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Evaluation of Antileishmanial Activity of *Osteospermum ecklonis* Extract of Aerial Parts against *Leishmania donovani: in vitro*(Conference Paper)

Hind M. Jewelee*,1 and Thukaa Z.Abdul-Jalil *

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*Department of Pharmacognosy and Medicinal Plants, College of Pharmacy, University of Baghdad, Baghdad, Iraq

Abstract

Lack of safe available non-resistant treatment for visceral leishmaniasis (Kala-azar) keeps limiting the complete cure of this disease, drugs that have toxic side effects or lack of effectiveness have led to disease relapse, all these factors have lightened the way to the search for alternative drugs from natural resources that have been shown to have antileishmanial activity through literature survey

In the present study, the comparative in vitro anti-leishmania activity of various fractions of *Osteospermum ecklonis* aerial parts fractions have been evaluated. Extracts were prepared through maceration and Soxhlet apparatus using 85% methanol and fractionation was done by separating the active constituents according to the differences in their polarities using four solvents in different polarities (petroleum ether, chloroform, ethyl acetate, and finally n-butanol) ،Two of the resultant fractions(petroleum ether as well as n-butanol fractions) were chosen to test the effective inhibition of *Leishmania donovani*, Results prove with no doubt that the petroleum ether fraction of the maceration aerial parts in a concentration of 2.5 mg/ml had better antileishmanial activity than other concentrations of tested samples and the result coincided with the anti-leishmaniasis activity of official treatment (pentostam®) ,This finding can be attributed to the terpene nature of the materials used to be existed in such fraction. These observations have paved the road to step in for extended studies in relation to the conventional herbal medicines for better and safe alternatives to available synthetic chemical drugs.

Keywords: ***Osteospermum ecklonis,*** **Anti-leishmania activity,** ***Leishmania donovani,*** **Maceration, Soxhlet apparatus**

تقييم النشاط المضاد لليشمانيا لمستخلص الأجزاء الهوائية للأوستوسبيرموم إكلونيس ضد الليشمانيا دونوفاني: في المختبر (بحث مؤتمر)#

هند محمد جولي *،١ و ذكاء زهير عبد الجليل *

 # المؤتمر العلمي العاشر لكلية الصيدلة، جامعة بغداد ٢ – ٣ حزيران٢٠٢٢

* فرع العقاقير والنباتات الطبية، كلية الصيدلة، جامعة بغداد، بغداد العراق .

الخلاصة

عدم وجود علاج كفوء لمرض الليشمانيا الحشويه (حمي الكالازار) بالإضافة الى وجود الاثار الجانبية للأدوية المستخدمة ذات التأثير السام او قليل الفعالية، كلها عوامل تؤدي عادة الى انتكاسة المرض. كل هذا عبد الطريق للبحث عن ادويه بديله مستخلصه من الموارد الطبيعية لاحتوائها على تأثير مضاد للإمراض الطفيلية من خلال البحث العلمي.

في هذه الدراسة تم تقييم النشاط المقارن المضاد لليشمانيا في المختبرللاجزاء الهوائية لنبتة الاقحوان الافريقي (Osteospermum ecklonis) للتأكد من وجود فعالية مضادة لليشمانيا تم اعداد المستخلصات بطريقتي النقع وبجهاز السكسوليت باستخدام ٨٥٪ من الميثانول وتمت التجزئة للمكونات للمستخلصات بالاعتماد على اختلاف قطبياتها باستخدام مذيبات مختلفة القطبيه (الاثير البترولي، الكلوروفورم، الكحول البيوتانولي والاثيل اسيتيت). اثنان من المتجزأت الناتجه تم اختيارهما (متجزأة الاثير البترولي وكذلك الكحول البيوتانولي) لاختبار قابليتها على التثبيط الفعال لطفيلي الليشمانيا(*Leishmania donovani*). بلا شك اثبتت النتائج ان متجزآ الاثير البترولي للأجزاء الهوائية ذات التركيز ٢.٥ ملغم/ مل اظهر نشاط مضاد لليشمانيا أفضل من التركيزات الأخرى للعينات المختارة لتزامن نشاط العينة مع نشاط العلاج التقليدي لليشمانيا(®pentostam) والذي يمكن ان يعزى الي طبيعة المواد التربينيه الموجودة في هذا المتجزأ.

