Journal of Experimental Pharmacology

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ORIGINAL RESEARCH

In vitro Antileishmanial Activity of Some Ethiopian Medicinal Plants

This article was published in the following Dove Press journal:
Journal of Experimental Pharmacology

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Introduction: Leishmaniasis is a group of diseases caused by protozoan parasites, which remains a burden for developing countries. The lack of a vaccine as well as the emergence of resistance toward the recommended drugs pose a challenge for the control of the disease. This urges the demand for new antileishmanial agents to prevent and treat this disease. Consequently, four Ethiopian plants were selected and tested for their antileishmanial activity against two Leishmanial parasites.

Methods: Methanol (80%) was used to macerate the plant materials. In vitro antipromastigote activity of the crude extracts was then tested against promastigotes and axenically cultured amastigotes of *Leishmania aethiopica* and *Leishmania donovani* clinical isolates using Alamar Blue assay, and cell viability was measured fluorometrically. 1% DMSO and the media were used as a negative control while amphotericin B was used as a positive control. Furthermore, preliminary phytochemical analysis of the extracts was performed.

Results: From the four plants' extracts, *Ferula communis* and *Otostegia integrifolia* showed better activity with IC_{50} value of 11.38±0.55 and 13.03±0.87 µg/mL against *L. aethiopica*, respectively. However, the same plant extracts exhibited lower activity against *L. donovani* with IC_{50} values of 23.41±2.32 and 17.24±1.29 µg/mL, respectively. *O. integrifolia* exhibited highest effect against amastigotes of *L. aethiopica* (IC_{50}: 16.84±0.65) and *L. donovani* (IC_{50}:14.55±0.38). *F. communis* resulted second highest in growth inhibition against amastigotes of *L. aethiopica* and *L. donovani* with IC_{50} value of 14.32±0.54 and 31.12±0.19, respectively. The phytochemical analysis of the extracts indicated the presence of phenol, flavonoids, tannins, saponins, terpenoids, and alkaloids.

Conclusion: The findings from this study demonstrate that crude extracts of *F. communis* and *O. integrifolia* showed promising antileishmanial activity against *L. aethiopica* and *L. donovani* that may be attributed to the presence of different secondary metabolites.

Keywords: antileishmanial activity, in vitro, *Leishmania aethiopica*, *Leishmania donovani*, medicinal plants

Introduction

Leishmaniasis is a protozoan parasitic disease caused by the genus *Leishmania*. There are more than 20 *Leishmania* species[1] which are mainly transmitted to humans via the vector of infected female *Phlebotomus* and *Lutzomyia* sand-flies.[2] More than 70 animal species including mammals serve as a host or reservoir, including humans, in the transmission of the parasite.[3]

The disease is endemic in 98 countries, most of which are developing countries.[4] According to World Health Organization's fact sheet on leishmaniasis, approximately 20,000–30,000 deaths and 0.7–1 million new cases of leishmaniasis occur per annum.[1]

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submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/JEP.S285079

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From the three main forms of the disease, an estimated 50, 000 to 90, 000 new cases of visceral leishmaniasis (VL) and 600, 000 to 1 million new cases of cutaneous leishmaniasis (CL) occur worldwide annually. Furthermore, more than 90% of mucocutaneous leishmaniasis (MCL) cases occur in Bolivia, Brazil, Ethiopia and Peru. In Ethiopia, the disease burden is huge, where the country is highly affected by both VL and MCL, with an estimated 4500 to 5000 new cases of VL per year.[5] Besides, the four *Leishmania* species *L. aethiopica, L. donovani, L. major*, and *L. tropica* are common in the country.[6]

Given that there is still no vaccine available for use in humans, the treatment of leishmaniasis mainly depends on chemotherapy. However, there has been development of resistance toward first line drugs. In addition, the second line drugs have severe adverse effects and are costly in resource-limited areas.[7] This urges the demand for new antileishmanial agents to prevent and treat this disease. Moreover, researchers' attention is being drawn toward naturally derived compounds used to treat parasitic diseases including leishmaniasis for identification of new lead compounds and/or new drugs against the disease which warrant investigation.[8]

