

Investigation the Activity of Iraqi *Agave attenuata* on *In Vitro* Growth of Cutaneous Leishmania Promastigotes

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ABSTRACT

In this research we concentrate on the study of the activity of Iraqi *Agave attenuata* root and leaves as antileishmania (cutaneous leishmania). Focusing on the phenolic content of the plant that detected in high concentration in ethyl acetate fraction obtained by extraction method using soxhlet apparatus. Also depending on the saponin content that *Agave* species rich with these constituents, in addition because of the previously studied effect of saponin as anti-parasite encourage us to extract steroidal saponin by a particular way. The result was the phenolic constituents in root have a great antileishmanial activity. As well as steroidal saponin root and leaves showed a considerable effect that is identical and also better than the drug used (pentostam) as a control. This study is the first to describe the antileishmanial activity especially on cutaneous leishmania and the result showed that the concentration that gave us perfect effect in ethyl acetate root fraction was at 1 mg/ml with IC_{50} (0.768 mg/ml), for steroidal saponin leaves and root fractions the maximum effect were at 1 mg/ml and 0.125 mg/ml respectively with IC_{50} (0.48 mg/ml) for leaves and (0.6 mg/ml) for root.

Key words: *Agave attenuata*, Phenolic compounds, Saponins, Cutaneous leishmania, Pentostam

HOW TO CITE THIS ARTICLE: Sherine Majeed Shah, Thukaa Z Abdul Jalil, Investigation the Activity of Iraqi *Agave attenuata* on *In Vitro* Growth of Cutaneous Leishmania Promastigotes, J Res Med Dent Sci, 2023, 11 (01): 114-120.

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Received: 01-Nov-2022, Manuscript No. JRMDs-22-66424;

Editor assigned: 04-Nov-2022, PreQC No. JRMDs-22-66424 (PQ);

Reviewed: 18-Nov-2022, QC No. JRMDs-22-66424;

Revised: 28-Dec-2022, Manuscript No. JRMDs-22-66424 (R);

Published: 05-Jan-2023

INTRODUCTION

The high toxicity of the currently available chemotherapeutic treatments makes it difficult to control leishmaniasis. The continued hunt for more powerful leishmanicidal chemicals has forced herbal medications into the spotlight as a safe and effective alternative to conventional therapies, which have a number of downsides. Leishmaniasis is a group of diseases caused by protozoan parasites belonging to the *Leishmania* genus. They are found in 98 countries and have a wide range of clinical manifestations, from disfiguring skin lesions known as Cutaneous Leishmaniasis (CL) to the fatal visceral variant of the illness known as Visceral Leishmaniasis (VL) or kala-azar [1].

Human to human transmission (anthroponotic transmission) or animal to human transmission (zoonotic transmission) are also possible ways for the disease to spread. Parasites (infective metacyclic promastigote forms) are transmitted during the hematophagy of the insect vector in both circumstances [2].

Infectious parasites are phagocytized by macrophages after being implanted in the dermis epidermis junction of vertebrate hosts and then begin the differentiation process to amastigotes, the evolutive form responsible for disease development and progression [3]. Because macrophages play such an important part in the infection as host cells, finding medicines that can trigger an effective antileishmanial response from these cells is critical.

The medications employed have varying efficacies and significant toxicity (cardiotoxicity, hepatotoxicity and nephrotoxicity, for example), which can cause a variety of side effects. The emergence of parasite drug resistance adds to the difficulty of controlling leishmaniasis [4].

Nowadays the prospection of plant species as an approach for discovering more effective compounds against leishmaniasis has been the attention of various research laboratories across the world.

The monocotyledonous family Agavaceae includes the genus *Agave*. Around 166 species of *Agave* exist [5,6]. The genus *Agave* originated in Mexico. This country has the most *Agave* species, with 125 species growing primarily in dry and semiarid zones. This genus' members produce a variety of secondary metabolites, which are employed in traditional medicine as anticancer, anti-inflammatory and anti-parasitic agents, among other things [7]. Saponins are abundant in *Agave* species and they are the most investigated secondary metabolites in that genus [8-13].