هذه الاستنتاجات عبدت الطريق للتوسع في دراسة الأدوية العشبية التقليدية لغرض الحصول على بدائل أكثر فعالية وامنا" من الأدوية المتاحة.

الكلمات المفتاحية: الاقحوان الافريقي ، النشاط المضاد لليشمانيا ، طفيلي الليشمانيا ،طريقة النقع ، جهاز السكسوليت .

[1]Corresponding author E-mail: hmchmc7jan@gmail.com
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Introduction

Leishmania donovani protozoan is known to be the causative parasite of what is called (Kala-azar) or visceral leishmaniasis, this disease is transmitted from animals (pets or rodents) or from person to another person through phlebotomize sand fly, being responsible for about 500000 cases per year worldwide it's characteristic symptoms are fever, weight loss, hepato-splenomegaly, diarrhea, vomiting and lymphadenopathy,
,it is endemic in the middle and southern governorates , the major incidence is among children and few among adults indicating age related improved immunity [1,2].

First line recommended therapies are pentavalent antimonial sodium stibogluconate (pentostam)®, amphotericin B (polyene antibiotic) that have serious side effects such as nausea, vomiting ,arthralgia ,hepatitis, cardiac dysrhythmias , pancreatitis and nephrotoxicity[3,4], however; the greatest limitation that the above medications have include the development of drug resistance organisms , a disadvantage that is clearly shown with sodium stibogluconate with failure rates up to 65% in areas of endemicity, [5] one of the suggested solution was the use of combination therapy to reduce the side effects and enhance effectiveness , the effectiveness of multidrug protocol was noticed to be almost similar to that of mono-drug therapy but this approach used to be indicated as less side effect outcome and was the essential motive to seek and quest for more secure and efficient antileishmanial agents [3] .

Osteospermum genus frequently used by the Arabian Bedouins for treatment of fever, stomach illness as well as liver disorders, being rich with triterpenes, glycosides, sterols, One of the important domiciliary plants cultivated in Iraq as an ornamental plant is ***Osteospermum ecklonis*** F. Asteraceae also known as African Daisy has been used over decades as a remedy for certain health issues, as cardiovascular[6] antimicrobial[7], anti-parasitic [8] , whitening, antitumor [7], and the flowers used as a relaxing aid not surprisingly as it was reported to have several phytochemicals of pharmacological importance such as flavonoids and phenols, terpenoids, essential oils, saponins, polysaccharides, coumarins [9], carotenoids [10] as well as phytosterols

Sesquiterpenes and triterpenes were isolated from the genus *Osteospermum* throughout a study that was conducted in 1983 [11] that paved the way for the conduction of this study, the *leishmania donovani* represent one of the most endemic diseases in Iraq ,that's why this study was conducted as a search for alternative remedy for this illness.

Materials and Methods

Collection and authentication of plant materials

Osteospermum ecklonis was obtained from an herbarium near Baghdad during March and the plant was authenticated by prof. Dr Sukaena Abbas\ Department of Biology\ College of the Science \University of Baghdad. Aerial parts were separated from roots and were washed carefully to remove any contaminants and then brought to dryness in a shady room for about a month, for conduction of the study, the dried plant from aerial parts, were grounded into a fine powder by, first, manually followed by electrical grinding

Extraction and fractionation of plant extracts

The dried, powdered aerial parts of the plant (100gm) was macerated with 750 ml of 85%mathanol for 15 days with stirring for 1hr daily, the macerate was filtered, and new solvent added, another 100 gm of powdered aerial parts plant material was extracted by Soxhlet apparatus with the same solvent mixture on a medium heat for a total of 20 hrs., the filtrate from both ways were evaporated and were kept aside.
The dried extract from the previously mentioned two methods, is then suspended in 250 ml distilled water and washed repeatedly and separately with another 250 ml of petroleum ether, chloroform, ethyl acetate, and finally n-butanol, in a separatory funnel for three times in each solvent, fractions of each solvent were collected separately all fractions (except for n-butanol)were dried over anhydrous sodium sulphate, filtered before been evaporated with the rotatory evaporator until dryness, as illustrated in the following schematic diagram, in Figure(1)