The use of plants as alternative medicine in Ethiopia has been a long-standing practice for centuries. In fact in Ethiopia, for 70%-80% of its people and around 90% of domestic animals, traditional medicines are used as a primary source of treatment.[9] Consequently, the following plants of interest were selected; *Discopodium peninervium* Hochst (Solanaceae) also called "Ameraro" in Amharic,[8] *Ferula communis* L. (Apiaceae) identified colloquially as "Doge",[10] *Otostegia integrifolia* Benth (Lamiaceae) locally recognized as "Tinjuit",[11] and *Urtica simensis* Hochst. ex. A. Rich. (Urticaceae) also called "Samma" in Amharic.[12]

The aforementioned herbs were selected for their traditional use as treatment for skin and wound infection which is ostensibly similar to the manifestations of leishmaniasis.[10,12,13] Besides, similar antileishmanial activities were reported within the genus of these plants.[8,14–16] Therefore, in this study, in vitro antileishmanial activity and phytochemical analysis of 80% methanolic extracts of each plant were investigated.

Materials and Methods

Collection and Authentication of Plant Materials

Fresh leaves of *D. peninervium, O. integrifolia, U. simensis* and roots of *F. communis* were collected from Semien Shewa Ensaro woreda (Latitude: 9° 49′ 59.99" N Longitude: 39° 00′ 0.00" E) 139 km away from Addis Ababa, in September, 2019. The identification and authenticity of the plant materials was confirmed at the National Herbarium, Department of Biology, Addis Ababa University. The voucher specimen; *D. peninervium* (HA001), *O. integrifolia* (AD001), *U. simensis* (HA003) and *F. communis* (HA002) was deposited for future reference. The collected parts of plants were separated, washed with tap water, and left to dry in shade. Dried parts of plants were grounded to a coarse powder and stored in a desiccator at room temperature until further use.

Preparation of Plant Extracts

The powdered plant materials (250 g) were macerated using 80% methanol (3 × 2 L, 72 h each). Then the plant extracts were filtered and the methanol in the filtrate was removed using a rotary evaporator (Buchi, Switzerland). The concentrated extract was dried with a lyophilizer (Wagtech, Denmark). Finally, the crude extracts of *D. peninervium, O. integrifolia, U. simensis* and *F. communis* yielded 12.5%, 16.6%, 24.4%, and 10.15% respectively.

In vitro Antileishmanial Activity Test

Cell Culture

In vitro antipromastigote activity test was carried out against the promastigote stage of clinical isolates of L. aethiopica and L. donovani. The two clinical isolates were grown in tissue culture flasks containing RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU penicillin/mL (Sigma-Aldrich, Germany) and 100 µg/mL streptomycin (Sigma-Aldrich, Germany) solution at 22°C for L. aethiopica and 24°C for L. donovani.[17] A cell-free medium was used to grow parasites in vitro and to set up the test system for determination of the IC_{50} values.

From late stationary phase promastigotes (3×10^6 cells/mL) axenically cultured amastigotes were acquired. Using medium 199 with Hank's salts supplemented with 20% FBS, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin, the cells were centrifuged and then resuspended. After that, by using 1 N HCl, the pH was made to 5.5. Following incubation of cells at 31°C for *L. aethiopica* and 37°C for *L. donovani* at 5% CO_2, amastigote-like rounded morphology together with loss of flagella and cell clumping started appearing within 24 h. The parasites were kept for a week as some motile parasites with intermediate forms and short flagella were detected.

The human monocytic leukemia cell line THP-1 cells were incubated in RPMI 1640 medium plus 10% hi-FCS and 20 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) at 37°C and 5% CO_2 for 72 h. A cell-free medium was used to grow parasites in vitro and to set up the test system for determination of the IC_{50} values of the extracts.

