



In Vitro Anti-Leishmanial Activity of Glucosinolate Fraction from *Alyssum linifolium* Steph. ex Willd (Brassicaceae)

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ABSTRACT

Objectives: The intracellular parasitic protozoan, *Leishmania* spp., causes several forms of diseases in humans. Cytotoxicity and emergence of new strains resistance to the current anti-leishmanial drugs have encouraged researchers to focus on new resources. Glucosinolates (GSL) are found mainly in the Brassicaceae family with potential cytotoxic and anti-parasitic properties. The present study reports *in vitro* antileishmanial activity of the GSL fraction from *Alyssum linifolium* seeds against *Leishmania major*.

Materials and Methods: The GSL fraction was prepared by ion-exchange and reversed-phase chromatography. For the assessment of antileishmanial activity, the promastigotes and amastigotes of *L. major* were treated with different concentrations of the fraction (75-625 µg/mL).

Results: The IC₅₀ was 245 µg/mL for anti-promastigote effect of the GSL fraction and 250 µg/mL for its anti-amastigote effect that had a significant difference ($p < 0.05$) with both glucontime and amphotericin B. The selectivity index of the GSL fraction (15.8), to glucontime and amphotericin B, was greater than 10, indicating the selective effect of this fraction against *L. major* amastigotes. Glucoiberberin was the major constituent of the GSL fraction characterized using nuclear magnetic resonance and electron ionization-mass spectrometry spectra. Based on gas chromatography-mass spectrometry data, iberberin and iberberin nitrile, the hydrolysis constituents from glucoiberberin, included 76.91% of the total seed volatiles.

Conclusion: The results suggest that GSLs like glucoiberberin could be considered a new promising candidate for further studies on antileishmanial activity.

Key words: *Alyssum linifolium*, glucosinolate, Brassicaceae, antileishmanial activity, *Leishmania major*

INTRODUCTION

The genus *Alyssum* contains about 175 species and belongs to the Brassicaceae (previously Cruciferae) family. Its main habitat is in temperate and mountainous regions of Asia, Africa, America, and Europe. 27 *Alyssum* species are distributed in Iran that their seeds are known as “qodume / ku:doome/” and are used for medicinal purposes.^{1,2} Flax-leaf *Alyssum* (*Alyssum linifolium* Steph. ex Willd.) is an annual weedy plant commonly distributed in semi-arid areas of Iran.³

Traditionally, mucilage of the plant seeds is used for respiratory diseases such as cough and hoarseness.⁴ Several bioactive glucosinolates (GSLs),⁵ phenolic compounds⁶, and fatty acids⁷ have been identified from *Alyssum* species with potential cytotoxic,⁵ neuroprotective,⁷ anti-parasitic,⁸ antioxidant,⁵ and antimicrobial⁶ activities.

GSLs and their enzymatic hydrolysis products are groups of compounds with sulphur atoms found abundantly in seeds and other parts of Brassicaceae plants. Recently, the promising

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chemoprotective, anti-tumor,⁹ anti-parasitic,¹⁰ and biopesticide activities of GSL compounds have been reported.¹¹

The anti-parasitic activities of GSLs and their hydrolysis products (such as isothiocyanates) have previously been demonstrated, including the nematicidal¹² and antiprotozoal¹³ effects. Based on the literature reviews, no study has yet examined the effect of GSLs against *Leishmania* species. The intracellular parasitic protozoan, *Leishmania* spp. are mostly distributed in North Africa, Middle East, and Central Asia. The female sandflies that are infected by *Leishmania* spp. can disseminate the parasites. It causes several forms of diseases in humans such as cutaneous, mucocutaneous, and visceral leishmaniasis.¹⁴ The major clinical manifestations of leishmaniasis include fever, jaundice, dyspnea, and hematologic disorders.¹⁵ Due to the high cytotoxicity and drug resistance to the available medications for treating leishmaniasis,¹⁶ recent investigations have been focused on the new resources such as active ingredients from medicinal plants.