However, *Agave* species produce a large number of phenolic chemicals, which are both diverse and abundant. Salm-Dick *Agave attenuate*, this is one of *Agave* species that has recently been studied for its phenol and flavonoid content as well as its biological activity [14].

Because *Agave* species are rich in phenolic compounds and steroidal saponins and there are a lot of studies about the effect of saponins as anti-parasite and especially steroidal saponins in addition to other evidence that phenolic compound have a good effect as antileishmaniasis that encourage us to discover this important activity in *Agave attenuate* leaves and root that never studied before [15-17].

MATERIALS AND METHODS

Plant material

From local botanical garden in Baghdad fresh leaves and root were collected. Then these fresh leaves and root were identified by department of biology/biology university Baghdad college and authentication done by Dr. Zainab Abed Aoun PhD in plant identification.

Preparation of plant extract

500 grams of fresh leaves and 250 gm of fresh root of *Agave attenuata* were washed to remove other external materials, dried in shade place with natural air until completely dried, then powdered by using electric blender and weighed for extraction method.

Extraction method for different phyto constituents by using soxhlet apparatus (for phenolic and flavonoids phyto constituents)

80 grams of powdered *Agave attenuate* leaves and root were packed in a thimble and extracted using 750 milliliters of n-hexane for four hours for the leaves and root, respectively. The extract was then dried until the crude extract weighed 1.9 gm (leaves) and 0.4 gm (root) and the plant was then allowed to dry before continuing extraction with another solvent. Ethyl acetate was the other solvent used. Place the dried plant in a thimble and extract with 750 ml of ethyl acetate for 7 hours, or until the extract turns pale in color. The crude extracts were also dried and weighed, yielding 0.8 gm (leaves) and 0.9 gm (root). The plant was also allowed to dry to carry on with the extraction process. The final solvent was methanol and the dried plant was packed in a thimble with 800 ml of methanol and the extraction began and lasted for 20 hours until the color turned pale. After drying, the crude extract weighed 10.9 gm (leaves) and 2.7 gm (root) [18].

Extraction method for steroidal saponin

30 gram dried powdered leaves and root suspended in 400 ml distilled water, refluxed for 3 hours to separate the polysaccharides, the aqueous extract was filtered and dried before being re suspended in an ethanol: water (80:20) solution and left at 25°C for 18 hours. Filtration was used to separate the polysaccharides and the hydro

alcoholic extract was dried. By separator funnel, the concentrated hydro alcoholic was partitioned with ethyl acetate (1:2, v/v, 2 times) and the ethyl acetate layer was dried.

The ethyl acetate extract was then hydrolyzed in an 80 percent hydro ethanolic solution under reflux for 3 hours (30 ml containing 5 ml of concentrated HCL).

The reaction mixture was neutralized with 10% NaOH before being extracted twice with ethyl acetate using a separating funnel (150 ml first time and then 100 ml).

The ethyl acetate layer was evaporated till dry, yielding 0.4 gram leaves and 0.6 gm root, which were subsequently identified [19,20].

Antileishmanial activity

Preparation of inoculum: Cutaneous leishmania cells obtained from many confirmed cases collected from hospitals in Baghdad and brought to Al-Nahrain university's college of science's center for biological technology research. The antileishmanial activity of plant extract was tested by first propagating the organism in log phase in RPMI media (Roswell Park Institute Park Memorial) enriched with 12% serum from calf fetal, at 25°C for 3 days until an average of 10^5 parasites/ml in haemocytomer was reached [21,22].