In vitro Antileishmanial Assay

Each of the plant extracts was added to a separate 96-well microtiter plate containing 100 µLcomplete culture medium to achieve a final concentration of 100 µg/mL. Then, a suspension of 100 µL of the parasites (3.5×10^6 promastigotes of *L. aethiopica* or *L. donovani*) obtained from the previous culture was added to each well. After that, parasites were incubated for 72 hrs at room temperature for promastigotes of both strains, at 31°C and 37°C for axenically cultured amastigotes of *L. aethiopica* or *L. donovani* respectively, in the presence of various concentrations of extract. Next, resazurin (0.125 mg/mL) was added to 20 µL suspension (10% of the total volume of each well). The mixture was covered with aluminum foil, and left at the previously stated temperature. Fluorescence intensity was measured using Victor 3 Multilabel Counter (PerkinElmer, MA, USA) at excitation wavelength of 544 nm and emission wavelength of 590 nm. The assay was conducted in triplicate and compared to negative controls (1% DMSO and Media alone) and reference drug (Amphotericin B, Sigma-Aldrich, Germany). During the assay, cell viability was monitored by measuring fluorescent signal. The fluorescence intensities produced are proportional to the number of viable cells.[17]

Cytotoxicity Study in THP-1 Monocyte

Onto 96-well plates, THP-1 monocytes were plated at a density of 4×10^4 cells per well (in 200 volume) in the presence or absence of plant extracts, and plates were incubated at 37°C, 5% CO_2 for 72 h. Then Alamar Blue was added, during the last 3 h of incubation, cell viability was measured fluorometrically as described previously.[17]

Phytochemical Screening

The 80% methanol extracts of each plant were investigated for the presence or absence of secondary metabolites such as alkaloids, flavonoids, phenols, saponins, tannins, and terpenoids following standard procedures.[18]

Determination of Total Phenols

Folin–Ciocalteu's method was used to determine the total phenolic content of the extracts. Serial dilutions of the standard (gallic acid) were prepared in distilled water with concentrations 100, 50, 25, 12.5, 6.75 and 3.375 µg/mL, to establish calibration curve. 1 mL of the standard was then transferred into test tubes. After that, 5 mL of distilled water and 0.5 mL of Folin–Ciocalteu's reagent were added into the test tubes. 5 minutes later, 1.5 mL of Na_2CO_3 (20%) was added and the volume made up to 10 mL with distilled water. It was allowed to stand for 90 minutes at ambient temperature. The absorbance of the solution was measured at 760 nm using a UV spectrophotometer (Jenway Model 6500, England). Every experiment was conducted in triplicate. Similar procedure was also followed for the extracts (100 µg/mL) and the blank solutions. The total phenolic content was determined using a standard curve of gallic acid ($y = 0.0075x - 0.0862$, $R2 = 0.9665$). The calculated total phenolic results were expressed as mg of gallic acid equivalent per 100 g of extracts.[19]

Determination of Total Flavonoid Content (TFC)

Aluminum chloride complex forming assay was employed to determine the total flavonoid content of the extracts. Serial dilutions of the standard (Quercetin) were prepared in methanol with concentrations 1, 0.50, 0.25, 0.125 and 0.065 mg/mL, to establish calibration curve. 1 mL of the standard was then transferred into test tubes. 0.3 mL 5% $NaNO_2$ was added and left for 5 minutes. Another 0.3 mL of 10% $AlCl_3$ was mixed with the solution and allowed to stand for 5 minutes. After that, 2 mL solution of 1M NaOH was added into the solution followed by quantity sufficient distilled water to make up the volume to 10 mL. Finally the solution was incubated for 30 minutes at ambient temperature. The absorbance of the solution was recorded at 510 nm on UV spectrophotometer (Jenway Model 6500, England). The same procedure was repeated with the extract (1 mg/mL) and the blank solutions. The total flavonoid content was determined using a standard curve of quercetin ($y=0.5957x - 0.0055$, $R2 = 0.9982$). The calculated total flavonoid results were expressed as mg of quercetin equivalent per 100 g of extracts. All the procedures were performed in triplicate.[20]

Data Analysis

Antileishmanial activity (IC_{50}) values were calculated from sigmoidal dose-response curves of percent inhibition

using the computer software GraphPad Prism 8.4.3 (GraphPad Sofware, Inc., CA, USA), and Microsoft excel; values were expressed as mean ± SD of triplicate experiments.

