Original Article

Investigation the effect of the aqueous extract of *Chara vulgaris* (L.) on visceral leishmaniasis

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Abstract

Background: Visceral leishmaniasis (VL) is a parasitic disease that affects public health. It is described by weight reduction, irregular fever bouts, anemia, and amplification of the spleen and liver.

Materials and Methods: Three concentrations (15.6, 31.2, and 62.5 µg/mL) were used to find the potency of an aqueous extract of *Chara vulgaris* algae in the treatment of VL. A cytotoxicity assay was performed to show the cytotoxic effect of this extract on human cells. High-performance liquid chromatography (HPLC) test was done to determine the active compounds in the extract. Histopathological sections for infected liver and spleen were performed, as were liver function tests (alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase), which were assessed after 1 month of treatment.

Results: As cytotoxicity assay, results showed that there were no significant differences between the cells treated and those not treated with the extract. HPLC test demonstrated that phenolic and terpene compounds are the main active compounds in the extract. P-coumaric acid and ursolic acid present the highest percent among other phenolic and terpene compounds (21.84%, 17.82%), respectively. Histopathological sections showed that this extract had a significant effect in the treatment of infected tissues, and this effect was very clear after the end of the treatment period. As for the liver function tests, a significant increase ($P < 0.01$) in the studied liver enzymes was found in the infected group of mice compared to the healthy group, whereas in the infected and treated groups, a clear and gradual decrease in the level of enzymes was observed.

Keywords: Algae, aqueous extract, high-performance liquid chromatography tests, *Leishmania donovani*, leishmaniasis, liver enzymes

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INTRODUCTION

Leishmaniasis is a disease that affects public health; it is a gathering of infectious diseases with clinical importance and epidemiological variety.[1] Leishmaniasis is a parasitic disease, clinically divided into three types: cutaneous, mucocutaneous, and visceral.[2] Visceral leishmaniasis (VL) is described by weight reduction, irregular fever bouts, anemia, and amplification of the spleen and liver. Worldwide, between 25% and 45% of new cases of VL are reported to the World Health Organization.[3] The options for treating leishmaniasis are restricted to a limited number of medications with a range of side effects and uneven efficacy.[4]

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Research is being done on new medications to find a medication that is more effective, selective, and has fewer side effects.[5] Numerous studies regarding the biological activities of extracts from marine algae have been reported in the literature[6] and have also demonstrated notable antibacterial, antiviral, antitumoral, anti-inflammatory, anticoagulant, and antiparasitic properties.[7] Nevertheless, research on seaweeds' ability to combat leishmaniasis is scarce.[8]

A stem with elongated single-celled multinucleate internodes separated by multicellular nodes makes up the *Chara* (thallus) plant. The genus *Chara* plant axis or "stem" is differentiated into nodes and internodes. Every node has a whorl of branchlets, which are made up of several cells that stop growing at a specific length. One long cell with a large vacuole can make up the internode; cortex cells surround the internodal cell, which contains the vacuole.[9] In Iraq, this alga is widespread in all regions. It prefers fresh, low-flowing water and thrives and reproduces during the spring months [Figure 1].[10]

Marine and freshwater algae are photosynthetic organisms, and besides their use in food, pharmaceuticals, and cosmetic industries, they are also known to be used in bioremediation and biological isolation.[11] Macroalgae can be used to produce large quantities of biomass in a short time and less space; therefore, there is an increasing use of the biochemicals derived from algae biomass.[12] Furthermore, algae can produce many chemical compounds with therapeutic effects such as proteins, carbohydrates, alkaloids, phenols, and flavonoids, as well as sulfated fucan polymers.[13]

The aim of this study is first to determine the active compounds in the aqueous extracts of *Chara vulgaris* and second to determine the effect of this extract in the treatment of VL.

MATERIALS AND METHODS

Algae collection

This research was conducted from October 2021 to October 2022. *C. vulgaris* was collected from Salah al-Din Governorate in Iraq in March 2019 from a pond with a water depth ranging between 0.5 and 1 m and then diagnosed depending on the sources of classification,[14,15] by A.P. Dr. Al-Magdamy Buthaina A. H., and it was brought to the laboratory in a plastic container containing a lot of water to prevent evaporation. *C. vulgaris* was washed several times with tap water to get rid of the remains of epiphyte and rock, then washed with distilled water and dried at room temperature.

Preparation of concentrations of aqueous extract

One hundred grams of dried *C. vulgaris* were soaked in 250 mL of distilled water for 6 h on a hot plate before being homogenized to create the aqueous extract of this algae. For the following experiments, 10 g of plant powder was suspended in 100 mL of phosphate buffer saline. After being filtered and sterilized with a sterile Millipore filtering system of 0.2 μM, the stock solution was kept in sterile containers at 4°C until it was used.[16] To make the studied concentrations (15.6, 31.2, and 62.5) μg/mL and doses of treatment, the below formula was used and then done serial dilutions for the stock solution.