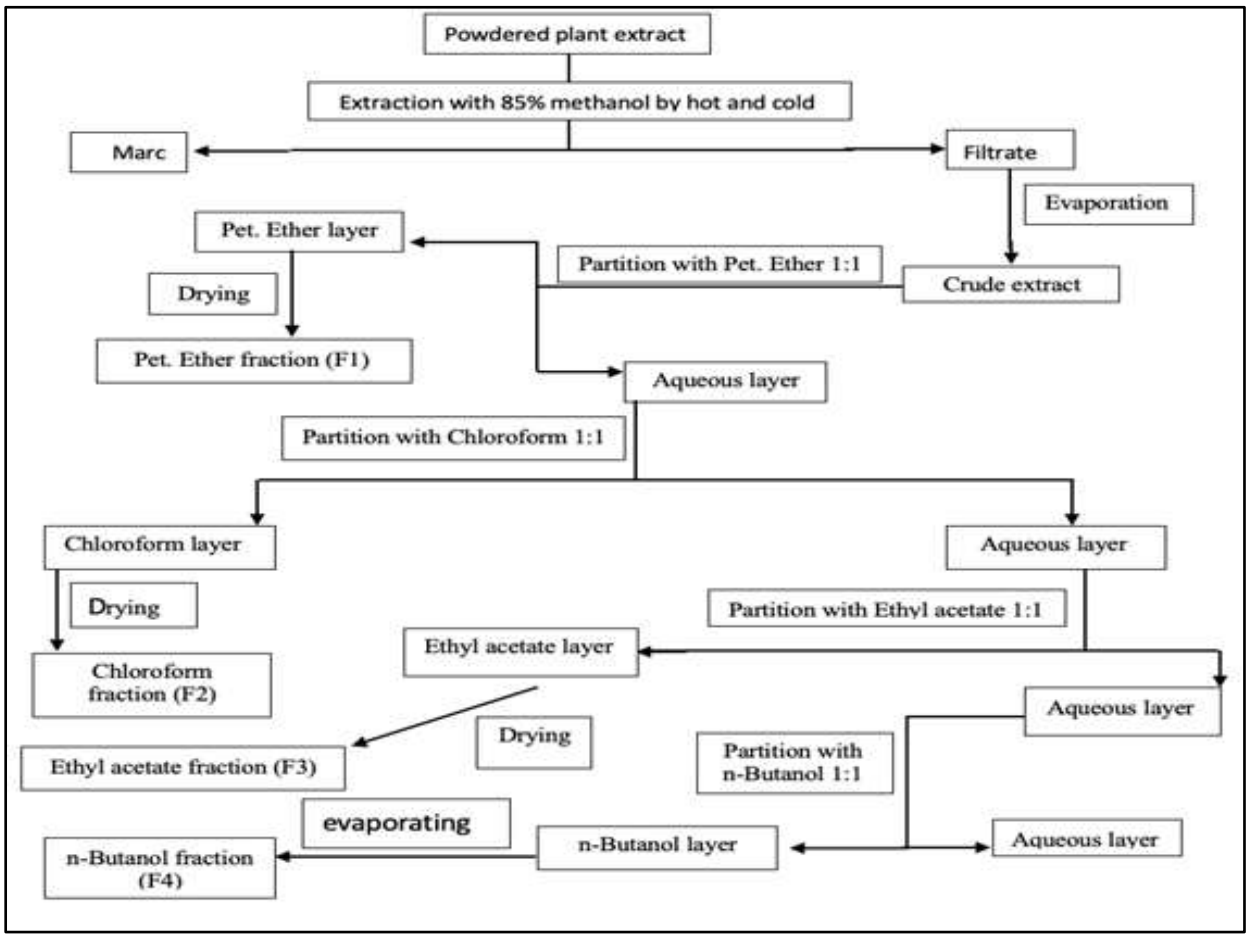

Figure 1. Schematic diagram illustrates the process of extraction and fractionation[12–17]

Percentage yield

The weight of each obtained fraction was measured ,divided by the weight of the sample ,the percentage yield of each fraction was calculated based on the following equation [18] **:**

$$\textbf{Percentage yield} = \frac{\textbf{weight of eaxtract}}{\textbf{weight of sample}} \times 100$$