Results

Antileishmanial Assay

The methanolic extracts of all the plants showed activity against promastigotes and axenically cultured amastigotes of *L. aethiopica* and *L. donovani* with varying IC_{50} values (Tables 1 and 2, Figures 1 and 2).

Of all the extracts, *O. integrifolia* exhibited highest antipromastigote activity with percentage inhibition of 71.78% and 71.40% (Table 1) with maximum tested concentration (ie, 100 µg/mL) against *L. aethiopica* and *L. donovani* respectively. At this concentration, *O. integrifolia* exerted similar effect against the amastigotes of *L. aethiopica* and *L. donovani* with percentage inhibition of 67.31% and 70.58% respectively (Table 2). *F. communis* also exerted comparable antipromastigote activity with percentage inhibition of 68.47% and 63.57% against *L. aethiopica* and *L. donovani* respectively. While the same plant exerted slightly lower activity against the amastigotes of both Leishmanial species (Table 2).

However, all the extracts showed lower activity as compared to amphotericin B which exhibited IC_{50} of 1.21±0.11µg/mL and 1.31±0.065 µg/mL (Table 1) against *L. aethiopica* and *L. donovani* correspondingly.

Preliminary Phytochemical Analysis

The result found from the investigations revealed the presence of different secondary metabolites such as alkaloids, flavonoids, phenols, saponins, tannins and terpenoids in the plant extracts as summarized in Table 3.

Table 1 Antipromastigote Activity IC_{50} Values and Percent Inhibition of the Plant Extracts Against *L. aethiopica* and *L. donovani*

Plant Extracts	% Inhibition (100 µg/mL) Against *L. aethiopica*	% Inhibition (100 µg/mL) Against *L. donovani*	IC_{50} (µg/mL)[a] Against *L. aethiopica*	IC_{50} (µg/mL)[a] Against *L. donovani*
D. peninervium	62.88±0.94	58.27±0.92	13.38±1.20	63.62±1.84
F. communis	68.47±1.12	63.57±1.55	11.38±0.55	23.41±2.32
O. integrifolia	71.78±0.62	71.40±1.10	13.03±1.29	17.24±0.87
U. simensis	53.91±0.76	61.99±0.72	63.78±0.89	44.37±1.61
Amphotericin B (Reference)	–	–	1.21±0.11	1.31±0.065
Media alone (NC)	0.00[b]	0.00[b]	0.00[b]	0.00[b]
1% DMSO (NC)	0.00[b]	0.00[b]	0.00[b]	0.00[b]

Notes: Values are expressed as mean ± SD; n = 3. [a]Effective concentration required to achieve 50% growth inhibition in µg/mL; [b]no effect.
Abbreviations: NC, negative control; DMSO, dimethyl sulphoxide.

Table 2 IC_{50} Values and Percent Inhibition of the Plant Extracts Against Axenically Cultured Amastigotes of *L. aethiopica* and *L. donovani*

Plant Extracts	% Inhibition (100 µg/mL) Against *L. aethiopica*	% Inhibition (100 µg/mL) Against *L. donovani*	IC_{50} (µg/mL)[a] Against *L. aethiopica*	IC_{50} (µg/mL)[a] Against *L. donovani*	Cytotoxic Effect in THP-1 LC_{50} (µg/mL)
D. peninervium	60.98±0.34	56.6±0.78	15.13±2.31	71.52±0.32	*186.01± 0.08*
F. communis	64.64±0.51	61.39±1.28	14.32±0.54	31.12±0.19	175.22± 0.72
O. integrifolia	67.31±0.10	70.58±0.32	16.84±0.65	14.55±0.38	144.55± 0.49
U. simensis	50.35±0.28	60.39±0.03	82.37±1.63	47.30±1.08	155.11± 0.51
Amphotericin B (Reference)	-	-	1.22±0.17	1.461±0.01	12.32± 0.23
Media alone (NC)	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00
1% DMSO (NC)	0.00[b]	0.00[b]	0.00[b]	0.00[b]	0.00

Notes: Values are expressed as mean ± SD; n = 3. [a]Effective concentration required to achieve 50% growth inhibition in µg/mL; [b]no effect.
Abbreviations: NC, negative control; DMSO, dimethyl sulphoxide.

