratio ($p \leq 0.05$), where** ($p \leq 0.01$)		

Figure 3. Inhibitory percentages under the influence of Volatile Oil of the plant *Saliva officinalis*

Returning to the results presented in Table (3), we will notice that the volatile essential oil of sagebrush has a high effectiveness for inhibiting the growth of the parasitic flagella, especially when used in its low and sub-high overhang with study (Ultee et al., 2002). As for the reason for the ineffectiveness of the higher concentrations of the volatile oil of *Saliva officinalis*, the reason may be due to the emergence of a reaction of the parasitic cell membrane against the higher concentrations and thus prevented penetration of the cell membrane, while the reason is likely to be due to the effective compounds contained in the volatile oil, which can interact differently with Mitochondrial membranes to generate free radicals that oxidize the large molecules of the parasite, which leads to its death through programmed cell death, and this is what the study agreed with (Tariku et al., 2010).

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