Preparation of plant extract concentrations

Anti-parasitic activity was examined in four fractions and compared to a commonly used antileishmania medicine. Because of the availability of high quantities of phenolic phyto constituents, the ethyl acetate fraction for root and leaves obtained by hot method extraction (soxhlet apparatus) and labeled as (A) for Ethyl Acetate Leaves (EAL) and as (B) Ethyl Acetate Root (EAR) were chosen. The steroidal saponin fractions obtained from the fore mentioned method of extraction were labeled as (C) Steroidal Saponin Leaves (SSL) and as (D) Steroidal Saponin Root (SSR). Each fraction was weighed at 1 mg and kept for five repeated dilutions. Dimethyl Sulfoxide (DMSO) in 100% v/v was added as a solubilizing agent to each of the four plant extracts in an amount that did not exceed 20 µL, followed by distilled water until the required concentration (1 mg/ml) was reached, which was then used to prepare successive dilutions. 1000 µg/ml, 500 µg/ml, 250 µg/ml, 125 µg/ml and 62.5 µg/ml were the concentrations that were obtained.

Preparation of positive control

The positive control was GlaxoSmithKline/UK pentavalent antimonial (sodium stibogluconate injection 100 mg/ml), which is a typical antileishmania medication. The sodium stibogluconate (100 mg/ml) was made by diluting 1 ml of medication in 10 ml distilled water to a concentration of 10 mg/ml (10000 µg/ml). In each well, six microliters of RPMI medium and one microliter of inoculum were inoculated separately. To ensure reproducibility, the experiments were repeated three times [23].

Evaluation of the sample fractions against cutaneous leishmaniasis

To assess antileishmanial activity, flat bottom plate that contained 96 wells, (Figure 1) were 200 μ L (x3) were taken from each concentration that were previously prepared, a total of 60 wells were occupied, another 12 wells with culture only as negative control, twelve wells with the positive control of sodium stibogluconate and the last twelve wells were a blank which only contained 100 μ l of culture medium without any plant extract, drug or parasite. The plate was incubated at 25°C \pm 1°C for 24 hours after which a 10 μ L 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°C \pm 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].

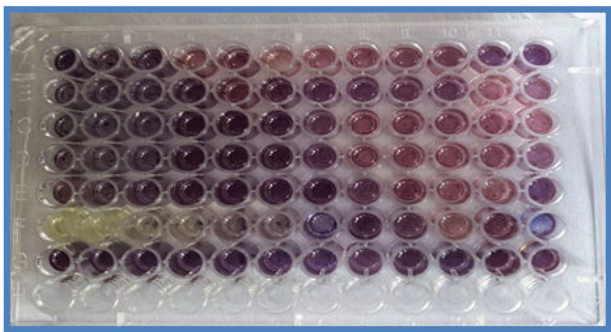


Figure 1: 96 wells plate that is ready for scan with ELISA.

Scanning

The ELISA (Enzyme Linked Immuno sorbent Assay) spectrophotometer instrument detects the optical density in each well at a wavelength of 490 nm and the higher the number of living matter, the more purple the color presented and the higher the ELISA reading absorbance.

Statistical analysis

The significance of the data was verified using a mean comparison after calculating the inhibition rate percentage of each mean for the three fraction concentration gradients used. Using IBM software's one way ANOVA test, the Least statistically Significant Differences (LSD) and p value to determine whether the inhibition rate is significantly different from that of sodium stibogluconate.

Calculating of IC₅₀

While it's essential to know how to distinguish between different types of inhibitors, much of the drug development process is focused on establishing whether one inhibitor is more effective than another. This requires determining the potency of an inhibitor. The IC₅₀ is the most commonly used to quantify an inhibitor's effect. The

IC₅₀ value is the inhibitor concentration required to lower the rate of an enzymatic process by 50% [24].

The IC₅₀ of each analytical sample was calculated using the following procedure: at each of the five locations, inhibition ratios (y) were plotted against sample concentrations (x) and the associated regression line (y=ax+b) was generated. It was not necessary for the regression line to pass through the origin. Because the inhibition curve was not precisely straight but slightly curved, we can determine the IC₅₀ value using interpolation by connecting the two points surrounding the 50% inhibition point with a straight line. After selecting two points surrounding a 50% inhibition ratio, a regression line (y=ax+b) was created. The regression line did not have to pass through the origin; instead, x (sample concentration) was determined by substituting 50 for y in the regression equation y=ax+b [25].

