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Phytochemical Screening, in Vitro Antileishmanial Activity of *Conyza Canadensis* Extract by Neopterin

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ABSTRACT

Aims Leishmaniasis is a parasitic disease caused by the *Leishmania* parasite, transmitted through the bite of an infected sandfly. The disease presents in four primary clinical forms: visceral, cutaneous, diffuse cutaneous, and post-kala-azar dermal leishmaniasis. The clinical manifestations vary depending on the *Leishmania* species and the host's immune response. Current treatments include antileishmanial drugs such as amphotericin B, antimonials, sitamaquine, pentamidine, paromomycin, and miltefosine. However, these drugs present challenges, including resistance to pentavalent antimonials and nephrotoxicity. The World Health Organization recommends exploring plants as therapeutic agents due to their efficacy and affordability. *Conyza Canadensis*, a biennial plant, has been utilized for wound healing and has shown efficacy against both gram-positive and gram-negative bacteria. This study aimed to investigate the anti-leishmanial effect of extracts from C. canadensis on the Iraqi strain of *Leishmania* tropica.

Materials & Methods In this experimental study, the extracts were prepared as stock solutions and sub-cultured in RPMI-1640 medium. The cells were incubated in tissue culture flasks and treated with various concentrations of SSG and aqueous/alcoholic extracts. The anti-leishmanial activity of these extracts was evaluated using the sandwich enzyme-linked immunosorbent assay (ELISA) technique. The findings indicated that the extracts possessed anti-leishmanial activity.

Findings The Iraqi *C. canadensis* plant extract contained alkaloids, gallic acid, apigenin, chlorogenic acid, caffeic acid, quercetin, p-Coumaric acid, and glutathione. These compounds are known to reduce neopterin levels and enhance the immune response against *L. tropica*.

Conclusion The extracts of *C. canadensis* demonstrate anti-leishmanial activity, correlated with neopterin levels, showing promise as an alternative therapy for leishmaniasis. Further research is necessary to establish their effectiveness.

Keywords Apigenin; Caffeic Acid; Chlorogenic Acid; Gallic Acid

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Introduction

Leishmaniasis is a parasitic disease caused by the Leishmania parasite, transmitted to humans through the bite of an infected sandfly [1]. In humans, leishmaniasis can manifest in various forms, ranging from superficial, inflammatory lesions on the skin to more severe and sometimes lethal infections of the internal organs. There are four primary clinical manifestations of the disease: visceral (LV or kala-azar), cutaneous (LCM), diffuse cutaneous (DCL), and post-kala-azar dermal (PKDL), which depend on the Leishmania *spp.* [2].

The Leishmania *spp.* has two life stages: promastigote (PRO) and amastigote (AMS). The Phlebotomus sandfly serves as both an intermediate host and a vector. Different Leishmania types elicit various immune responses [3]. During the life cycle of Leishmania, the AMS undergoes binary fission in the sandfly's midgut and eventually transforms into a PRO. The PRO then migrates to the pharynx of the sandfly and prepares for transmission to a new host. When the sandfly feeds on the blood of a vertebrate host, the PRO is injected into the host's skin [4, 5]. A sandfly's life cycle is believed to last approximately ten days [6].

During blood-feeding, the sandfly injects metacyclic PRO and saliva into the host's bloodstream. The saliva contains biologically active components that modify the immune response and influence parasite infection. Neutrophils and monocytes/macrophages then infiltrate the bite site [7].

When PRO comes into contact with host cells, macrophages engulf them. Once inside, PRO undergoes a differentiation process into AMS and proliferates within phagolysosomes. Leishmania must adapt metabolically and resist host immune system assaults [8]—eventual macrophage lysis and the subsequent release of infectious stages that can invade other cells. The parasite's life cycle within the human host persists until another sandfly acquires a blood meal from the afflicted individual, completing the cycle [9].

Activation of macrophages and monocytes triggers the biosynthesis of NEO, a pteridine derived from guanosine triphosphate, reflecting an overdriven cellular immune response. The pro-inflammatory IFN-γ produced after T-lymphocyte activation is the primary factor that triggers NEO formation [10]. When the parasite infection is active, NEO release increases visceral leishmaniasis due to activated macrophages and an increase in macrophage load. However, as the infection wanes, NEO release decreases [11].

The artificially produced NEO directly correlates with the quantity of IFN-γ and indirectly suggests an elevation in TNF-α. There is a strong connection between the excessive synthesis of NEO and the stimulating effects of these cytokines on the metabolic activity of immune cells. Additionally, NEO plays a role in the mechanism by which activated macrophages exert their cytotoxic actions. NEO levels indicate the interaction among various cytokines in the monocyte/macrophage population [12].