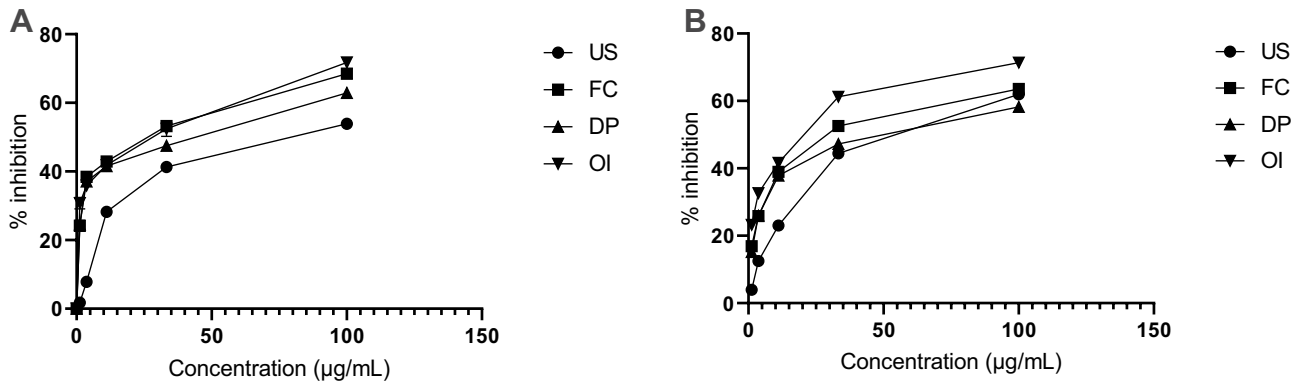

Figure 1 Antipromastigote activity of the plant extracts against *Leishmania aethiopica* (**A**) and *Leishmania donovani* (**B**).
Abbreviations: DP, *Discopodium peninervium*; FC, *Ferula communis*; OI, *Otostegia integrifolia*; US, *Urtica simensis*.

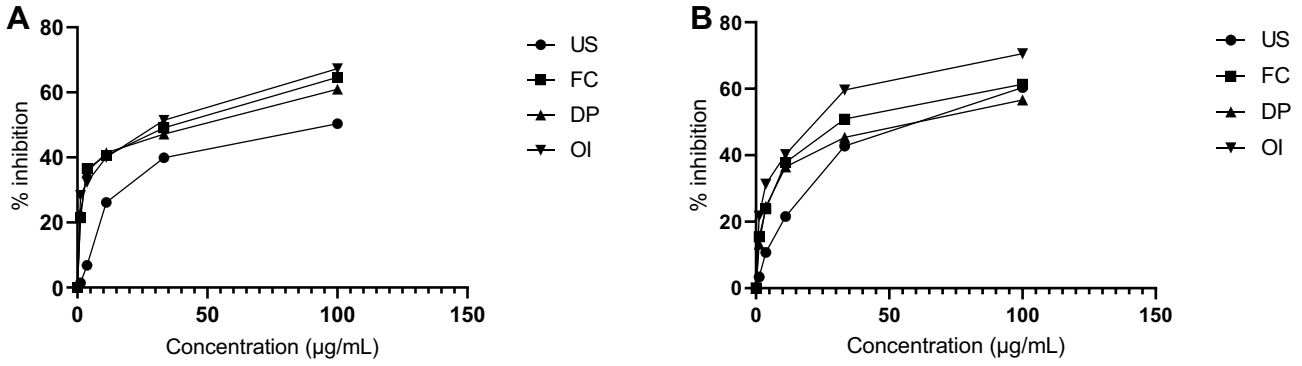

Figure 2 Effects of plant extracts on axenically cultured amastigotes against *Leishmania aethiopica* (**A**) and *Leishmania donovani* (**B**).
Abbreviations: DP, *Discopodium peninervium*; FC, *Ferula communis*; OI, *Otostegia integrifolia*; US, *Urtica simensis*.