RESULTS AND DISCUSSION

Extraction and fractionation

The composition of the plant material, the solvent used, the pH of the solvent, the temperature and the solvent to sample ratio all influence the extraction method chosen. It also relies on how the final products will be used. The type of plant, the section of the plant to be extracted, the nature of the bioactive components and the availability of solvent all influence the solvent choice. In general, polar solvents like water, methanol and ethanol are used in polar compound extraction, while nonpolar solvents like hexane and dichloromethane are used in nonpolar compound extraction [26]. To extract all phytochemicals that found in the Iraqi *Agave attenuata* we used soxhlet apparatus with increasing polarity solvents.

Steroidal saponins, commonly known as C-27 steroidal saponins, are mainly found as glycosides. The hydro alcoholic extract's ethyl acetate partition was found to be selective for saponins as aglycone. Saponins can be extracted using non-polar solvents or supercritical fluids after chemical, enzymatic, or hydrothermal hydrolysis of crude saponins and/or saponin rich extracts. Mineral acids (such as HCL and H₂SO₄) are typically employed in the traditional approach. The liquid-liquid extraction with ethyl acetate demonstrated good selectivity for saponins. The limited solubility of saponins as an aglycone in aqueous solution could explain the ethyl acetate extract's high selectivity for obtaining saponins.

Antileishmanial activity test

The percent of organisms killed by each concentration of each tested fraction was calculated using the Optical Density (OD) data collected from ELISA, using the equation:

$$\% \text{ Inhibition Rate} = \frac{(\text{OD Control} - \text{OD Test})}{\text{OD control}} \times 100$$

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

The % inhibition rate was calculated for each concentration of each fraction and compared to the computations of the positive and negative controls, as well as ANOVA analysis to determine the concentration

that there is no significant mean difference between this and the positive control (the null hypothesis is to be achieved) at (p>0.05).

Fraction 1 (EAL): The calculated inhibition rate and mean for ethyl acetate leaves' five concentrations revealed that there is low activity as antileishmaiasis comparing with the positive control inhibition rate that is 56.25% (Table 1).

Table 1: Mean of % IR for each concentration gradient for fraction A.

Sample name	Concentrations	% IR Means ± SD
A1	1 mg/ml	9.745471 ± 0.279610443
A2	0.5 mg/ml	24.84904 ± 0.085359371
A3	0.25 mg/ml	37.38439 ± 0.030214051
A4	0.125 mg/ml	36.4366 ± 0.123776681
A5	0.625 mg/ml	38.88252 ± 0.090691173

Fraction B (EAR): The % IR and mean comparison calculations revealed that concentration at 1 mg/ml (B1)

has killing effect to the cells more than that of positive control (% IR 56.24856684) and % IR for B1 is 71.32156233% (Table 2).

Table 2: Mean of % IR for each concentration gradient for fraction B.

Sample name	Concentrations	% IR Means ± SD
B1	1 mg/ml	71.32156233 ± 0.162735641
B2	0.5 mg/ml	17.23610793 ± 0.245993677
B3	0.25 mg/ml	21.94450814 ± 0.562203403
B4	0.125 mg/ml	2.835741038 ± 0.038212854
B5	0.625 mg/ml	7.360697088 ± 0.043135446

Fraction C (SSL): The % IR and mean comparison calculations revealed that concentration at C1 (1 mg/ml) has the inhibition rate greater than that of positive control, the %IR of +ve is 56.24856684% and % IR of C1

is 73.40059619% also at concentration C3 (0.25 mg/ml) and C4 (0.125 mg/ml) show activity close to that of positive control (Table 3).

Table 3: Mean of % IR for each concentration gradient for fraction C.