According to anti-parasitic potential of Brassicaceae plants and the widespread distribution of *Alyssum linifolium* in Iran, we evaluated the anti-leishmanial activity of the GSL fraction from *A. linifolium* seeds in both promastigote and amastigote stages of *Leishmania major* parasites. The purification and identification of the major GSL constituents of *A. linifolium* seeds were performed by chromatographic techniques (ion-exchange and reversed-phase chromatography), electron ionization-mass spectrometry (EI-MS) and nuclear magnetic resonance (NMR) spectroscopy. For further investigation of the volatile constituents in *A. linifolium* seeds (isothiocyanates and nitriles), the gas chromatography-mass spectrometry (GC-MS) method was performed.

MATERIALS AND METHODS

Experimental instruments

Ultraviolet-visible spectrophotometer: PG Instruments (T80+ series, Lutterworth, UK). EI-MS: (Agilent Technologies, Santa Clara, USA). NMR spectroscopy (¹H- and ¹³C-NMR): Bruker Advance III Spectrophotometer, (Billerica, MA, USA).

Preparation of glucosinolate fraction and identification of the major glucosinolates

The seeds of *A. linifolium* were collected and authenticated by Dr. Yousef Ajani from Karaj, Iran (May 2019). The voucher herbarium specimen (105795) was deposited at the Research Institute of Forests and Rangelands, Tehran, Iran. The GSL fraction was prepared according to our previous study.¹⁷ Briefly, the ground seeds were extracted with hot methanol and the resulting extract was loaded onto the DEAE Sephadex A-25 column. The sulfatase (S9626, Sigma) solution was added to the column for the desulfation of GSLs. For further purification, the GSL fraction was loaded onto a vacuum-liquid chromatography (VLC) column packed with C18-reversed phase silica gel and eluted with aqueous CH₃CN. The separation of GSLs was achieved using high performance liquid chromatography (HPLC). The data from EI-MS and ¹H-¹³C-NMR spectroscopy were used for the structural analysis.

HPLC analysis

The semi-preparative purification of the desulfo-GSL fraction was performed on Knauer equipment including the WellChrom pump K-1800 and C18 gravity-SB column (250 × 20 mm). Separation of the desulfo-GSLs was carried out using water (A) and acetonitrile (B) programmed as follows: 0-1 min, 1% B; 1-25 min, linear gradient up to 22% B; 25-29 min, linear gradient drops down to 1% B; flow rate: 8 mL/min; injection volume: 5 mL; wavelength: 229 nm.¹⁸

Determination of total glucosinolate content

The quantification was done according to Jezek et al.¹⁹ In this method, GSLs are hydrolyzed by sodium hydroxide to release 1-thioglucose and the latter compound reacts with a potassium ferricyanide solution. The absorbance of the solution was measured at 420 nm using a spectrophotometer. Various concentrations of sinigrin (0-1 mg/mL) were applied for plotting the standard curve.¹⁹

Volatile fraction preparation

Hydrodistillation was achieved using a Clevenger-type apparatus. *A. linifolium* seeds were used for the extraction of volatile compounds. Hydrodistillation was achieved for 3 h and the volatiles were trapped by pentane. Anhydrous sodium sulfate was used to dry the volatile fraction. The concentrated fraction was analyzed using GC-MS.²⁰ An Agilent 6890 gas chromatograph consisting of a BPX-5 MS capillary column (30 m × 0.25 mm) was employed under the following conditions; temperature gradient: 50°C: for 5 min, 240°C at 3°C/min, 300°C at 15°C/min; carrier gas: helium, flow rate: 0.5 mL/min. MS mode: EI; detector voltage: 70 eV; mass range: 40 to 500 *m/z*.