C1 V1 = C2 V2

C1: 10 g (serial dilution was made to reduce the concentration of the stock solution, to reach concentration to the milligram unit), V1: 100 mL, C2: (15.6, 31.2, and 62.5) μg/mL, V2 : (0.1, 0.2, and 0.4) ml (dose of treatment).

Identification and quantification of *Chara vulgaris* aqueous extract compounds by high-performance liquid chromatography test

To identify the active compounds of *C. vulgaris* aqueous extract, 1 g of the algae samples was homogenized, grinding into a fine powder, and left to dissolve in 3% H2SO4 at room temperature for 2 h. Following filtration through 2.5 um filter paper, the supernatants were applied to Extrelut (Merck) columns after being rendered alkaline with 25% NH4OH (pH 9.5). After the alkaloids were eluted with CH2Cl2 (6 mL/1 g Extrelut), the extracts were dried using a nitrogen stream. To further high-performance liquid chromatography (HPLC) analysis, the obtained residues were resolved in 1 mL CH3OH in accordance with the optimum separation of genuine standards.[17]

To extract the percentage concentration of each compound, the following equation was adopted:

$$\text{concentration of sample}\left(ug/mL\right)=\frac{\text{area of sample}}{\text{area of standard}}\times \text{conc. of standard}\times\text{dilution factor}$$

Cytotoxicity assays

Normal human fibroblasts (NHF) derived from adipose tissue were used to test the cytotoxic effect of *C. vulgaris* aqueous extract on human cells.

On 96-well plates, the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) cell viability test was carried out. One hundred microliter of cell suspension was added per well of the 96-well microplate (1×10^4 cells/well), the experiment was carried out using the previously mentioned concentrations (15.6, 31.2, and 62.5) μg/mL in triplicate, and then these cells were used to seed cell

lines. A confluent monolayer was achieved after 24 h. The experiment was carried out in triplicate, and then cells were treated with the aforementioned concentrations of algae extract. To assess the viability of the cells, the medium was removed after the 72-h treatment period, 28 μL of a 2 mg/mL MTT solution was added, and the cells were incubated for 1.5 h at 37°C. After the MTT solution was removed, 130 μL of dimethyl sulfoxide was added to each well to dissolve any leftover crystals. The mixture was then incubated for 15 min at 37°C while being shaken.[18] Using a microplate reader, the absorbance was determined at the test wavelength of 492 nm. The percentage of cytotoxicity, or the inhibition rate (IR) of cell growth, was calculated using the following formula:[19]

$$\text{Inhibition rate (IR)} = A - B/A \times 100$$

A is the optical density of the control, and B is the optical density of the samples.

Culture of parasite

The Baghdad University College of Science provided the *Leishmania donovani* strain (DUAA/IQ/2005/MRU15), which was cultivated at 25°C–26°C in Novy–MacNeal–Nicolle medium supplemented with 100 IU/mL gentamycin.[20]

Ethical statement

This research was conducted at the Iraqi Center for Cancer Research and Medical Genetics/Al-Mustansiriya University. This center follows the Ethical Guidelines for the Use of Animals in Research, provided by the National Committee for Research Ethics in Science and Technology, 2018. This center has been established since the beginning of 1998 and is interested in conducting advanced scientific research on cancerous diseases, their causes, consequences, mechanisms of occurrence, and follow-up their treatment. The research was conducted based on book number 2872 on June 12, 2022.

In vivo study

Thirty male BALB/c mice were obtained from the Cancer Research Center/Al-Mustansiriya University. The weight of all mice was 25–28 g and aged 10–12 weeks. The motility and growth of mice were very normal during the research period, with the exception of the infected and untreated group (positive control), whose motility began to gradually decrease over time after infection. All mice were inoculated intraperitoneally with 2×10^7 promastigote of *L. donovani*, then mice were divided into five groups, and each one consisted of 6 mice as follows:

1. Uninfected group (negative control group)
2. Infected and untreated group (positive control group)
3. Three infected groups were then treated with different concentrations of the algae aqueous extract (15.6, 31.2, and 62.5) μg/mL.[21]

The treatment was given twice daily and orally for a month. After the end of the treatment period, blood was drawn from the mice to make liver function tests, and then the mice were dissected and the liver and spleen separated for histological sections. The method of anesthesia (formalin) was used to kill mice to minimize the pain they feel during this procedure. Furthermore, as few mice as possible were used to conduct the research.

Histology processing steps

The specimens were handled carefully, and they were fixed a short time after dissection, then the steps of histology processing were done in the sequence (fixation, dehydration, clearing, wax infiltration, embedding, sectioning, and staining).[22,23]

Liver function tests

Estimation of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in serum.

Mixed the working reagent and the samples, then incubated at 37°C for 1 min. Measured the change in absorbance per minute (Δ OD/min) during 3 min [Table 1].[24]

Calculation:

- SGOT (AST) activity (U/L) = (Δ OD/min) × 1745
- SGPT (ALT) activity (U/L) = (Δ OD/min) × 1745
- ALP activity (U/L) = (Δ OD/min) × 2750.