Current antileishmanial drugs include amphotericin B, antimonials, sitamaquine, pentamidine, paromomycin, and miltefosine. Immunomodulatory antileishmanial drugs enhance the innate immune system [13]. The treatment regimen, however, has significant drawbacks. Pentavalent antimonials are the primary treatment for visceral leishmaniasis. Commercially available as sodium stibogluconate (SSG) and meglumine antimoniate (MA), these pentavalent antimonials (SbV) have seen reduced efficacy due to resistance [14].

Despite its effectiveness in treating visceral leishmaniasis resistant to pentavalent antimony, the injection and adverse side effects of these pharmaceuticals render them toxic and sometimes fatal [15]. The leishmanicidal efficacy of amphotericin B (AmB) reduces treatment failures and relapses. However, this drug is not recommended as a first-line treatment due to its nephrotoxicity and the need for parenteral administration [9, 17].

The World Health Organization (WHO) asserts that using plants represents the only viable path toward developing a safe, effective, and affordable therapeutic agent to address various health issues. Clinical trials have provided empirical evidence demonstrating the efficacy of specific indigenous flora in combating cutaneous leishmaniasis and their potential utility in facilitating wound healing through herbal ointments. When native plants are abundant, they may offer a more cost-effective alternative to synthetic medications. This advantage is particularly significant in developing countries where the disease is prevalent [16].

The herb *Conyza canadensis* grows naturally in various regions, including north of Baghdad, Baquba, Kut, Rustam, Mosul, Abu Ghraib, Rowanduz, Za'franiya, Pushtashan, Qerna Qaw valley, northeast of Zakho, and 50 km from Basra to Nassiriya [18]. It is a biennial plant [18]. HPLC analysis of its extract confirms the presence of quercitrin, quercetin, apigenin, p-coumaric acid, and caffeic acid [19].

C. canadensis has been used for managing wounds, swellings, arthritis-related pain, inflammation, diarrhea, and microbial infections such as urinary and respiratory tract infections [19, 3].

Various studies have demonstrated the efficacy of the plant's ethanolic, methanolic, chloroform, and ethyl acetate fractions against gram-positive and gram-negative bacteria. Additionally, the plant exhibits notable pharmacological activities, including anti-inflammatory, anticoagulant, anti-gastric ulcer, anti-diabetic, antioxidant, anti-cancer, and anti-mutagenic properties [19].

The study aimed to determine the antileishmanial properties of extracts from the Iraqi *C. canadensis* against *Leishmania tropica*.

Materials and Methods

This experimental study was conducted in the Pharmacology and College of Medicine of the University of Babylon from September 2022 to September 2023, in which the U937 monocyte was supplied by the National Cell Bank of Iran. The cells were grown in Roswell Park Memorial Institute (RPMI-1640) liquid medium (Gibco, UK) containing 10% fetal bovine serum (Gibco, UK) and phorbol myristate acetate (PMA) (Invevo Genes, USA). Stibogluconate (Pentostam) (GSK, UK) and *C. canadensis*, harvested from the mountains of Sulaymaniyah at the end of winter, were used. Neopterin (NEO) levels were measured using an ELISA Kit (Elabscience/USA). The Iraqi strain of *L. tropica* was obtained from the parasitology laboratory, graduate studies, Department of Biology, College of Science, University of Baghdad. The strain was then cultured in a biphasic medium (Nove-MacNeal-Nicolle) [20].

Phytochemical compounds in the *C. canadensis* extract

Total alkaloid content

A total of 20g of plant material was subjected to extraction using methanol for 24h, utilizing Soxhlet equipment. The extract underwent filtration, and the methanol was removed by evaporation using a rotary evaporator set at a temperature of 45°C.

Qualitative estimation (test for alkaloids): Alkaloids were confirmed using Dragendorff's method. The extract was dissolved in dilute HCL, and Dragendorff's reagent was added, producing a crystalline precipitate indicating the presence of alkaloids. Positive samples were then quantitatively evaluated [21].

Quantitative estimation of alkaloid: The residue was dissolved in 2N HCl, filtered, and combined with a Bromocresol Green (BCG) solution and phosphate buffer. The resulting mixture was washed with chloroform, and its pH level was adjusted using 0.1N NaOH. To create the standard curve, an atropine standard solution was mixed with phosphate buffer and BCG solution, then shaken with the extract and chloroform. The resulting solution was collected, diluted with chloroform, and its absorbance was measured at 470nm in a UV-Spectrophotometer against the blank [22].

Determination of total phenolic compounds

Phenolic compounds were detected in an ethanolic extract using the Folin-Ciocalteu reagent. The mixture consisted of the extract, the reagent, and sodium carbonate. After 2 hours, the phenolic content was estimated by measuring the absorbance at 765nm against a calibration curve made with gallic acid (GA) [23].

Analysis of total flavonoid content

The flavonoid content was assessed using the aluminum chloride colorimetric method. A sample of the crude extract was mixed with $NaNO_2$ and $AlCl_3$ solutions, and NaOH was added to bring the final volume to 10 ml. After 15 minutes, the absorbance was measured at 510nm, and the flavonoid content was calculated as mg rutin equivalent per gram of dry weight [24].