Parasite culture

The promastigotes of *L. major* (MRHO/IR/75/ER) were cultured in NNN medium, then sub-cultured in RPMI-1640 medium (Gibco) supplemented with 10% heat inactivated fetal bovine serum, 100 µg streptomycin, and 100 IU penicillin (Gibco, Scotland, UK).²¹

Anti-leishmanial activity

In vitro anti-promastigote activity assay

The evaluation was carried out for 72 hours incubation in 96 well plates.²² The 2-fold serial dilutions of the GSL fraction were prepared at the concentrations of 15-125 µg/100 µL of RPMI-1640 medium. As positive controls, amphotericin B and glucantime were diluted serially in two-fold steps at concentrations of 0.98-7.8 and 140-18.700 µg per 100 µL of RPMI, respectively. Next, 100 µL of each concentration of GSL fraction or standard drugs was added to the wells in triplicate. Finally, 100 µL of RPMI-1640 medium supplemented with 1 × 10⁵ promastigotes, was added to wells (final volume of 200 µL). All above concentrations were calculated per mL for IC₅₀. The number of 3 untreated wells containing the parasite was used as the negative control. After incubation (72 h), the amount of 20 µL of each well was mixed with an equal volume of 2% formaldehyde solution in phosphate-buffered saline (PBS). The promastigotes/mL were calculated in a hemocytometer under a light microscope (400× magnification) by mixing 20 µL

of each well content with an equal volume of 2% formaldehyde solution in PBS, pH 7.2. The following formula was used for measuring the death rate (DR):

$$\text{DR (\%)} = [(\text{NC} - \text{DT})/\text{NC}] \times 100$$

Here, NC and DT denote the promastigotes number in negative control and treated wells, respectively.

In vitro anti-amastigote assay

The macrophage cell line (murine J774A.1) from the Pasteur Institute (Tehran, Iran) was cultured in RPMI medium and added to a 96 well plate (2×10^5 cells/well) containing 200 μL of RPMI medium. After the incubation (5 h), the supernatants were discarded and 200 μL of RPMI containing the promastigotes was added to each well at a ratio of 1:10 (cell:promastigote) and incubated (24 h). RPMI was used for removing free parasites. Then, 200 μL of different concentrations of reference drugs or GSL fraction (as described in promastigotes treatment) was added to each well and incubated (72 h). The number of 3 untreated wells were used as negative controls. Next, the supernatants were removed, and the cells were incubated with 50 mL MTT (Sigma, Lyon, France) solution (stock solution: 5 mg/mL in PBS). After the incubation, dimethyl sulfoxide (100 mL) was added to wells, rotated for 30 min and centrifuged ($700 \times g$, 5 min). The supernatants were transferred to the new plates and scanned at 570 nm for measuring the optical absorbance using a well spectrophotometer (BioTek, Winooski, VT, USA). The cell DR was measured by the formula below: $1 - (\text{AT}/\text{AC}) \times 100$

where, AT means the absorbance of wells treated with different concentrations of reference drugs or GSL fraction; AC means the absorbance of negative control wells.²²

Cytotoxicity and selectivity index (SI) evaluation

The DR and SI were determined for each concentration of reference drugs or GSL fraction according to our previous study. The SI >10 represents the safety of the medication.²¹

Statistical analysis

The IC_{50} and CC_{50} were analyzed by GraphPad Prism software (version v6). The IBM SPSS v20 software was used for two-tailed *t*-test analysis.

RESULTS

Identification of the major GSLs

The GSL fraction, obtained from anion exchange chromatography, was purified using VLC. The number of 5 fractions (A1-A5) were resulted. Based on HPLC peaks, the fraction A3 showed two peaks (Figure 1), where its major desulfo-GSL constituent was isolated by semi-preparative HPLC and identified as 3-methyl thiopropyl-GSL (glucoiberverin) (Figure 2) using NMR and EI-MS spectroscopy. $^1\text{H-NMR}$: 1.90 (2H, *m*, $\text{CH}_2\text{-2}'$), 2.1 (3H, *s*, S-CH_3), 2.55 (2H, *m*, $\text{CH}_2\text{-3}'$), 2.66 (2H, *t*, $J_{1,2'} = 7.2$, $\text{CH}_2\text{-1}'$), 3.2-3.4 (4H, *m*, H-2,3,4,5), 3.58 (1H, *dd*, $J_{5,6a} = 5.2$, $J_{6a,6b} = 17.6$, H-6a), 3.77 (1H, *dd*, $J_{5,6b} = 3.7$, $J_{6a,6b} = 17.6$ H-6b), 4.82 (1H, *d*, $J_{1,2} = 9.6$, H-1). $^{13}\text{C-NMR}$: 14.56 (C-4'), 26.75 (C-2'), 30.77 (C-3'), 32.97 (C-1'), 61.66 (C-6), 69.98 (C-4), 72.87 (C-2), 78.16 (C-3), 80.24 (C-5), 81.53 (C-1), 152 (C-7). EI-MS: The major mass fragments (*m/z*): 147 (R-N=C=S), 115 (R-CN), 72 (CH_2NCS^+), 61 ($\text{CH}_3\text{SCH}_2^+$), 45 (CH_3S^+).²³