Statistical analysis

The statistical analysis was performed for the results using SPSS program (V.28. IBM, Chicago, IL, Delaware, U.S.) with one-way analysis of variance using Least Significant Difference (LSD) to determine the significant differences between the studied groups. The results were presented as mean ± standard error and *P* value at $P < 0.01$ were considered significant differences between the means of the studied groups.[25] An unpaired *t*-test and GraphPad Prism 6 were used for the statistical analysis of the cytotoxicity assay results. The values were shown as the triplicate measurements' mean ± standard deviation.[26]

RESULTS

High-performance liquid chromatography separation of phenolic compounds, steroidal alkaloids, and terpenoids in *Chara vulgaris* extract

The main terpenoids, alkaloids, and phenolic compounds (PCs) were separated on the fast liquid

chromatographic column under ideal conditions. The results are shown in Tables 2-4.

HPLC technique was used to identify and quantify the major PCs in the aqueous extract of algae. From the HPLC chromatogram [Figure 2 and Table 2], seven different chemicals were identified from the aqueous extract. The compound that represented the highest percent of PCs extracted was p-coumaric acid (21.84%) of the total identified compounds, whereas the compound that represented the lowest percent was ferulic acid (7.07%).

HPLC chromatogram [Figure 2 and Table 3] identified two alkaloid compounds from the aqueous extract, the highest percent compound was diosgenin (59.57), followed by hecogenin (40.43).

From the HPLC chromatogram [Figure 2 and Table 4], 10 different chemicals were identified from the aqueous extract. The compound that represented the highest percent of terpenoid compounds extracted was ursolic acid (UA) (17.82%) of the total identified compounds, whereas the compound that represented the lowest percent was terpenoids (4.35).

The effect of algae extract on the normal human fibroblast

Statistical analysis of the results of NHF treated with aqueous extract showed that after 72 h of incubation, there were no significant differences between the cells treated with the extract and those that were not treated with it. There was no cytotoxicity of the extract on cells at all used concentrations [Figure 3a and b].

Table 1: Test parameters for normal procedure

	AST and ALT	ALP
Mode of reaction	Kinetic	Kinetic
Slope of reaction	Decreasing	Increasing
Wavelength (nm)	340	405
Temperature (°C)	37	37
Factor	1745	2750
Linearity (U/L)	350	700
Blank	DI water	DI water
Delay (s)	60	60
Number of reading	3	3
Interval (s)	60	60
Sample volume (µL)	100	20
Reagent volume (µL)	1000	1000
Cuvette	1 cm light path	1 cm light path

AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, ALT: Alanine aminotransferase, DI: Deionized water

HISTOPATHOLOGICAL RESULTS

Liver

As it is known, hepatocytes, which make up 70%–80% of the liver's cytoplasmic mass, make up the majority of the liver's important cells. Hepatocytes are arranged in plates and are separated by vascular channels called sinusoids. The Kupffer cells, a type of cell found in the hepatic sinusoids, phagocytose the aged erythrocytes [Figure 4a]. The section of the infected liver with VL shows a focal necrotic area with inflammatory cell infiltration (mononuclear cells) and dilated hepatic sinusoids [Figure 4b].

After mice were infected with VL, they were treated with an aqueous extract of algae at concentrations (15.6, 31.2, and 62.5) µg/mL. Mice were dissected in two stages: first, after 2 weeks and second, after 4 weeks. In both periods, the liver and spleen were separated, and then the treatment progress was compared after the end of these two periods.

Treated liver with extract after 2 weeks

Sections (c, e, and g) are sections of infected liver tissue that were treated with (15.2, 31.6, and 62.5) µg/mL of algae

Figure 1: *Chara vulgaris* algae

Table 2: The main phenolic compounds separated by high-performance liquid chromatography

Sequences	Compounds	Retention time (min)	Area	Concentrations (0.1 mg/mL)	Percentage of compounds
1	Catechuic acid	2.408	120,629	3.366	16.52
2	Tannic acid	3.097	126,225	2.853	14.00
3	Caffeic acid	4.66	157,269	4.315	21.18
4	Ferulic acid	6.518	122,152	1.442	7.07
5	p-coumaric acid	7.177	126,216	4.450	21.84
6	Sorbic acid	8.075	140,860	2.211	10.85
7	Rustic acid	9.163	139,642	1.738	8.53

Table 3: The main alkaloid compounds separated by high-performance liquid chromatography

Sequences	Compounds	Retention time (min)	Area	Concentrations (0.1 mg/mL)	Percentage of compounds
1	Diosgenin	2.90	159,223	4.770	59.57
2	Hecogenin	4.145	172,023	3.238	40.43

Table 4: The main terpenoid compounds separated by high-performance liquid chromatography