Analysis of amino acids

Solid samples weighing approximately 5mg with a precision of 0.01mg and liquid samples weighing approximately 100mg with an accuracy of 0.01mg were hydrolyzed with 1ml of 6M hydrochloric acid solution at 100°C±20°C for 24 hours. After hydrolysis, the amino acid residues were dissolved in 100µl of acetonitrile and derivatized with 100µl of OPA. The sample was then injected ten times (100µl per injection) into a gas chromatograph with a C18-ODS column and a fluorescence detector (Ex=445nm, Em=465nm) using an isocratic flow of 50/50 (v/v) water (pH=7.0) and acetonitrile at a flow rate of 1.0mL/min [25].

Analysis of total glycosides

To ascertain the presence of glycosides, the extracted substance was mixed with Baljet's reagent and allowed to sit for an hour. It was then mixed with water, and the absorbance was measured at a wavelength of 495 nm [26].

Preparing the stock solution: *C. canadensis* aqueous extract stock solution (AqCC) involved dissolving 1µg of extract in 10ml of pyrogen-free Distel water. Subsequently, six serial dilutions were created with 62.5, 125, 250, 500, 1000, and 2000µg/ml concentrations. To create the alcoholic extract of *C. canadensis* stock solution (AqCC), 1mg of extract was dissolved in three milliliters of methanol. Six serial dilutions, each with concentrations of 62.5, 125, 250, 500, 1000, and 2000µg/ml, were then created. A stock solution of stibogluconate (Pentostam) SSG was prepared at 100mg/ml, followed by six serial dilutions at 62.5, 125, 250, 500, 1000, and 2000µg/ml concentrations.

Cell line preparation and sub-culturing: Monocyte cell lines were sub-cultured as an in vitro model to evaluate the antileishmanial activity of AlCC and AqCC. The media used was RPMI-1640, supplemented with antimicrobial drugs such as gentamicin or penicillin (50µg/ml) and 5% fetal bovine serum. Afterwards, the cells were incubated at 37 degrees Celsius for twenty-four hours [27].

The development of a "macrophage-like" state in U937 monocyte: To activate NF-κB, we dissolved 5mg of PMA powder in 1.5 ml of endotoxin-free water, followed by storing at -20°C, protecting from light, avoiding repeated freeze-thaw cycles, adding 1 µl of PMA solution to the U937 monocyte cell lines, and incubating at 37°C for 24 hours [28]. PRO was used to infect macrophages from U937 in a stationary growth phase at a ratio of 20:1. The mixture was incubated in tissue culture flasks at 34°C with 5% CO2 and 95% relative humidity. After 12 hours, non-internalized PRO was removed by washing the cells five times with plain RPMI. The cells were further

incubated for 96 hours in RPMI supplemented with 10% FCS. After treating the cell line with different concentrations of SSG and aqueous/alcoholic extracts, it was incubated at 37°C for 24 hours [29].

Detection of NEO by Enzyme-Linked Immunosorbent Assay (ELISA): The study assessed the antileishmanial activity of extracts by introducing U973 macrophage cells into individual wells of a 96-well microplate. Following overnight treatment, promastigotes of *L. tropica* were administered to the macrophages and cultured at 37°C for 96 hours. Subsequently, the cell line was exposed to varying doses of SSG, as well as aqueous and alcoholic extracts, and then incubated at 37°C for another 24 hours. After this incubation period, the treated macrophage cells' liquid portion was collected from each well and transferred into separate Eppendorf tubes designated for each group. The nitric oxide and NEO levels were then measured using the sandwich enzyme-linked immunosorbent assay (ELISA) method [30, 31].

Statistical analysis

The data were analyzed using SPSS 26 software and the student's t-test. The threshold for statistical significance was set at a p-value of ≤0.05.

Findings

HPLC analysis of the *C. canadensis* extract

Table 1 shows the active compounds in the extract. The analysis revealed six distinct peaks in the chloroform fraction of the Iraqi *C. canadensis* plant, as shown in Figures 1 and 2 for the aqueous and alcoholic extractions, respectively.