Total glucosinolate content

The total GSL content was calculated as 23.28 ± 0.7 mmol equivalent of sinigrin/kg of dry sample by the following equation: $y = 0.361x + 0.164$, $R^2 = 0.9916$.

Identification of the volatile compounds using GC-MS

Based on the results, 76.91% of the total volatiles were identified as glucoiberverin hydrolysis products (Figure 3). The major compounds were recognized as 4-methyl thiobutyl nitrile (62.91%) and 3-methyl thiopropyl isothiocyanates (iberverin 14.01%), which were characterized by their retention time and MS spectra (Table 1).

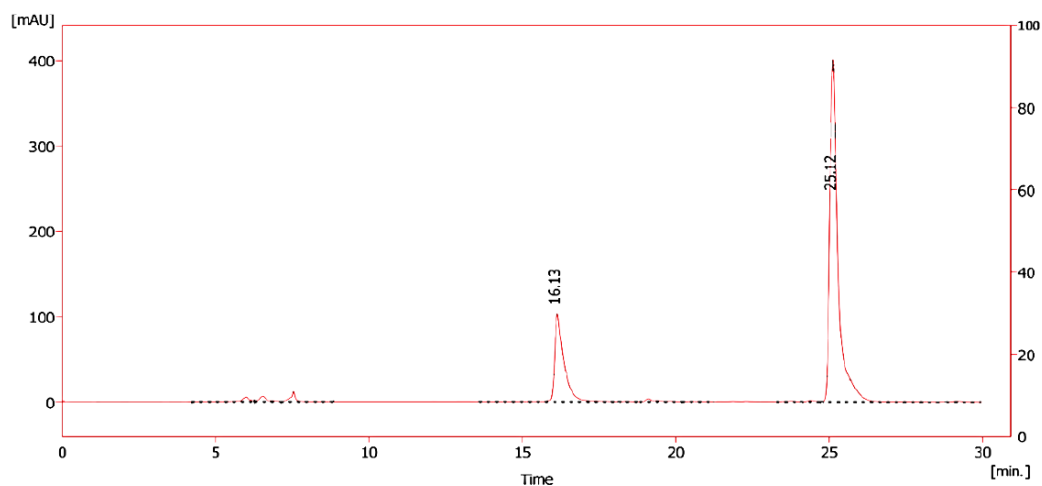


Figure 1. HPLC profile of desulfo-glucosinolates isolated from seeds of *Alyssum linifolium*. The peak in Rt of 25.12 min was identified as glucoiberverin according to NMR and mass spectrometry data

HPLC: High performance liquid chromatography, Rt: Retention time, NMR: Nuclear magnetic resonance

Antileishmanial activity

GSL fraction-treated promastigotes

The highest DR for the GSL fraction was found at a concentration of 75 $\mu\text{g/mL}$ (20%). The concentrations of 39 and 4.9 $\mu\text{g/mL}$ of amphotericin B showed the highest (73.8%) and lowest DR (38.4%), respectively. The highest DR for glucantime was 68.2% at a concentration of 93.500 mg/mL and the lowest was 24.5% at a concentration of 700 $\mu\text{g/mL}$. A significant difference ($p < 0.05$) was observed between the concentrations of the GSL fraction and standard drugs (amphotericin B and glucantime). The IC_{50} of amphotericin B and glucantime were calculated 8 and 95 $\mu\text{g/mL}$, respectively. This was determined 245 $\mu\text{g/mL}$ for the GSL fraction (Figure 4).