Phytochemical analysis

Qualitative investigation of secondary metabolites existence was conducted based on the standard tests that is described in Harborne [19]**.**

Qualitative identification by TLC

Thin layer chromatography was held for the aerial parts to compare between the fractions for the same plant part and it was performed using the chromatographic system with silica gel GF_{254} as stationary phase, the following solvents were utilized:

(S1) Toluene: Ethyl acetate (93:7)

(S2) Toluene: ethyl Acetate: Formic acid: Acetic acid (20:10:10:7.5)

Preparation of inoculum

Leishmania. donovani was obtained from the bone morrow of an infected child at a hospital in Baghdad and brought to the center of Biological Technology Research at collage of science in Al-Nahrain University. Evaluation of the antileishmanial activity of plant extract started by first propagating the organism by incubating in the log phase in RPMI media (Roswell Park Institute Park Memorial) this culture media is enriched with 12% serum from calf fetal, at 25 °c for 5 days until reaching an average of 10^5 parasites \ml in haemocytomer [20,21].

Preparation of plant extract concentrations

Based on the percentage of yield two fractions were nominated to test presence of antiparasitic activity that is comparable to conventional therapies. From the aerial parts (G)extract that was obtained by hot (S) and cold (M) methods, petroleum ether (PE) and n- butanol fractions (n-b) were the nominated ones thus there was a total of four samples to conduct the experiment, 1 mg was weighed from each sample and was set aside to prepare five successive serial dilutions, samples were denoted as follows:

Sample A= (M\G\PE), Sample B= (S\G\PE), Sample C= (M\G\n-b), Sample D= (S\G\n-b).

For each of four samples, first Dimethyl sulfoxide (DMSO) in 100%v/v [20] was added as solubilizing agent in an amount that doesn't exceed 20 µ L to 1mg extract then complete the volume with distilled water till reaching required concentration (1 mg/ml) which was successively utilized to prepare successive dilutions ended up with the following

concentrations 1000µg\ml, 500µg\mL, 250 µg\ml, 125µg\ml, and 62.5 µg\ml[22] .

Preparation of positive control

The positive control was the pentavalent antimonial (sodium stibogluconate injection 100 mg\ml) from GlaxoSmithKline\UK, which is the classic drug for kala-azar fever caused by *L. donovani*, Sodium stibogluconate (100 mg\ml) was diluted several times until obtaining a concentration of 100µg\ml, then 6 µl were inoculated in each well that was already containing 1 ml of RPMI and 1ml of *L. donovani* inoculum which was applied into two wells successively to assess variance.

Evaluation of samples activity against L. donovani

To assess antileishmanial activity, flat bottom plate that contained 96 wells was used, as shown in Figure (2), leishmania culture was added to all of them, then 90 wells were filled with 10µL (x3) of the fraction concentrations that were previously prepared, another three wells were used as negative control i.e no added materials to the lieshmania culture , then two wells were filled with positive control of sodium stibogluconate and the last well was supplied with 50% DMSO thus reaching a total of 96wells.

The plate was incubated at 25 ± 1°C for 24 hours after which a 10µL MTT dye (3-(4,5-dimethyl thiazo-2-yl)-2,5-diphenyltetrazolium bromide) was added in each well then incubated again for further 4 hours at 25 ± 1° C to assess metabolic activity, followed by addition of DMSO to each well as a solubilizing agent that will target the MTT purple dye inside the living matter release and making scanning process possible [23].