Table 1. Active compounds present in the aqueous (AqCC) and alcoholic (AlCC) extracts of *C. canadensis*

Parameter	AqCC	AlCC
Total phenolic content (mg Gallic/gm)	140.15	98.58
Total flavonoid content (mg Rutin/gm)	98.2	62.15
Total alkaloid content (%)	25.6	15.99
Total glycoside content (%)	11.2	6.58
Gallic acid (µg/gm)	111.5	74.5
Apigenin (µg/gm)	80.9	42.9
Chlorogenic acid (µg/gm)	74.6	52.1
Caffeic acid (µg/gm)	30.5	16.5
Quercetin (µg/gm)	65.9	43.9
p-Coumaric acid (µg/gm)	151.4	108.9
GSH (µg/gm)	63.5	41.9

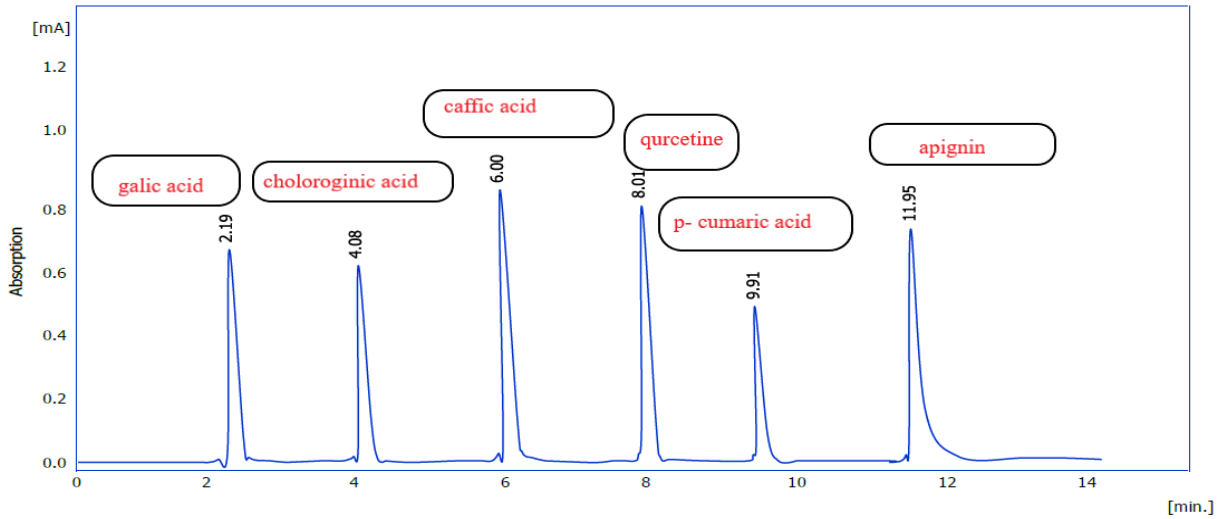

Figure 1. HPLC chromatogram of the aqueous *C. canadensis* extract

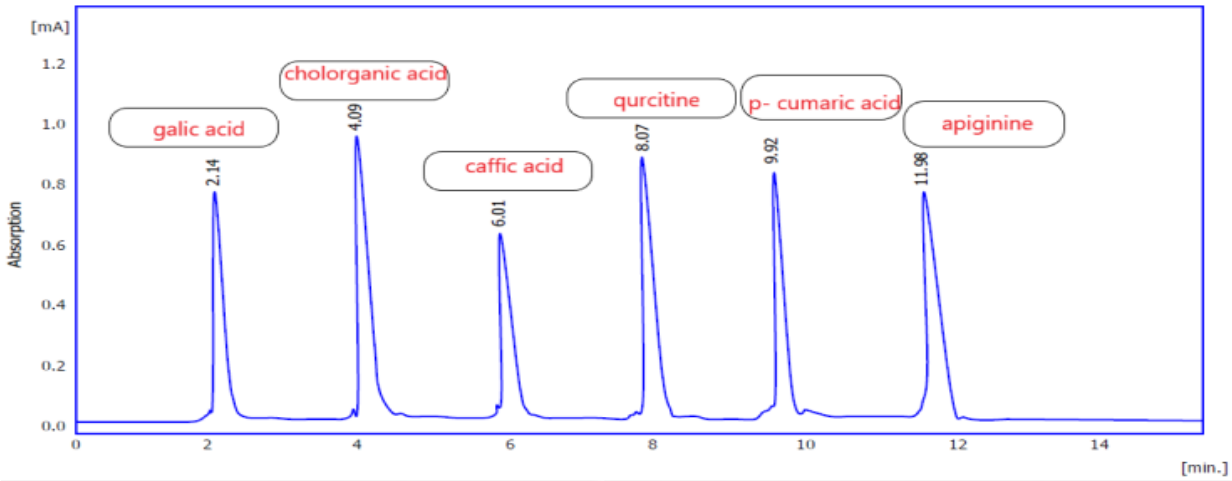

Figure 2. HPLC chromatogram of alcoholic *C. canadensis* extract

Each peak represents a different type of active compound present in the plant. The retention times of each peak in the chloroform fraction were compared with those of a standard (Figure 3).