Sample name	Concentrations	% IR Means ± SD
C1	1 mg/ml	73.40059619 ± 0.072350996
C2	0.5 mg/ml	28.05931361 ± 0.117252813
C3	0.25 mg/ml	55.78995643 ± 0.183798803
C4	0.125 mg/ml	52.7325537 ± 0.283033959
C5	0.625 mg/ml	24.3598563 ± 0.430743801

Fraction D (SSR): The % IR and mean comparison calculations revealed that the concentrations at D3 (0.25 mg/ml), D4 (0.125 mg/ml) and D5 (0.625 mg/ml) showed greater activity than positive control rate. And at

concentration D2 (0.5 mg/ml) displayed very close effect to positive control (Table 4).

Table 4: Mean of % IR for each concentration gradient for fraction D.

Sample name	Concentrations	% IR Means ± SD
D1	1 mg/ml	34.4187113 ± 0.173305126
D2	0.5 mg/ml	56.61545517 ± 0.203592731
D3	0.25 mg/ml	65.81823741 ± 0.024931016
D4	0.125 mg/ml	66.24627379 ± 0.116610463
D5	0.625 mg/ml	61.99648399 ± 0.296154614

In Figure 2 we can see the inhibition rate % IR against concentrations and comparing the result with the positive control used (Figure 2).

locations, inhibition ratios (y) were plotted against sample concentrations (x), and a regression line (y=ax+b) was generated.

Unfortunately, the computed IC₅₀ for fraction ethyl acetate leaves EAL did not exist since the fraction lacks the ability to kill 50% of infected cells.

According to the straight line equation, the computed IC₅₀ for second fraction ethyl acetate root EAR is 0.768 mg/ml.

The IC₅₀ for steroidal saponin leaves SSL is 0.48 mg/ml calculated from straight line equation.

IC₅₀ for the last fraction, steroidal saponin root SSR, is 0.6 mg/ml (Figures 3 and 4).

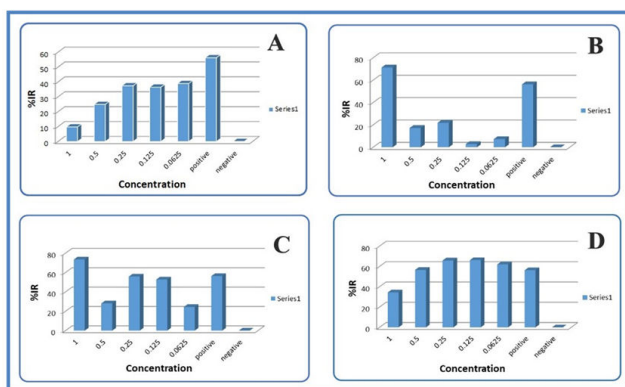


Figure 2: % IR of the four fractions against concentration (A for ethyl acetate leaves, B for ethyl acetate root, C for steroidal saponin leaves and D for steroidal saponin root).

ANOVA analysis revealed equivalent and non-significant differences between fractions and positive control, which is the goal in this study to achieve a similar effect to that of the control (Figure 3).

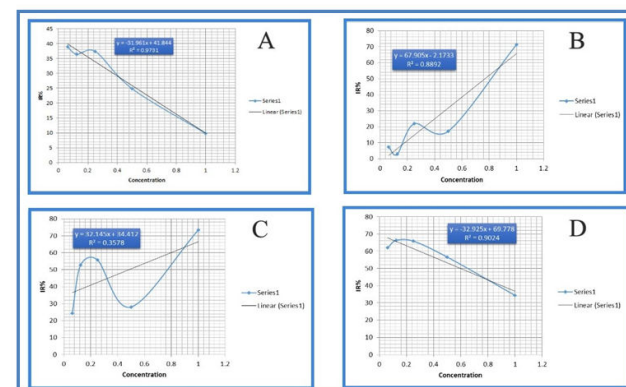


Figure 4: IC₅₀ for the four fractions (A for ethyl acetate leaves, B for ethyl acetate root, C for steroidal saponin leaves and D for steroidal saponin root).

For the first time, antileishmanial activity was estimated for the plant *Agave attenuata* leaves and root for fractions rich in phenolic phyto constituents (ethyl acetate for leaves and root), as well as because *Agave* species are full of steroidal saponin and the activity of saponin as an anti-parasitic caught our attention, so we extracted steroidal saponin in a special method for leaves and root and tested for antileishmania effect. These fractions have antileishmanial activity, and in certain amounts (Table 5).