Sequences	Compounds	Retention time (min)	Area	Concentrations (0.1 mg/mL)	Percentage of compounds
1	Terpenoids	2.768	156,895	1.301	4.35
2	Camphor	3.907	159,683	2.387	7.98
3	Terpinene-4 ol	5.153	145,932	3.829	12.81
4	p-coumaroyl alphitolic acid	6.06	176,593	2.791	9.33
5	Terpinolene	7.18	156,905	2.613	8.74
6	Amyris	8.827	146,898	2.783	9.31
7	Ursolic acid	9.818	143,806	5.327	17.82
8	Oleanolic acid	10.655	149,958	3.239	10.83
9	Palasonin	11.76	146,496	2.193	7.33
10	Caryophyllene	13.18	170,978	3.438	11.50

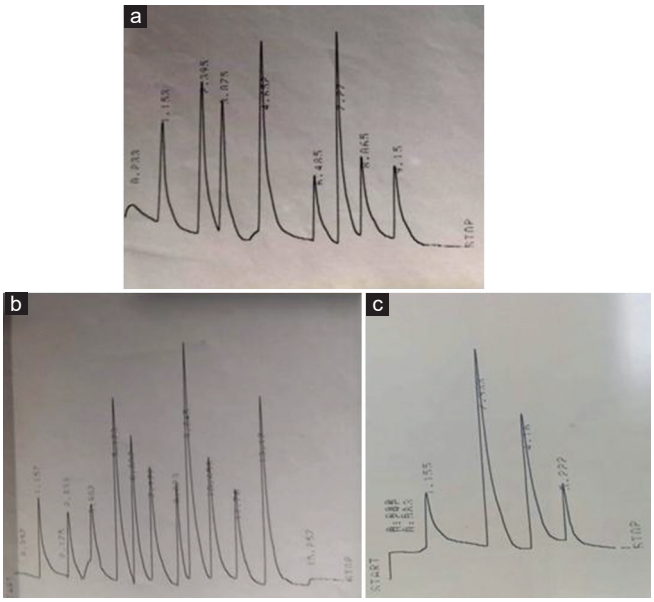

Figure 2: High-performance liquid chromatography chromatogram recorded at 280 nm for the main compounds identified in the *Chara vulgaris* aqueous extracts: (a) Phenols, (b) terpenoids, (c) alkaloids

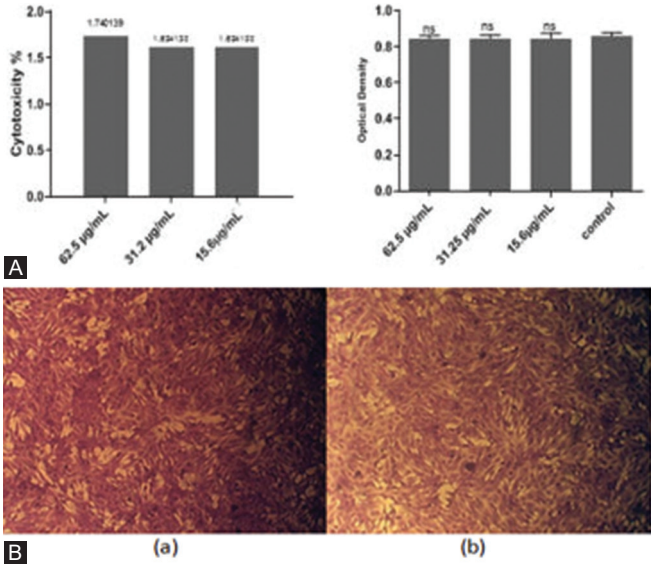

Figure 3: (A) Statistical analysis of normal human fibroblast (NHF) treated and not treated with the extract, (B) NHF before and after treated with the extract. a: Untreated (standard), b: treated (sample)

aqueous extract, respectively. The first section shows that liver tissue looks like near the normal for hepatic tissue, but still, there are focal areas of necrosis and inflammatory cell infiltration with mild sinusoidal dilatation [Figure 5a]. The second section showed that there was still a focal area of necrosis and inflammatory cell infiltration, mild dilatation of the sinusoid, and mild fatty changes inside the cells [Figure 5c]. In the last section, we observed that the liver tissue is look like normal histological structure appearance [Figure 5e].

Treated liver with extract after 4 weeks

Sections (d, f, and h) are sections of infected liver tissue that were treated with (15.2, 31.6, and 62.5) µg/mL of aqueous extract, respectively. The first section revealed mild depletion of glycoprotein and increment of Kupffer cells [Figure 5b]. The second section showed that liver tissue is look like normal, in addition to Kupffer cell hyperplasia [Figure 5d]. At the end, the last section showed mild depletion of glycoprotein and dilatation of the sinusoid [Figure 5f].

Spleen

In mice and people, the spleen comprises white pulp submerged in red pulp [Figure 6a]. The section of the infected spleen with VL showed a widening of white pulp with a narrowing of red pulp [Figure 6b].