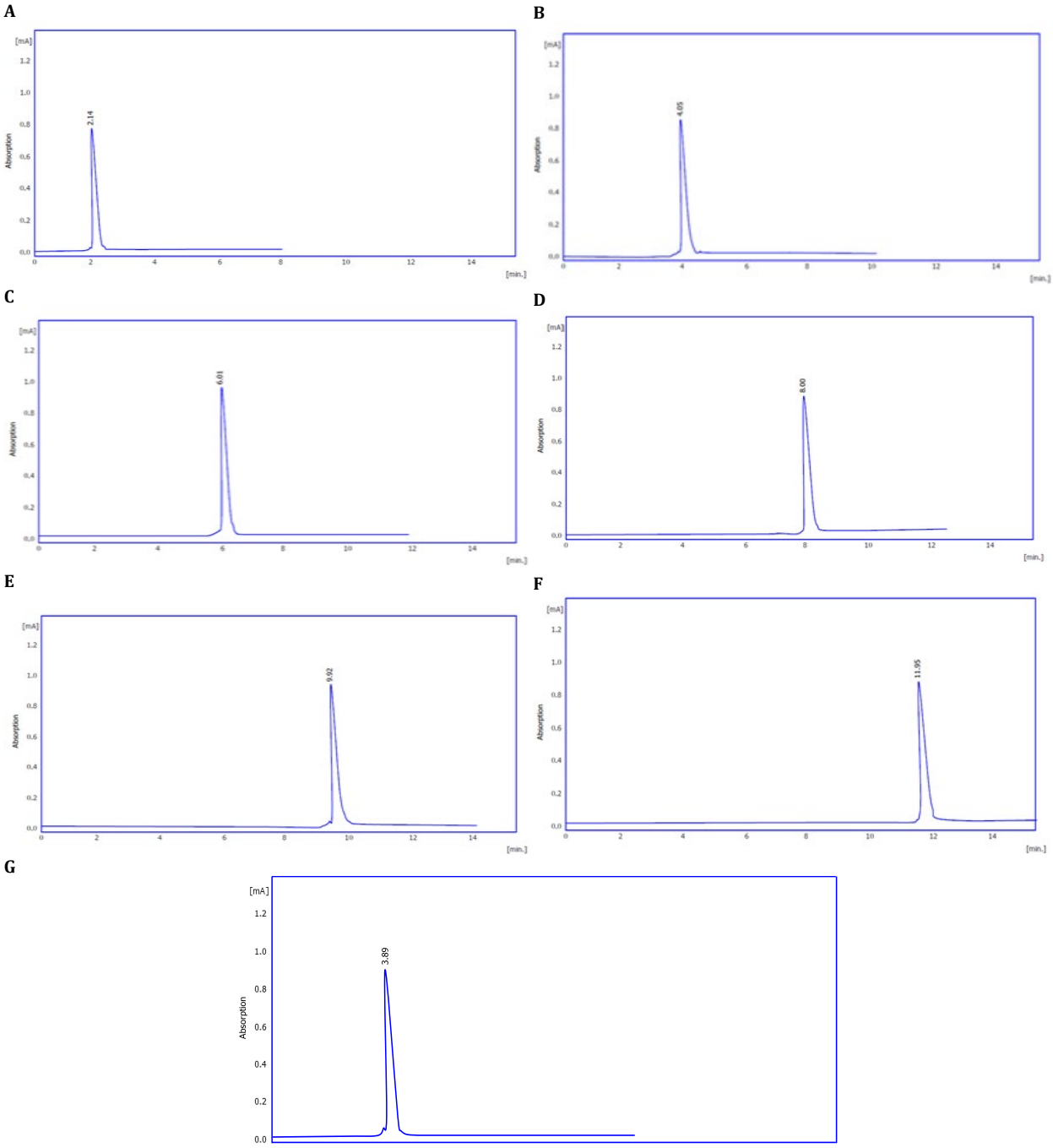

Figure 3. HPLC chromatogram of the Iraqi *C. canadensis* compounds compared to the standard (A: gallic acid; B: chlorogenic acid; C: caffeic acid; D: qurecetin; E: P-cumaric acid; F: apigenin; G: GSH)

Table 2 presents the active compounds' retention time compared with the standard.

Table 2. The retention time (minute) of the active compounds in comparison with the standard

Compound	Standard	Alcoholic	Aqueous
Gallic acid	2.14	2.14	2.19
Apigenin	11.95	11.98	11.95
Chlorogenic acid	4.05	4.09	4.08
Caffeic acid	6.1	6.01	6.00
Quercetin	8.00	8.07	8.01
p-Coumaric acid	9.92	9.92	9.91
GSH	3.89	3.88	3.80

Additionally, samples exhibited three peaks, as depicted in Figures 4 and 5, at retention times ranging from 3.5 to 8 minutes. Compared with the standard curve of glutathione (GSH), as shown in Figure 3G, the presence of GSH was confirmed.

Effect of the extract compounds on NEO

Effect of SSG on NEO in U937 macrophage cell line

The administration of SSG led to a significant decrease in NEO levels in the U937 macrophage cell line compared to the control group ($p<0.05$; Figure 6).