Determination of Total Phenols and Flavonoid Content

The highest total phenol content was found from *D. peninervium* (0.43 mg of GAE/g extract) followed by *O. integrifolia* (0.39 mg of GAE/g extract) as indicated in Table 4 and Figure 3 A. Similarly, the highest amount of flavonoids was measured with *D. peninervium* (0.38 mg of QE/g extract) while *F. communis and O. integrifolia* contained comparable amounts of flavonoids, 0.2 and 0.18 mg of QE/g extract respectively as depicted in Table 4 and Figure 3B.

Discussion

Leishmaniasis is a widespread health burden in developing countries like Ethiopia. This is partly attributed to undernourishment as a result of poverty, compromised immunity, as well as unresponsiveness of the disease to recommended drugs.[6,21] Therefore, it is vital to search for alternative drugs from medicinal plants. Accordingly, the present study was carried out to search for the antipromastigote potential of some Ethiopian medicinal plants that are used in folk medicine.

The four crude extracts showed antipromastigote activity against *L. aethiopica* and *L. donovani*. However, all the plant extracts exerted lower activity than the reference drug; amphotericin B (Table 1, Figure 1A and B).

The highest antipromastigote activity was recorded for *F. communis* (IC_{50}=11.38±0.55 µg/mL). The extract also halted the growth of *L. donovani* (IC_{50}=23.41±2.32 µg/mL) (Table 1). There are studies that stated similar activities of members of the genus *Ferula*. The hydroalcoholic extract of *F. asafetida* and essential oil from *F. galbaniflua* arrested the growth of *L. major* (IC_{50} =11.8 µg/mL)[22] and *L. amazonensis* (IC_{50}/24hr=95.70 µg/mL) respectively.[23]

O. integrifolia exhibited highest effect against *L. donovani* (IC_{50}=17.24±0.87 µg/mL) as compared to other extracts. In addition, the extract displayed activity against *L. aethiopica* (IC_{50}=13.03±1.29 µg/mL) (Table 1). Mothana et al reported growth inhibition of hydroalcoholic

Table 3 Phytochemical Screening of 80% Methanol Extracts of Tested Plants

Phytochemicals	*D. peninervium*	*F. communis*	*O. integrifolia*	*U. simensis*
Alkaloids	–	+	–	+
Flavonoids	+	+	+	+
Phenol	+	+	+	+
Saponins	–	–	+	–
Tannins	–	+	–	–
Terpenoids	+	+	+	–

Note: (+) present, (−) not present.

Table 4 Total Phenolic and Flavonoid Content of 80% Methanol Extracts of Tested Plants

Plant	Phenol (mg of GAE/g)	Flavonoid (mg of QE/g)
D. peninervium	0.43±0.0031	0.38±0.0059
F. communis	0.26±0.0032	0.2±0.0012
O. integrifolia	0.39±0.0025	0.18±0.0032
U. simensis	0.2±0.0021	0.078±0.0003

Note: Values are expressed as mean ± SD; n = 3.

extract of *O. fruticosa* against *L. infantum* (IC_{50} >64.0 µg/mL).[24]

D. peninervium showed comparable activity against *L. aethiopica* (IC_{50}=*13.38±1.20* µg/mL) as that of *O. integrifolia,* while it exhibited lower activity against *L. donovani* (IC_{50}=*63.62±1.84* µg/mL) (Table 1). In another study, the essential oil from the same plant exhibited better activity against *L. aethiopica* (IC_{50} =12.62 µg/mL) and *L. donovani* (IC_{50}=19.93 µg/mL).[25]

On the other hand, *U. simensis* showed lowest result with IC_{50} value of 63.78±0.89 µg/mL against *L. aethiopica* and 44.37±1.61 µg/mL against *L. donovani* (Table 1). The aqueous extract of *U. dioica* halted the growth of *L. major* promastigotes with IC_{50} of 4500 µg/mL.[15]

However, no study has reported on the effect of the studied plant extracts against *L. aethiopica* and L. *donovani* axenic amastigotes.