Treated spleen with extract after 2 weeks

Section (c) is a section of infected spleen tissue that was treated with 15.2 µg/mL of algae aqueous extract. This

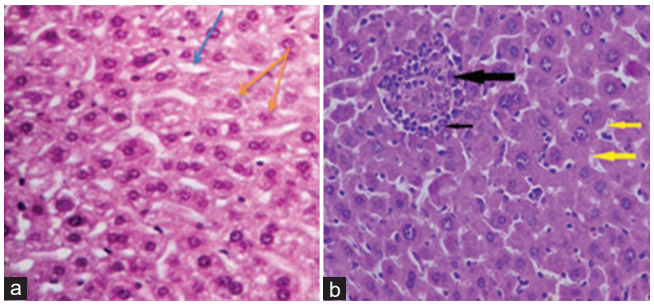

Figure 4: Section of normal and infected liver. (a) Blue arrow: sinusoid, Orange arrow: Hepatocyte, (b) Black arrow: Necrosis and inflammatory cell, Yellow arrow: Sinusoidal dilatation (H and E, ×40)

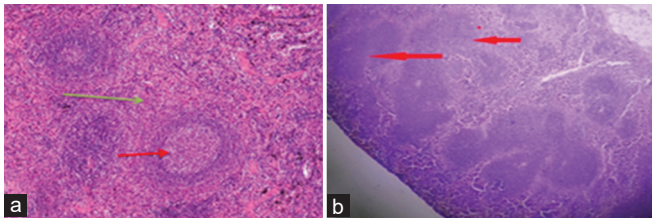

Figure 6: Section of normal and infected spleen, (a) red arrow: white pulp, green arrow: red pulp (b) red arrow: widening of white pulp (H and E, ×40)

section showed a widening of red pulp and a reduction in white pulp [Figure 7a]. While sections (e) and (g) are sections of infected spleen tissue that were treated with (31.6 and 62.5) μg/mL of aqueous extract, respectively, both sections showed a widening in the white pulp [Figure 7c and e].

Treated spleen with extract after 4 weeks

The sections (d), (f), and (h) are sections of infected spleen tissue that were treated with (15.2, 31.6, and 62.5) μg/mL of aqueous extract, respectively. All these sections showed a widening in the white pulp [Figure 7b, d, and f].

Liver function tests

The liver function tests were performed for all study groups (negative, positive control, and treated groups) after the end of the treatment period; three enzymes were included ALT, AST, and ALP. The results found that there is a significant increase in enzyme levels in the infected group as evidence of the damage to the liver as a result of infection. On the other hand, the results revealed that this rise gradually began to decrease with the start of treatment in a dose-dependent manner. The statistical analysis showed that there were significant differences between the infected and treated groups, as well as between the concentrations used ($P < 0.01$) [Table 5 and Figure 8].

DISCUSSION

Leishmaniasis treatment includes four main options: pentavalent antimony, polyene amphotericin B,

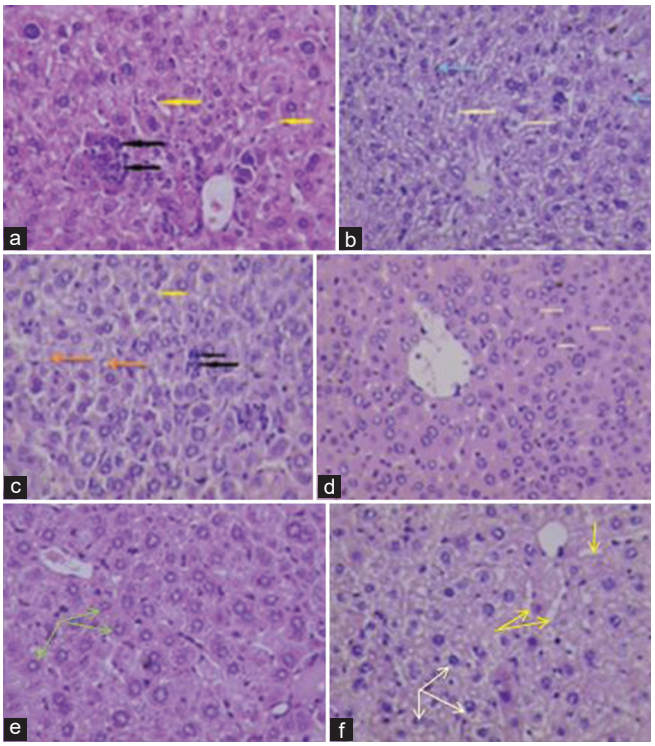

Figure 5: Section of the liver in the studied groups. (a) Black arrow: Focal areas of necrosis, Yellow arrow: Sinusoidal dilatation, (b) Light blue arrow: Kupffer cell, Light yellow arrow: Depletion of glycoprotein, (c) Black arrow: Focal areas of necrosis, Yellow arrow: Sinusoidal dilatation, Brown arrow: Fatty changes, (d) Light yellow arrow: Kupffer cell hyperplasia, (e) green arrow: normal liver cell approximately, (f) Light yellow arrow: Mild depletion of glycoprotein, Yellow arrow: Sinusoidal dilatation (H and E, x 40)