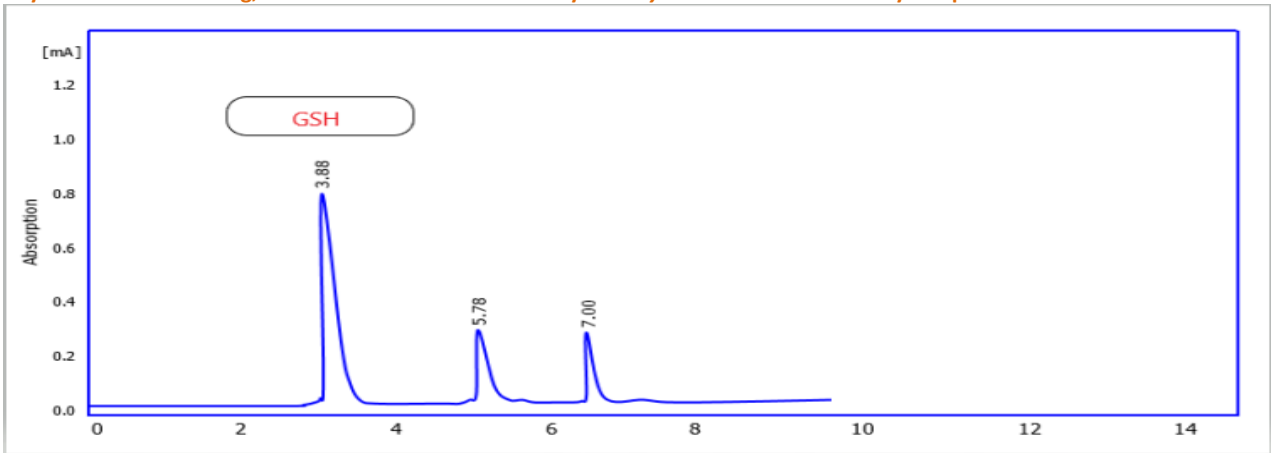

Figure 4. HPLC chromatogram of alcoholic *C. canadensis* extract for amino acid

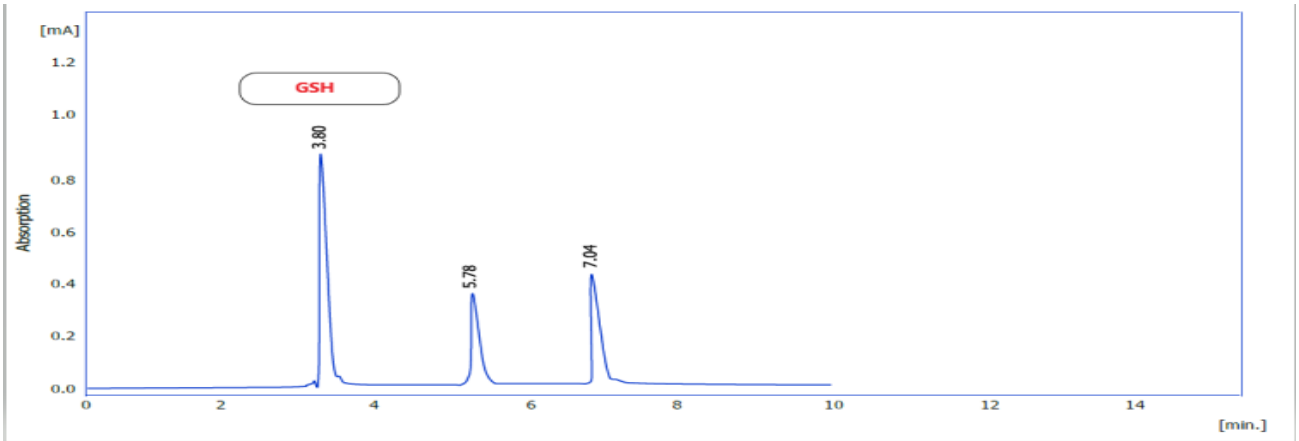

Figure 5. HPLC chromatogram of aqueous *C. canadensis* extract for amino acid

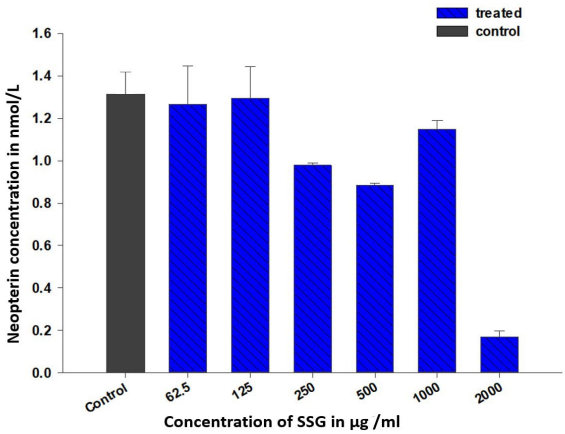

Figure 6. Effect of SSG on U937 macrophage cell line

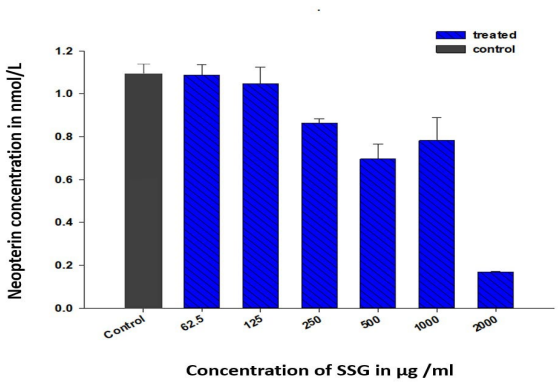

Figure 7. Effect of SSG on U937 macrophage cell line infected with *Leishmania tropica*

Effect of SSG on NEO in U937 macrophage cell line infected with L. tropica

The experiment revealed a substantial decrease in NEO production at high concentrations (250, 500, 1000, and 2000µg/ml) compared to the control group after 24 hours of incubation (Figure 7).

Effect of AqCC on NEO in U937 macrophage cell line

AqCC significantly reduced NEO levels in macrophages compared to the control group. The decline in NEO was statistically significant at elevated concentrations (125, 250, 500, 1000, and 2000µg/ml; $p < 0.05$; Figure 8).