GSL fraction-treated amastigotes

The concentrations of 625 and 75 $\mu\text{g/mL}$ of GSL fraction showed the highest (59.3%) and lowest (31%) death rate, respectively. The highest DR for amphotericin B was 78% at

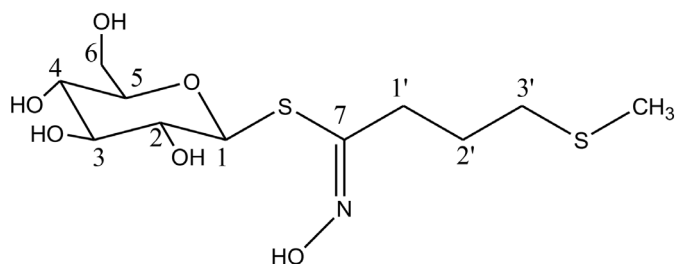


Figure 2. The structure of the purified desulfo-glucoiberverin

a concentration of 39 mg/mL and the lowest was 41.8% at a concentration of 4.9 $\mu\text{g/mL}$. For glucantime, the highest and lowest death rates were 70.2% (93.500 $\mu\text{g/mL}$) and 26.8% (700 $\mu\text{g/mL}$), respectively. No significant difference was observed between different concentrations of the GSL fraction and standard drugs ($p > 0.05$). IC_{50} was also 5.5 $\mu\text{g/mL}$ for amphotericin B, 165 $\mu\text{g/mL}$ for glucantime and 250 $\mu\text{g/mL}$ for GSL fraction (Figure 5).

Cytotoxicity test and selectivity index

Cytotoxic concentration 50 (CC_{50}) and SI were 105 $\mu\text{g/mL}$ and 19.09 for amphotericin B, 2650 $\mu\text{g/mL}$, and 16.06 for glucantime, and 3950 $\mu\text{g/mL}$ and 15.8 for the GSL fraction, respectively.

DISCUSSION

This study reports the isolation of the main GSL constituent from *A. linifolium* seeds by ion-exchange and reversed-phase chromatography and identification of the isolated compound using EI-MS and NMR spectra for the first time. The major constituent of the GSL fraction was identified as glucoiberverin, a methyl thioglucosinolate, which was previously identified in some other *Alyssum* species such as *A. sibiricum* (as the main GSL constituents) and *A. peltarioides*.²⁴ NMR and MS data of the isolated compound were confirmed with the published spectroscopic studies.^{25,26} A survey of the literature revealed that the methylthio and methylsulphinyl GSLs are found to be the common constituents in *Alyssum* species^{27,28} and the present results agree with these findings. The chemical characterization of the volatile fraction from *A.*

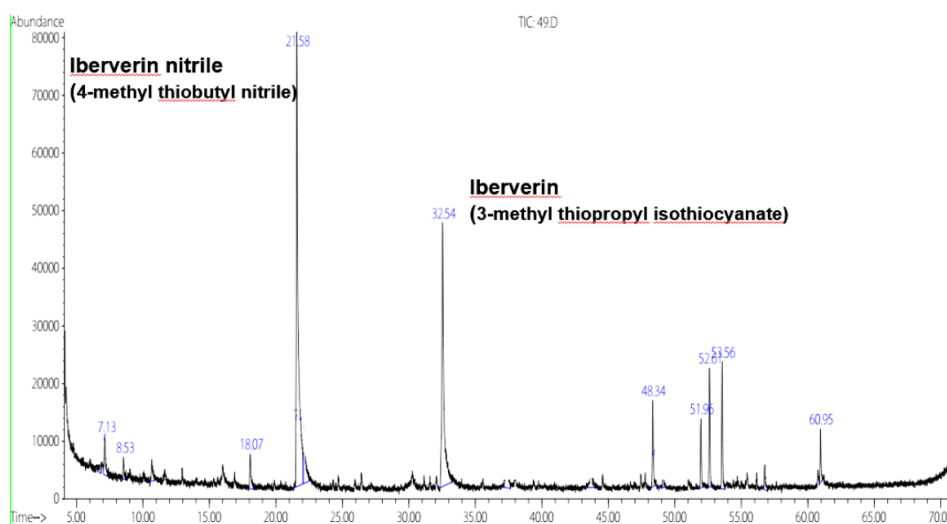


Figure 3. GC chromatogram of the volatile fraction from *Alyssum linifolium* seeds