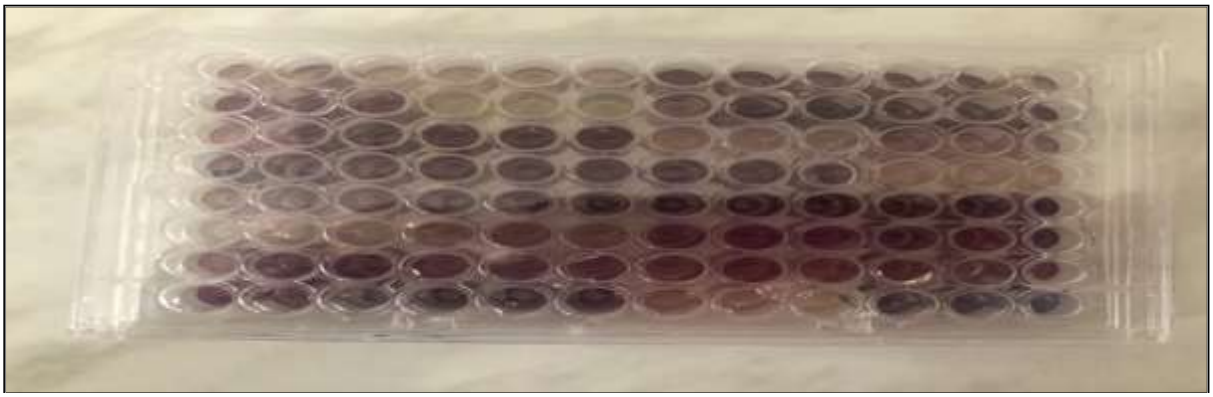

Figure 2. 96 wells plate that is ready for scan with ELISA

Spectrophotometric assessment by ELIZA

Metabolic activity observed with the aid of ELISA spectrophotometer apparatus that measures the optical density in each well at wave length of 490 nm, the higher the number of living matter the more purple color shown and the higher absorbance of ELISA reading [21–25] .

Statistical analysis

Statistical analysis was performed by calculating inhibition percentage of each mean for the three fraction concentrations gradients applied, then significance of results was tested via mean comparison utilizing one-way ANOVA test of IBM software, least statistical differences (LSD) and p value to indicate significant or non-significant difference of inhibition rate from that of the sodium stibogluconate[26].

Results and Discussion

Extraction and fractionation

Two different methods were used for the extraction in this study: the Soxhlet (hot) method and maceration (cold) method. to reach a level of certainty about who yields best percentage, the yield of each fraction with its percentage is illustrated in the Table (1):

Table 1. The yield of aerial parts of the plant with the denoted method of extraction.

Extraction method / Fraction name	Aerial parts\ Soxhlet	% Yield	Aerial parts \maceration	% Yield
Petroleum ether (F1)	2.493 gm	2.493%	4.344gm	4.344%
chloroform (F2)	0.043 gm	0.043%	0.071gm	0.071%
Ethyl acetate (F3)	0.132 gm	0.132%	0.064 gm	0.064%
n-butanol (F4)	1.98gm	1.98%	1.23gm	1.23%

Qualitative phytochemical analysis revealed the presence of appreciable phytochemicals, terpenes and steroids existence was recorded at the pet. Ether fraction while tannins and flavonoids existence were recorded at the ethyl acetate as well as n-butanol fractions as shown in the Table (2)

both of pet. ether. fraction and n-but. Fractions obtained from the maceration and Soxhlet methods were chosen for this study due to higher yield while chloroform layer and ethyl acetate layer were neglected due to low yield.

Table 2. Preliminary chemical tests.

Fraction name / Test name	Aerial parts/maceration				Aerial parts/Soxhlet			
	F1	F2	F3	F4	F1	F2	F3	F4
Keler -Killiani test for cardiac glycosides	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Dragendroff's test for amin compounds	-ve	+ve	-ve	-ve	-ve	=ve	-ve	-ve
Mayer's test for alkaloids	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Salkowiski test for terpenoids	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
Lieberman test for steroids	+ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve
Braeman's test for tannins	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve
Alkaline test for flavonoids	-ve	-ve	+ve	+ve	-ve	-ve	+ve	+ve
Saponin test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Qualitative identification by thin layer chromatography

TLC of petroleum ether for aerial parts of plant was done by using the solvent systems (S1) And was visualized by spraying with H2SO4 (5%) spray reagent followed by heating. Petroleum ether showed spots of numbers of steroidal components, with the same R_f value similar to that of ß-sitosterol as illustrated in the Figure (3).