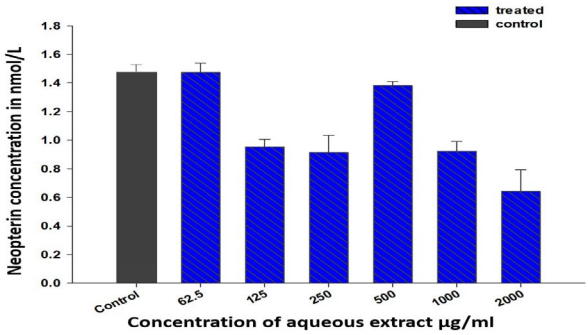

Figure 8. Effect of aqueous *C. canadensis* extract on U937 macrophage cell line

Effect of AqCC on NEO in U937 macrophage cell line infected with L. tropica

AqCC at 1000 and 2000µg/ml caused a substantial drop in NEO levels compared to the control group ($p \leq 0.05$; Figure 9).

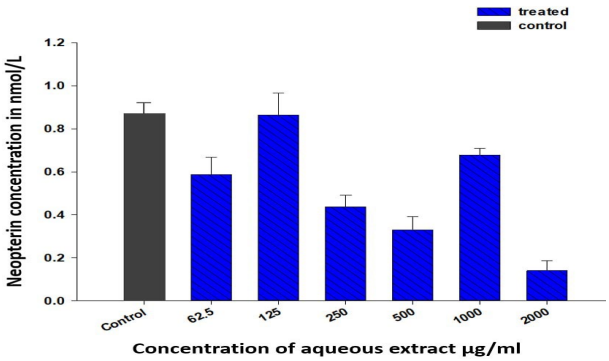

Figure 9. Effect of aqueous *C. canadensis* extract on U937 macrophage cell line infected with *L. tropica*

Effect of AlCC on NEO in U937 macrophage cell line

AlCC markedly decreased the levels of NEO in the U937 macrophage cell line compared to the control group (Figure 10).

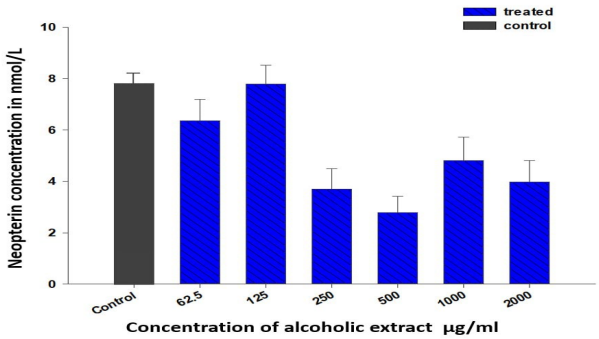

Figure 10. Effect of the aqueous *C. canadensis* extract on the U937 macrophage cell line

Effect of AlCC on NEO in U937 macrophage cell line infected with L. tropica

In the U937 macrophage cell line, AlCC significantly reduced NEO levels compared to the control group, demonstrating its role in the immune response against *L. tropica* ($p<0.05$; Figure 11).