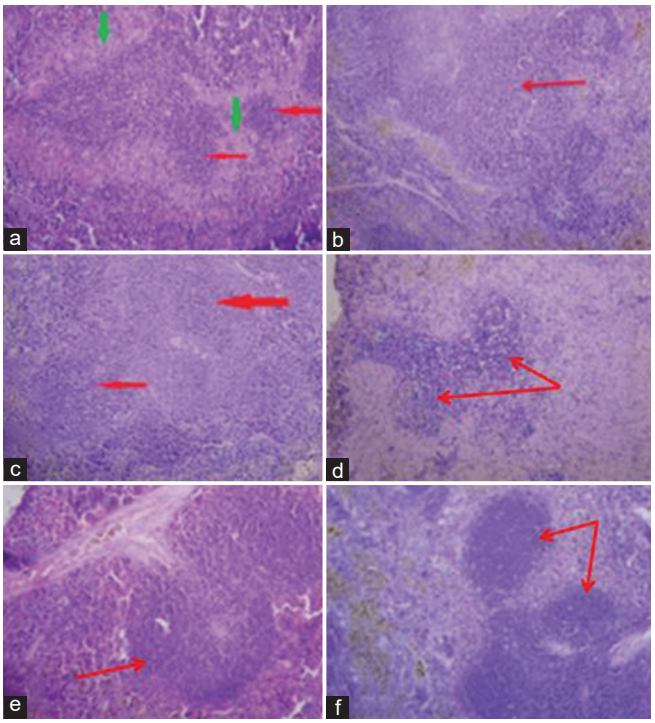

Figure 7: Section of the spleen in the studied groups. (a) red arrow: reduction of white pulp, green arrow: widening of red pulp, red arrow in sections (b,c,d,e,f): widening of white pulp (X40) (H&E)

Table 5: The levels of alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase compared with positive and negative controls

Groups	ALT (mg/dL)	AST (mg/dL)	ALP (U/L)
Negative control	20±1	70.5±4	42.5±1
Positive control	54.5±2.5	166.5±4	152.5±2
15.6	41±1	142.5±4	127±2
31.2	33.5±5	126±1	95±2
62.5	25.5±1	102.5±5.5	72.5±3

Results represent as (mean±SD). SD: Standard deviation, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, ALT: Alanine aminotransferase

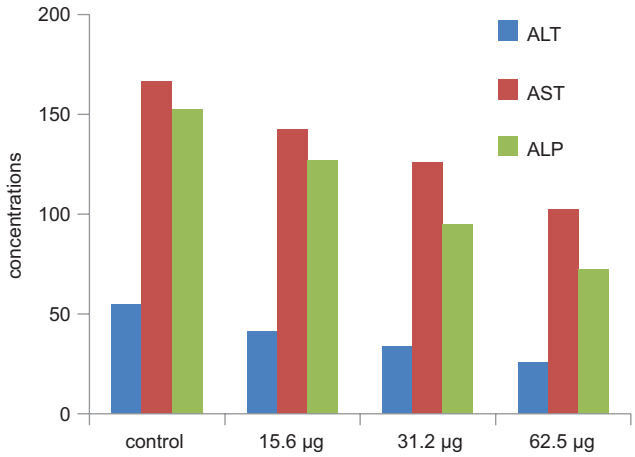

Figure 8: The levels of alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase compared with positive control. ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase

alkylphosphocholine miltefosine, and aminoglycoside paromomycin.[27] These drugs used in leishmaniasis treatment have serious side effects;[28] therefore, a lot of research has been conducted to search for natural compounds that have leishmanicidal activity against leishmaniasis.

Generally, mammals have fibroblasts in their conjunctive tissue. For cytotoxicity tests, NHF is typically chosen due to its ease of maintenance and ability to yield results with a strong correlation to biological findings. They constitute the predominant cell type in regeneration and are also present in wounds.[29]

One of the natural resources in the marine ecosystem is marine algae. A variety of biologically active substances that have served as sources of food, feed, and medicine are present in it.[30] For example, one study demonstrated that the efficacy of *Chlorococcum humicola* algae extracts against some fungi in the gramineae family. The results demonstrated that the highest inhibition zone of 13.67 mm for the *Fusarium graminearum* fungus at a concentration of 32 mg/mL. Subsequently, at a concentration of 32 mg/mL, the methanol extract displayed the highest inhibition zone of 11.0 mm for the *Alternaria triticina*. This was greater than the 8.0 mm inhibition zone recorded in response to the ketoconazole.[31] Because microalgae have a high protein and amino acid content, it is a protein-rich and alternative source of protein. As a result, amino acids isolated from *C. humicola* have been shown to have antibacterial and antifungal activity.[32] Similarly, other studies have shown the antibacterial activity of *C. humicola* extract against *Klebsiella pneumonia* and *Pseudomonas aeruginosa*.[33] The inhibition of phosphatase protein enzyme, which is crucial to the process of inserting substances into the organism' body, by the algal extract explains its inhibitory effect.[34]