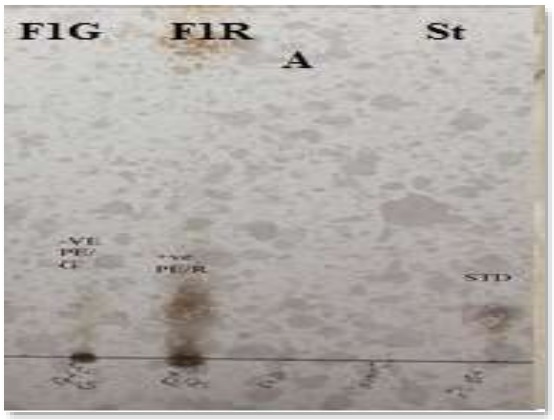

Figure 3.TLC chromatogram of pet. Ether / aerial parts and ß-sitosterol standard, analyzed using solvent system S1

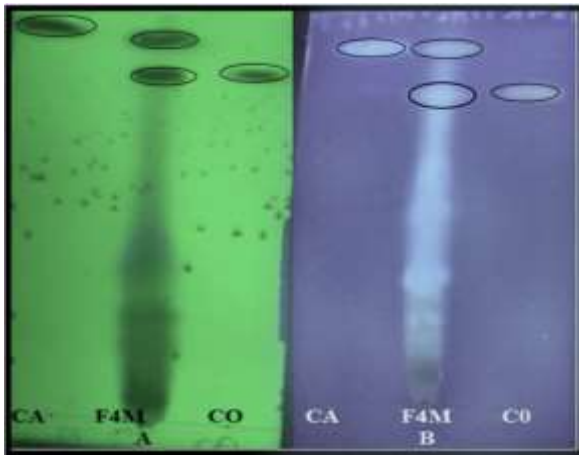

Figure 4. TLC chromatogram obtained by S2 for analyzed fractions (n-but) with caffeic acid and p-coumaric acid standards under UV light, A:254 nm and B: 366 nm.

TLC for n-butanol fraction was performed using the solvent system (S2)

Spots were detected under UV light (254,366 nm). The TLC analysis revealed the presence of chlorogenic acid in n- butanol as shown in Figure (4)

Antileishmanial activity test

The optical density (OD) data obtained from ELIZA were utilizes to calculate the % of organisms died by each concentration of each tested fraction, according to the equation: % of Inhibition Rate (OD control-OD test /OD control)*100 [22]

$$\% \text{ IR } = \frac{\text{OD Control} - \text{OD Test}}{OD\ Control} \times 100$$

Calculation of the % of Inhibition Rate for each concentration of every fraction was conducted and compared to the calculations of he + ve and – ve controls, as well as ANOVA analysis was performed to find the conc. That has no significant mean difference from that of the +ve control (the null hypothesis is to be achieved) at (p> 0.005), calculations revealed the results that are illustrated in the following discussion:

Fraction A (M\G\PE)

Calculation of percentage of toxicity and mean comparison for the tested five concentrations of fraction A revealed that there is high coincidence between concentration 2.5 mg\ml and 5 mg/ml that gave IR % of 84.52 and 81 % respectively which are similar to that observed for the positive standard as shown in Figure (5) .

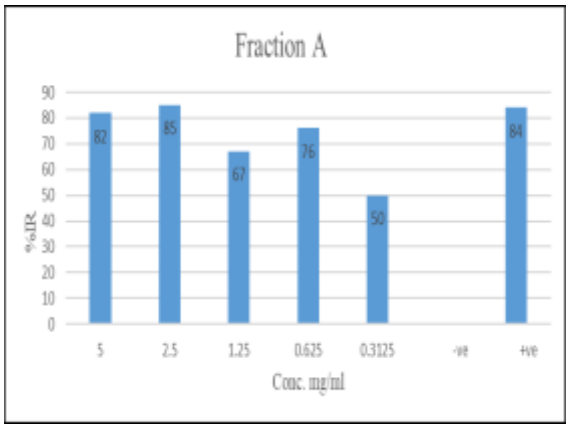

Figure 5. %IR for aerial maceration petroleum ether fraction (A) dilutions (M\G\PE)

ANOVA analysis of the results revealed non-significant differences between conc. A4 and that for +ve control, the result that confirms what stated before, that regarding this fraction conc. Of 2.5 mg/ml coincide to the results of +ve control with (p >0.005) next to A5, as illustrated in Figure (6).