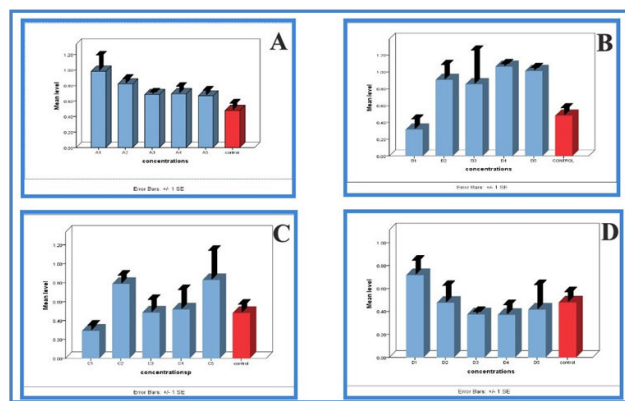


Figure 3: ANOVA diagram for different concentrations of four fractions (A for ethyl acetate leaves, B for ethyl acetate root, C for steroidal saponin leaves, D for steroidal saponin root).

IC₅₀: The IC₅₀ of each analytical sample was calculated using the following procedure: At each of the five

Table 5: Concentration of each fraction that showed best antileishmanial activity.

Fraction name	Fraction content	Concentration used	% IR
A	EAL	0.625 mg/ml	38.88%
B	EAR	1 mg/ml	71.32%
C	SSL	1 mg/ml	73.40%
D	SSR	0.125 mg/ml	66.25%

As previously stated, the antileishmanial activity of steroidal saponin in leaves and root is very good and phenolic phytoconstituents in root have a significant effect, making them a suitable candidate for further research as antileishmanial substances.

CONCLUSION

In this study, the Iraqi *Agave attenuata* leaves and root were discovered as a natural medicinal plant that contain many phyto constituents that proved their ability to act as antileishmania identical to that of pentostam treatment.

Four fractions used for this experiment depending on the high concentration of phenolic constituents that found in ethyl acetate fraction for leaves and root. The other two fractions, we focused on steroidal saponin content of *Agave attenuata* that the saponin has anti-parasitic activity. The result was the phenolic content in ethyl acetate fraction for root has a considerable activity at 1 mg/ml, and has IC₅₀ at 0.768 mg/ml. Both steroidal saponin fractions also showed a significant effect, for leaves the maximum activity was at 1 mg/ml and the IC₅₀ appear at 0.48 mg/ml and about the steroidal saponin fraction for root, the highest activity was at 0.125 mg/ml and IC₅₀ was at 0.6 mg/ml.