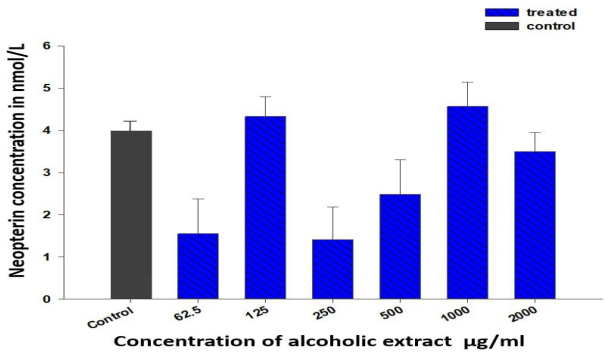

Figure 11. Effect of the aqueous *C. canadensis* extract on the U937 macrophage cell line infected with *L. tropica*

Discussion

According to this study, NEO levels in individuals infected with L. tropica decreased significantly in response to treatment with SSG, AlCC, and AqCC at all concentrations. However, a significant reduction in NEO concentrations was observed exclusively at elevated concentrations of 1000 and 2000µg/ml of AqCC. Serum NEO levels that exceed the established normal range's upper limit indicate cell-mediated immunity activation. It is important to recognize that serum NEO does not exhibit disease specificity. Monitoring and evaluating its concentrations in the blood serum throughout an infectious disease can provide valuable insights into the disease's severity and the effectiveness of the treatment [31]. Visceral leishmaniasis (VL) is characterized by an increase in the number and activation of macrophages, which would lead to a rise in NEO concentrations during the disease's active phase. Subsequently, as the parasite infection decreases, these levels would also decrease [11-13]. Recent studies have observed antileishmanial activity in AlCC and AqCC. This is confirmed by the presence of quercitrin, quercetin, apigenin, p-coumaric acid, and caffeic acid in the extracts, as determined by HPLC analysis [20]. The results of this research are consistent with those of Monzote *et al.*, who similarly documented the antileishmanial properties of p-coumaric acid [32]. This compound has been found to suppress the activity of three crucial enzymes involved in the progression of *Leishmania braziliensis*: Aldehyde dehydrogenase (ALDH), mitogen-activated protein kinase (MPK4), and DNA topoisomerase 2 (TOP2). This discovery aligns with prior investigations suggesting that SSG functions by impeding DNA topoisomerase. Conversely, measuring NEO levels in the blood serum throughout treatment for VL can help determine the efficacy of the therapy [10]. Elevated levels of reactive oxygen species (ROS) are associated with these measurements. Determining NEO concentrations enables an evaluation of the extent of oxidative stress and immunological activation [33]. Flavonoids, abundant in *C. canadensis*, exhibit various biological effects, such as regulating enzymes responsible for the elimination of ROS [34]. These chemical compounds can stimulate cellular apoptosis and autophagy and can inhibit the growth and penetration of cancerous cells. In healthy cells, flavonoids act as antioxidants, helping to control ROS concentrations. In cancer cells, however, they function as potent pro-oxidants. By inhibiting pro-inflammatory signaling pathways and stimulating apoptotic pathways, flavonoids regulate the balance of ROS [35]. GA has demonstrated significant immunomodulatory characteristics, including an increase in macrophage capacity to ingest and eliminate foreign particles, enlargement of lysosomes, release of nitrite, and elevated levels of calcium ions within macrophages [36]. Chlorogenic

acid (CGA) shows antileishmanial properties through its ability to eradicate parasites and disrupt their cell cycle, resulting in detrimental and inhibitory effects on the PRO. In vitro, CGA eradicates intracellular AMO, proving its efficacy in removing parasites from host cells. The enhanced functionality of macrophages facilitates the clearance process by concurrently elevating IL-12, TNF, and NO levels and reducing IL-10 synthesis. Majumder *et al.* propose that CGA may function as an innovative and non-toxic chemical agent to treat visceral leishmaniasis, presenting a viable alternative to chemotherapy [37]. Anke *et al.*, however, found that serum NEO concentrations did not increase in patients with CL [38]; their research contradicts this finding, suggesting that the two sources conflict.

Our findings support using natural plant extracts as alternative therapies for leishmaniasis. *C. canadensis* shows promise due to its bioactive components and various pharmacological effects. Further research is needed to understand the mechanisms of action and assess the treatment's effectiveness in animal models and clinical trials. Developing cost-effective and accessible therapies is crucial, especially in regions with limited resources. Continued exploration of *C. canadensis* as a therapeutic candidate could lead to effective and sustainable solutions for leishmaniasis.

Conclusion

This study demonstrates the significant antileishmanial activity of *C. canadensis* extracts and highlights the correlation between NEO levels and Leishmania infection.

Acknowledgments: We would like to express my deepest gratitude to everyone who has helped me throughout my time at Kufa University's College of Medicine, but especially to everyone in the Department of Pharmacology and Therapeutics.

Ethical Permissions: On May 1st, 2021, a local ethics commission at the College of Medicine at Kufa University approved both the study protocol and the informed consent statement.

Conflicts of Interests: Considering the presentation of research, there were no potential conflicts of interest.

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