The phytochemical screening of the four extracts revealed the presence of various phytochemical constituents, ie, phenols, flavonoids and terpenoids (Table 3). Phenols were reported to possess antipromastigote activity. For instance, rosmarinic acid tested against *L. donovani* promastigotes and intracellular amastigotes resulted in altered membrane integrity of the mitochondria and the cells.[26] Flavonoids were also found to exert significant antileishmanial activity against *L. donovani*.[27] In an attempt to decipher the mechanism of antileishmanial activity of flavonoids, catechins; type of flavonoid were found to form complexes with the parasite cell wall to influence processes requiring cell linking, and hence inhibit the parasite growth.[28] Meanwhile, intracellular activities were reported for quercetin that inhibited enzyme topoisomerase II and chelated iron which is used in the replication of the parasite within the macrophage phagolysosomes.[29]

Nerolidol, a terpenoid commonly found in many plants exerts antileishmanial activity against *L. amazonensis* via inhibition of various biochemical pathways such as biosynthesis of dolichol, ergosterol, and ubiquinones.[30] In

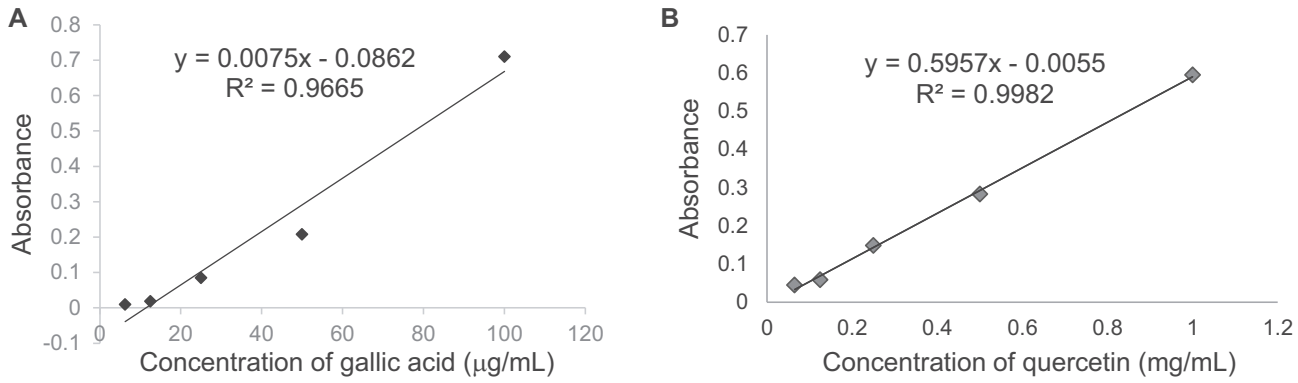

Figure 3 Total phenol (**A**) and flavonoid (**B**) contents of 80% methanol plant extracts.

addition, a triterpenoid dihydrobetulinic acid inhibited the growth of *L. donovani* amastigotes by targeting DNA topoisomerase I and II preventing DNA cleavage via forming enzyme–DNA complex, leading to apoptosis. Thus, the antileishmanial activity of the plant extracts, which are rich in phenols, flavonoids and terpenoids, may possibly be due to the specific components and/or synergistic interaction among various principles.[31]

Conclusion

The present study showed 80% methanol extracts from *O. integrifolia* and *F. communis* exhibited promising in vitro antileishmanial activity. This not only corroborates the traditional claim of the plants, it also provides clues for further examination of active principles of these plants for the development of effective and safe antileishmanial drugs.

Abbreviations

CL, cutaneous leishmaniasis; DMSO, dimethyl sulfoxide; IC_{50}, the half maximal inhibitory concentration; MCL, mucocutaneous leishmaniasis; VL, visceral leishmaniasis.

Data Sharing Statement

The data used to support the findings of this study will be available from the corresponding author on reasonable request.

Acknowledgments

The authors are thankful to Mr. Melaku Wondafrash, Addis Ababa University, for identification of the plant material. The authors are thankful to department of microbiology, immunology and parasitology, faculty of medicine, college of health sciences for providing the laboratory setting.

Author Contributions

All authors contributed to data analysis, drafting, or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Funding

No specific funding was granted for this project.

Disclosure

The authors report no conflicts of interest for this work and declare that there is no conflict of interest regarding the publication of this article.

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