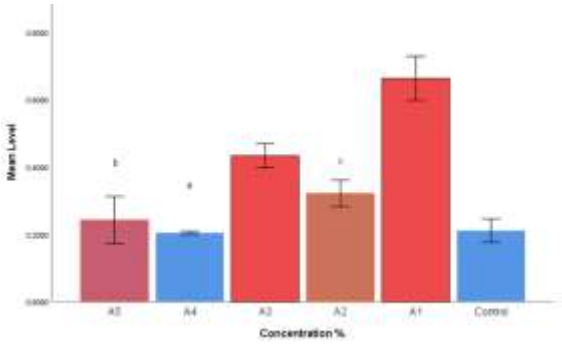

Figure 6. ANOVA diagram for different concentrations of fraction A.

Fraction B (S\G\PE)

Calculations of the percentage IR and mean comparison revealed that conc. 5mg/ml (B5) has close results to that of the +ve control as shown in Figure (7).

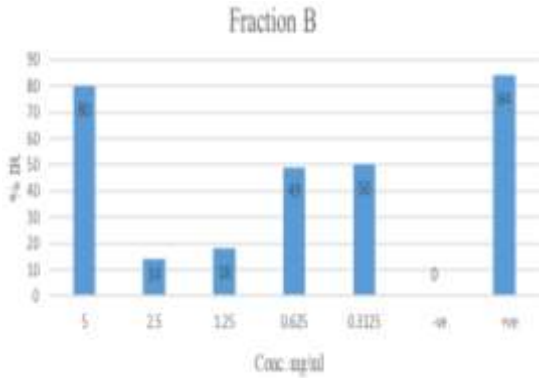

Figure 7. %IR for aerial hot petroleum ether fraction (B) dilutions (S\G\PE)

ANOVA analysis revealed comparable and non-significant differences of B5 and +ve control, as the non-significant mean differences is the one to be achieved in this study to get an effect similar to that of the +ve control.as was stated previously in this section and as illustrated in Figure (8).

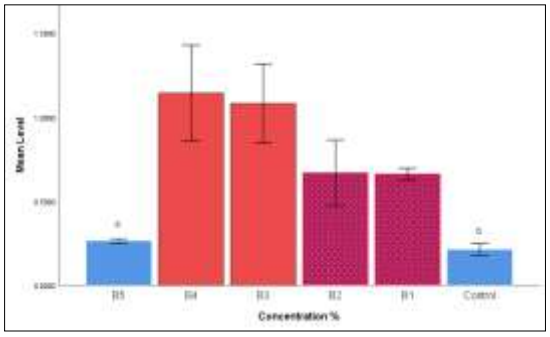

Figure 8. ANOVA diagram for different concentrations of fraction B.

Fraction C(M\G\n-b)

Percentage IR for conc. 5mg/ml (C5) revealed very close result to that observed for the +ve control as shown in Figure (9).

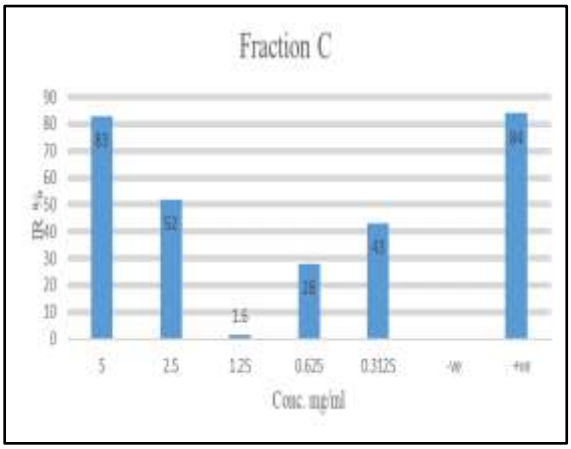

Figure 9. %IR for aerial maceration n-butanol fraction (C) dilutions (M\G\n-b)

ANOVA analysis revealed non-significant mean difference between the C5 and that of the +ve control as illustrated in the Figure (10) .

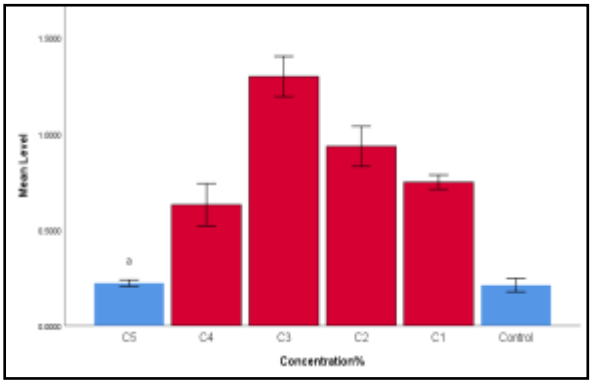

Figure 10. ANOVA diagram for different concentrations of fraction C

Fraction D (S\G\n-b)

Only conc. 5 mg/ml (D5) showed IR percentage similar to that of the +ve control as illustrated in Figure (11).