On the other hand, there are numerous studies of *C. vulgaris* algae as a source of many active compounds. In one of these studies, the growth of the potentially toxic cyanobacterium (*Microcystis aeruginosa*) is inhibited by extracts of *C. vulgaris*. The bioassay results showed that macroalgae ethyl acetate (MEA) extracts inhibit *M. aeruginosa* growth in a concentration-dependent manner. Day 4 of the experiment yielded the highest IR, surpassing 83%, and on day 7 of the experiment, it reached 97.98%. Seven PCs identified as effective allelochemicals were found by HPLC analysis. Overall, the data show that MEA extracts could be suggested as a possible allelochemical and used as an eco-friendly alternative algaecide to manage microcystis blooms in eutrophic water bodies.[35] The activity of *C. vulgaris* extracts against *Leishmania tropica* promastigotes is revealed by another study. This study demonstrated that the organic extract of *C. vulgaris* inhibited the growth of *L. tropica* promastigotes.[36] The presence of several biologically active compounds, including phenols, which are oxidizing compounds toxic to microorganisms, terpenes, which exhibit biological activity toward bacteria, fungus, viruses, and protozoa, and tannins, which work to stop bleeding and secretions and inhibit enzymes and proteins tanker in the cell membrane, is what gives *C. vulgaris* extracts their activity.[37]

The results of the HPLC test in the current research showed that the main active compounds in the aqueous extract of *C. vulgaris* are phenols and terpenes, meaning that the improvement that appeared in the infected liver and spleen after treatment with the aqueous extract of the algae is due to these two compounds. There are many studies that have confirmed the leishmanicidal activity of these two compounds. It has been reported that PCs have a variety of biological properties, including the ability to inhibit protozoan parasites.[38] Monzote *et al.*[39] evaluated the *in vitro* and *in vivo* activities of PCs against cutaneous

leishmaniasis. Their findings revealed that when PCs were used to treat animals, the lesion size was significantly reduced ($P = 0.05$) in comparison to both the positive and negative controls.

By reviewing several studies related to PCs (that were purified from the aqueous extract of algae through the HLPC test in this study), it was noted that caffeic acid (CA), p-coumaric acid, and tannic acid have more leishmanicidal effects than other PCs in the extract. CA is one of the cinnamic acid derivates, found in a wide range of plant and algae extracts that permit many structural modifications, such as the conversion into esters or amides with powerful biological activities.[40] Numerous studies have found that CA and its derivatives have strong anti-leishmanial effects, suggesting that these substances could 1 day be used as drugs to treat leishmaniasis. CA demonstrated IC50 values for promastigotes of 12.5 g/mL,[41] 16.0 μM against *Leishmania amazonensis* intracellular amastigotes[42] and 21.9 μM for *Leishmania infantum* intracellular amastigotes.[43] As a mechanism of action, CA's ability to change the morphology and volume of promastigote cells, along with the loss of mitochondrial integrity, an increase in the production of reactive oxygen species, the exposure of phosphatidylserine, and the loss of plasma membrane integrity, suggests an apoptosis-like process. In addition, CA raised levels of tumor necrosis factor (TNF) and nitric oxide (NO), whereas decreasing interleukin 10 (IL-10) levels and iron availability.[41] It is possible to draw the conclusion that CA has leishmanicidal effects, and its mode of action involves activating multiple targets that have an impact on the parasite's viability.[44]

Carter *et al*.[45] demonstrated that while cinnamic acid exhibits low toxicity to the host, its derivates like CA inhibit the leishmanial enzyme and modify the host's immune response to parasite defense. With IC50 values ranging from 1.5 to 11 μM, caffeic and rosmarinic acids inhibited the recombinant *L. amazonensis* arginase.[46] The ability of these two substances to inhibit the recombinant *L. infantum* arginase was also investigated. One hundred micrometer rosmarinic acid and CA inhibited the leishmanial enzyme by 71% and 57%, respectively. They were successful in eliminating *L. infantum* from RAW 264.7 macrophages.[43] In tumor-associated macrophages, CA has been demonstrated to modify cytokine expression and reduce arginase I activity.[47] The modulation of the host response most likely plays a role in CA's antileishmanial activity.[42]