REFERENCES

- Hartley MA, Ronet C, Zangger H, et al. Leishmania RNA virus: When the host pays the toll. *Front Cell Infect Microbiol* 2012; 2:99.
- Chakravarty J, Sundar S. Drug resistance in leishmaniasis. *J Glob Infect Dis* 2010; 2:167.
- de Pablos LM, Ferreira TR, Walrad PB. Developmental differentiation in leishmania lifecycle progression: Post-transcriptional control conducts the orchestra. *Curr Opin Microbiol* 2016; 34:82–89.
- Sundar S, Chakravarty J. An update on pharmacotherapy for leishmaniasis. *Expert Opin Pharmacother* 2015; 16:237-252.
- Good-Avila S V, Souza V, Gaut BS, et al. Timing and rate of speciation in *Agave (Agavaceae)*. *Proc Natl Acad Sci USA*, 2006; 103:9124–9129.
- Rocha M, Good Avila S, Molina Freaner F, et al. Pollination biology and adaptive radiation of Agavaceae, with special emphasis on the genus *Agave*. *Aliso* 2006; 22:329–344.
- Santos Zea L, Leal Diaz A, Cortes Ceballos E, et al. *Agave (Agave spp.)* and its traditional products as a source of bioactive compounds. *Curr Bioact Compd* 2012; 8:218–231.
- Bodeiko VA, Kintya PK. The structure of *Agave Saponins C' and D* from the leaves of *Agave americana*. *Steroid Sapon* 1975; 11:755–777.
- Debnath M, Pandey M, Sharma R, et al. Biotechnological intervention of *Agave sisalana*: A unique fiber yielding plant with medicinal property. *J Med Plants Res* 2010; 4:177–187.
- Pant G, Sati OP, Miyahara K, et al. Search for molluscicidal agents: Saponins from *Agave cantala* leaves. *Int J Crude Drug Res* 1987; 25:35–38.
- Tinto WF, Simmons Boyce JL, McLean S, et al. Constituents of *Agave americana* and *Agave barbadensis*. *Fitoterapia* 2005; 76:594–597.
- Yokosuka A, Jitsuno M, Yui S, et al. Steroidal glycosides from *Agave utahensis* and their cytotoxic activity. *J Nat Prod* 2009; 72:1399–1404.
- Eskander J, Lavaud C, Harakat D. Steroidal saponins from the leaves of *Agave macroacantha*. *Fitoterapia* 2010; 81:371–374.
- Rizwan K, Zubair M, Rasool N, et al. Phytochemical and biological studies of *Agave attenuata*. *Int J Mol Sci* 2012; 13:6440–6451.
- Sparg SG, Light ME, Van Staden J. Biological activities and distribution of plant saponins. *J Ethnopharmacol* 2004; 94:219–243.
- Dutta A, Ghoshal A, Mandal D, et al. Racemoside A, an anti-leishmanial, water soluble, natural steroidal saponin, induces programmed cell death in *Leishmania donovani*. *J Med Microbiol* 2007; 56:1196–1204.
- Antwi CA, Amisigo CM, Adjimani JP, et al. *In vitro* activity and mode of action of phenolic compounds on *Leishmania donovani*. *PLoS Negl Trop Dis* 2019; 13:1–22.
- Galanakis CM, Goulas V, Tsakona S, et al. A knowledge base for the recovery of natural phenols with different solvents. *Int J Food Prop* 2013; 16:382–396.
- Santos JDG, Branco A. GC-MS characterization of sapogenins from sisal waste and a method to isolate pure hecogenin. *Bio Resources* 2014; 9:1325–1333.
- Abraham RM, Awad ZJ. Phytochemical study of steroidal sapogenin tigogenin present in the leaves of *Agave americana* cultivated in Iraq. *Iraqi J Pharm Sci* 2015; 24:41-47.
- Bansal D, Sehgal R, Chawla Y, et al. *In vitro* activity of anti-amoebic drugs against clinical isolates of *Entamoeba histolytica* and *Entamoeba dispar*. *Ann Clin Microbiol Antimicrob* 2004; 3:1–5.

22. Sereno D, Lemesre JL. Axenically cultured amastigote forms as an *in vitro* model for investigation of antileishmanial agents. *Antimicrob Agents Chemother* 1997; 41:972-976.
23. Al-Ogaili N. Synergistic effect of *Lawsonia inermis* and *Peganum harmala* aqueous extracts on *in vitro* growth of *Leishmania tropica* promastigotes comparison to sodium stibogluconate. *Al-Qadisiyah Med J* 2016; 12:76-83.
24. Martinez Morales F, Alonso Castro AJ, Zapata Morales JR, et al. Use of standardized units for a correct interpretation of IC₅₀ values obtained from the inhibition of the DPPH radical by natural antioxidants. *Chem Pap* 2020; 74:3325-3334.
25. Abubakar AR, Haque M. Preparation of medicinal plants: Basic extraction and fractionation procedures for experimental purposes. *J Pharm Bioallied Sci* 2020; 12:1-10.
26. Gontijo VS, Espuri PF, Alves RB, et al. Leishmanicidal, antiproteolytic and mutagenic evaluation of alkyl triazoles and alkyl phosphocholines. *Eur J Med Chem* 2015; 101:24-33.