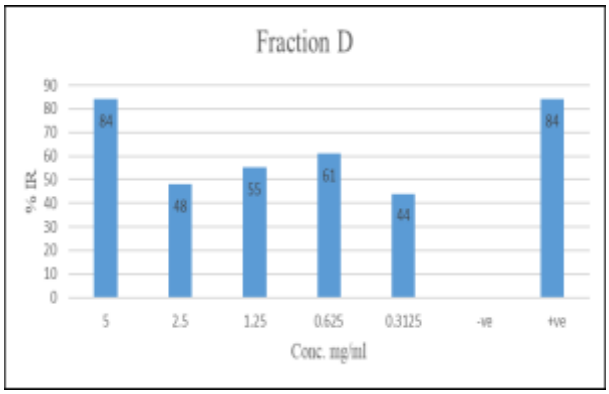

Figure 11. %IR for aerial hot n-butanol fraction (D) dilutions (S\G\n-b)

ANOVA analysis confirmed the above result and revealed that there are no significant mean differences between D5 and the +ve control, revealing that the fraction D at a conc. Of 5mg/ml has inhibition rate similar to that of the +ve control as illustrated in the Figure (12).

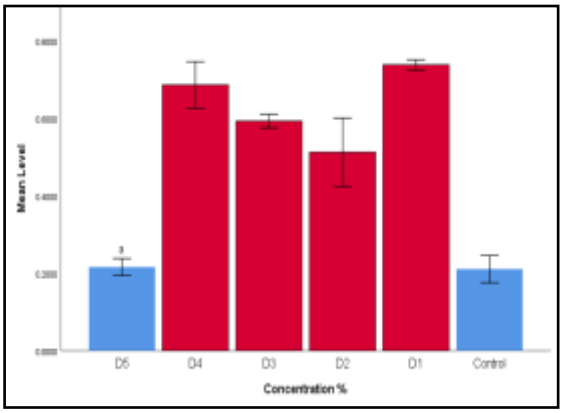

Figure 12. ANOVA diagram for different concentrations of fraction D

Estimating of antileishmanial activity was conducted for the first time for the plant ***O. ecklonis*** cultivated in Iraq, testing was carried for n-butanol and pet. Ether fractions of aerial parts (that was obtained by maceration and Soxhlet), referral +ve standard was pentostam®, coincidental antileishmanial activity was encountered in certain fractions and certain concentrations as illustrated in Table (3):

Table 3. Antileishmanial active concentration of each tested fraction

Name of fraction	Name of fraction	Antileishmanial active conc.mg/ml
A	Pet ether fraction from aerial parts maceration extraction	2.5
B	Pet ether fraction from aerial parts Soxhlet extraction	5
C	n-but. fraction from aerial parts maceration extraction	5
D	n-but. Fraction from aerial parts Soxhlet extraction	5

As shown above, stronger antileishmanial activity was encountered at the petroleum ether fraction of the Maceration aerial parts (A)as it was gained at a lower concentration (2.5 mg/ml) than the antileishmanial concentration of other tested samples, and as A represents pet. Ether fraction, that result of anti-leishmaniasis activity which coincided with the official treatment (pentostam)® activity can be attributed to the terpene nature of materials that are used to be existed in such fraction[19] .new researches have revealed that ß-sitosterol was capable of inhibiting the growth of leishmania amastigote and promastigote form. It also showed significant analgesic and anti-inflammatory activity that can helpful heeling secondary infections that might accompany leishmaniasis, hence it is a good candidate for further research as antileishmanial substance [(27)].

Conclusion

The results obtained in this study revealed that the pet. Ether fraction obtained by maceration had shown more compliable features to with that of pentostam® at in Vitro studies, however, further in Vivo studies at animal models and bioactivity guided analysis clues to be collected to assess and identify the active ingredient, site, and mode of action to be applied.

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