P-coumaric acid exhibits antiparasitic activity against *L. amazonensis*, and it also outperformed glucantime, the study's reference drug, in *in vitro* tests for cutaneous leishmaniasis.[39] Numerous possible mechanisms of action for this drug exist, such as increased accessibility to organelles, which are biological targets for leishmanicidal activity.[48] Lopes *et al*.[49] identify a group of 12 p-coumaric acid derivatives and assesses their leishmanicidal and antiplasmodial activity. Their findings demonstrated that the hexyl p-coumarate derivative had the greatest leishmanicidal potency against the amastigote form of *Leishmania braziliensis*. In addition, they demonstrated that the outcomes of the molecular docking study indicate that this substance inhibits DNA topoisomerase 2, mitogen-activated kinase protein, and aldehyde dehydrogenase, all of which are crucial enzymes in the growth of *L. braziliensis*. Moreover, eight derivatives of coumaric acid displayed moderate-to-good activity against *L. braziliensis* amastigotes. Chouhan *et al*.[50] showed that tannins (tannic acid is one of its derivatives) and associated substances eliminate *Leishmania* through a NO-mediated mechanism. Furthermore, Sen and Chatterjee[51] established the mechanism of action of a number of plant-derived substances, one of which is tannins, and they demonstrate that these substances increase NO production in infected macrophages and improve the expression of cytokines such as IL-10, IL-12, TNF, and interferon.

Terpenes are hydrocarbons created from isoprene units.[52] They are the most numerous and structurally varied class of secondary metabolites derived from natural materials. Terpenoids have the potential to treat protozoan parasitic diseases such as leishmaniasis, malaria, and trypanosomiasis, according to empirical data from a number of bioassays. The group's structural diversity is made possible by the parent terpenoid backbones' adaptability, which results in multiple cellular targets and consequently different antiparasitic action mechanisms.[53] For many parasite proteins with resolved crystal structures, interactions of different terpenoids with various parasite proteins have been predicted.[54] The researchers came to the conclusion that up to a point, various terpenoid classes' docking studies on *Leishmania* proteins have shown that larger molecules have a stronger binding affinity toward different *Leishmania* proteins.[55]

However, the major mechanism of protective immunity against *Leishmania*, macrophage activation as measured by increased NO,[56] has been examined as an index of antileishmanial activity of terpenoids. With some terpenoids, NO radicals production that led to parasite killing has been proven.[57] Terpenes can also easily pass through the lipid bilayer of cell membranes, altering the integrity of cellular structures as well as the mitochondrial membrane.[52]

Although the leishmanicidal effect of terpene compounds generally, several studies have shown that UA, a pentacyclic

triterpenoid, has the strongest effect compared with the rest compounds. According to reports, UA exhibits anti*L. amazonensis* activity against the parasite's promastigotes and amastigotes forms (IC50 [24 h] = 6.4 μg/mL and 27.0 μg/mL, respectively).[58] In addition, it was noted that UA is active against VL. The effects of UA are assessed in the *in vivo* model of VL (*L. infantum*) by Jesus *et al*.[59] The use of two different UA doses revealed that both doses were effective in reducing the parasite load in the spleen (over 92% reduction) and liver (over 96% reduction). Histopathological analysis of the spleen revealed less parasites than the infected, untreated control group, UA treatment preserved both white and red pulp, which was supported by INF-γ, IL-4, and IL-10 gene expression and splenic cell proliferation. The toxicological parameters were not affected by UA. UA causes mitochondria-dependent, caspase 3/7 independent programmed cell death. Through this mechanism, promastigotes were eliminated by UA. *In vivo* cutaneous leishmaniasis lesion size and parasite load were both decreased by this compound. In addition, UA was not toxic to BALB/c mice peritoneal macrophages and was effective at getting rid of intracellular amastigotes, which are linked to the production of NO.[58]

Terpinen-4-ol is one of the terpene compounds that was purified by HPLC in this research. In comparison to control wells, treatment with terpinen-4-ol at four concentrations reduced the viability of the amastigote forms inside macrophages. In addition, terpinen-4-ol demonstrated activity against *L. tropica* amastigote forms *in vitro*, with a minimal inhibitory concentration 50 value of 0.0416% (v/v).[60]

The antileishmanial property of β-caryophyllene (one of the caryophyllene derivatives that was purified by HPLC in this research) can now be added to the list of its activities.[61] The agonist activity of β-caryophyllene on the cannabinoid type 2 receptor expressed by macrophages may have mediated the NO inhibitory effect.[62] It is possible that macrophages metabolize β-caryophyllene into an active molecule with leishmanicidal properties.[63]

This study manifests that the extract used in this study: First, is safe for human cell, second, has a very good effect against VL. This opens new horizons for us to try to find a natural and safe treatment against VL.

CONCLUSION

It is necessary that there be continuous attempts by researchers to reach a drug composed of natural compounds used to treat VL. The present study has shown that phenols and terpenes were the main active compounds in the aqueous extract of *C. vulgaris*. In addition, from all the compounds purified from the aqueous extract, CA, and UA appear to have strong leishmanicidal effects, so we can use these two compounds to make an effective drug against leishmaniasis. This study represents a successful attempt to use the aqueous extract of *C. vulgaris* to treat VL *in vivo*.

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Conflicts of interest

There are no conflicts of interest.

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