GC: Gas chromatography

Table 1. *Alyssum linifolium* seed volatiles identified from GC-MS analysis

Compounds	R_t (min)	KI	Area (%)	MS spectral data (m/z)
4- Methyl thiobutyl nitrile	21.58	1128	62.91	115 (M^+), 74, 68, 61 (bp)
3- Methyl thiopropyl isothiocyanate	32.54	1359	14.01	147 (M^+), 101 (bp), 72, 61, 41

R_t : Retention time, KI: Kovats index, GC-MS: Gas chromatography-mass spectrometry, bp: Is indicated as the base peak (100%)

linifolium seeds showed 4-methyl thiobutyl nitrile (iberberin nitrile, 62.91%) and 3-methyl thiopropyl isothiocyanate (iberberin, 14.01%) that are normally the hydrolysis products of glucoiberberin.²⁹

The GSL fraction from *A. linifolium* seeds exhibited a considerable inhibitory effect against *L. major* promastigotes (45.7%) with a significant SI value of 15.8 compared to amphotericin B (19.09). Literature surveys report the anti-parasitic properties of Brassicaceae plants and their GSL constituents.^{30,31} Based on Montazeri et al.⁸, the hydroalcoholic extracts of some Brassicaceae plants, including *Alyssum homalocarpum*, provided considerable *in vitro* anti-toxoplasma activities. Spectrophotometric determination showed GSLs (24.37 mmol/kg dry weight) in *A. homalocarpum* seeds. Calzada et al.¹³ reported that the benzyl GSL isolated from *Lepidium virginicum* (Brassicaceae) root, possessed significant anti-

amoebic activity against *Entamoeba histolytica* trophozoites (IC₅₀: 100.1 µg/mL).¹³

The *in vitro* nematocidal activity of some GSL constituents from seeds of Brassicaceae plants was evaluated by Lazzeri et al.¹² The hydrolysis products of GSLs such as sinigrin, gluconapin, and glucotropaeolin showed lethal effects on the larval stage of *H. schachtii*.

Miltefosine, the first oral treatment of leishmaniasis, induces cell death in parasites by releasing Cyt c from mitochondria.³² Recent investigations showed that GSLs and their hydrolysis products (isothiocyanates, ITCs) induce apoptosis, mitochondrial release of Cyt c and induce the activation of different cellular proteases such as caspases upon apoptotic stimuli.³³ Therefore, it is hypothesized that GSLs and their hydrolysis products may cause the parasite death through the induction of apoptosis, and this issue needs further investigation.

Infection of cells with *Leishmania donovani* parasites leads to an increase in anti-apoptotic Bcl-2 expression, therefore, the Bcl-2 inhibition results in eliminating of the parasites.³⁴ Based on Aghaei et al.³⁵, downregulation of anti-apoptotic Bcl-2 protein in macrophages infected with *Leishmania* parasite, promotes the apoptosis process of infected-macrophages through the changes in the mitochondrial membrane and releasing Cyt c, resulting in the parasite death. The Bcl-2 expression is also altered by aliphatic GSLs such as sulforaphane through the downregulation of anti-apoptotic Bcl-2 proteins.³⁶ Therefore, this could be considered a potential mechanism of the parasite death induced by GSLs.

Different *Leishmania* species increase the activation of NF-κB in the host cell, resulting in the survival of the parasite and increasing the intracellular parasite burden. Therefore, NF-κB may be regarded as a target for treating leishmaniasis.³⁷ It has been reported that ITCs of Brassicaceae plants can inhibit NF-κB-mediated inflammatory process³⁸ and this could be considered a potential antileishmanial effect of *A. linifolium*.

The cell division cycle (CDC) proteins and mitogen-activated protein kinases (MAPK) play important roles in CDC and apoptosis.³⁹ Based on the recent investigation, the CDC2-related kinase (*CRK3*) genes are involved in the life cycles (promastigote and amastigote stage) of *Leishmania* parasites. It appears that *CRK3* is active in the G2 cell cycle of parasites and regulates the mitosis.⁴⁰ It was reported that benzyl isothiocyanate (BITC) and phenethyl ITC decrease the cyclin-dependent kinase 1, CDC2 and CDC25C proteins and suppress G2/M phases to induce apoptosis.^{39,41} Therefore, the *CRK3* inhibitor could be considered a novel antileishmanial drug.

The involvement of ITCs has been demonstrated in the activation of MAPK protein kinase and induction of apoptosis. Moreover, BITC induce the activation of two important MAPK signaling pathways, JNK and p38 MAPK, and promote apoptosis.⁵ Researchers observed that these two signaling pathways are involved in *Leishmania* disease.⁴² Moreover, several types of MAPK are involved in the life stages of *Leishmania* parasites.⁴³ Therefore, ITCs and BITC can be considered the effective antileishmanial compounds.

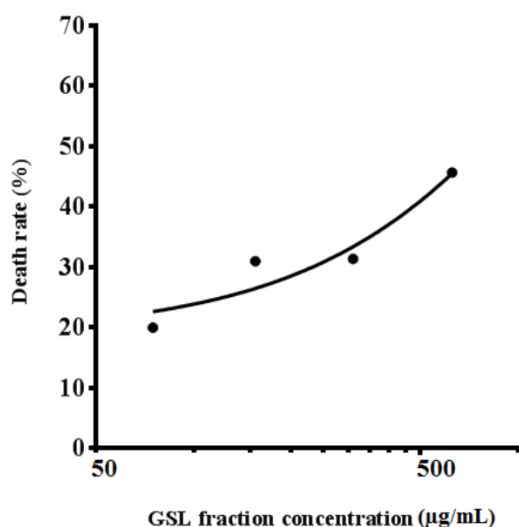


Figure 4. Treated promastigotes with different concentrations of GSL fraction from *Alyssum linifolium* seeds. The resulted IC₅₀ was 245 µg/mL
GSL: Glucosinolate

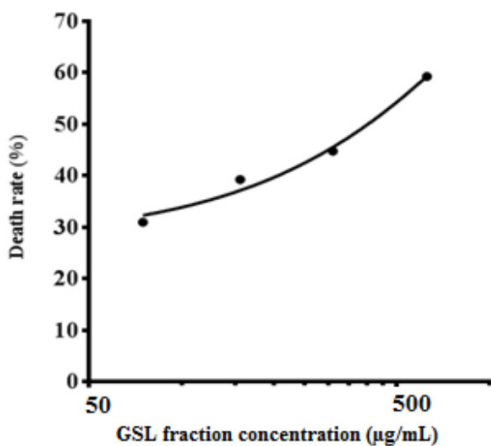


Figure 5. Treated amastigotes with different concentrations of GSL fraction from *Alyssum linifolium* seeds. The resulted IC₅₀ was 250 µg/mL
GSL: Glucosinolate

Based on the present results, it seems that GSL fraction from *A. linifolium* seeds might affect the *L. major* parasites through the above pathways.

CONCLUSION

Based on our findings, the GSL fraction from *A. linifolium* seeds have antileishmanial activity against *L. major*. Glucoiberberin was identified as the main GSL constituent of *A. linifolium* seeds. Due to anti-parasitic activities of GSLs, these compounds could be considered promising candidates for developing new antileishmanial drugs. Consequently, it needs more investigations on the inhibitory effects of GSLs such as glucoiberberin against *L. major* in animal models and more studies should be performed to identify the molecular pathways related to the efficacy of these compounds on *Leishmania* parasite.

Ethics

Ethics Committee Approval: IR.MAZUMS.REC.1397.297 and IR.MAZUMS.REC.1398.1069.

Informed Consent: Not applicable.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: S.S., Design: S.S., M.F., Data Collection or Processing: F.M., R.F., H.K., Analysis or Interpretation: S.S., M.F., Literature Search: F.M., R.F., Writing: F.M., R.F., S.S., H.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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