Butanol Fraction of *Kelussia odoratissima* Mozaff Inhibits the Growth of *Leishmania major* Promastigote and Amastigote

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ABSTRACT

Naturally derived compounds have been used to treat several infectious diseases including leishmaniasis. The study aimed to investigate the *in vitro* effects of *Kelussia odoratissima* Mozaff extract on *Leishmania major* promastigote and amastigote. Dried leaves of *K. odoratissima* were fractionated by 3 solvents including aqueous, butanol, and ethyl acetate. The results showed that the butanol fraction of *K. odoratissima* showed the highest anti-*Leishmania* effects against *L. major* promastigotes. Ninety four percent growth inhibition of the promastigote was observed when cells were treated with the 1,280 μg/mL butanol fractions. Moreover, 100% inhibition of amastigotes was detected after treatment with the butanol fraction. Half maximal inhibitory concentration (IC_{50}) of the butanol fraction in promastigotes and amastigotes was 264.1 and 154.1 μg/mL, respectively. The obtained results suggested the potential medicinal benefits of *K. odoratissima* butanol fraction as an alternative treatment for leishmaniasis caused by *L. major* infections.

Key words: Amastigotes, Butanol fraction, *Leishmania major*, *Kelussia odoratissima* Mozaff, Promastigotes

INTRODUCTION

Leishmaniasis is a group of infectious diseases caused by intracellular protozoa parasites of the genus *Leishmania* (Murray et al., 2005). Nowadays, leishmaniasis is prevalent in 98 countries, with up to 350 million people at risk of infection and an estimated 1.5 to 2 million new cases occurring annually (McGwire and Satoskar, 2014). Clinical manifestations of leishmaniasis include mainly cutaneous (CL), mucocutaneous (MCL) and visceral (VL) forms, with symptoms ranging from skin and mucosal to systemic ulceration which, if untreated, can lead to death. Cutaneous leishmaniasis (CL) is caused by *Leishmania major*, *L. tropica*, and *L. aethiopica*. In addition, the infection is usually limited to the skin and lymphatic system (Murray et al., 2005). A recent review focuses on the immune response displayed against cutaneous leishmaniasis, the most common zoonotic infection (Gabriel et al., 2019). *L. major* can infect the immune cells, including macrophages and dendritic cells. The organism has two infective stages of the life cycle, including promastigote and amastigote. Interestingly, the amastigote can survive within the phagosome of macrophages (Murray et al., 2005).

Pentavalent antimony compounds, such as meglumine antimoniate (glucantime ®), are being used as a first-line treatment for leishmaniasis (Oliveira et al., 2011). The use of these medications poses a health risk due to their intrinsic toxicity (Sundar and Chakravarty, 2010), especially when systemic therapy should be used for cutaneous leishmaniasis (Palumbo, 2009). A recent study interestingly reported the alarmingly increasing frequency of drug resistance to antimonial compounds in the treatment of *Leishmania* infections (Aït-Oudhia et al., 2011). Therefore, there are ongoing studies to develop and evaluate new medications for the treatment of leishmaniasis (Monge-Maillo and Lopez-Velez, 2013; Serakta et al., 2013).

In an attempt to overcome *Leishmania*-resistant antibiotics, medicinal plants have been used as alternative agents against *Leishmania* infection (Sen and Chatterjee, 2011). *Kelussia odoratissima* Mozzaf (wild celery; mountain celery) is a medicinal herb that belongs to the *Umbelliferae* family. This plant grows at high altitudes (more than 2000 meters above sea level). This self-growing plant, growing up to 50-200 cm, is found exclusively in Iran. In spring, the plant is

harvested from its habitat and presented on the market (Mahmoudi et al., 2014). The essential oil of *K. odoratissima* consists of bioactive components such as coumarins, flavonoids, and phthalides (Ahmadipour et al., 2015). *Kelussia* is one of the most valuable plants used in Iran, and it is traditionally consumed as a medicinal plant to treat analgesic, inflammatory, sedative, cough, and ulcer effects (Ahmadi et al., 2007). This medicinal herb shows the potential to prevent pulmonary hypertension (Ahmadipour et al., 2015). Recent findings confirm that flavonoid and phthalide compounds, as a major part of the plant, have anti-inflammatory, anti-viral, anti-diabetic, anti-cancer, and anti-toxin effects (Salehi et al., 2019). Limited studies have been done to test *K. odoratissima* extracts against *Leishmania* spp.

The aim of this study was therefore to determine *in vitro* anti-*Leishmania* activity of *K. odoratissima* fractions, including aqueous, ethyl acetate, and butanol fractions on promastigote and amastigote of *L. major*.

MATERIALS AND METHODS

Plant sample
The plant sample was purchased in dried form from the local grocery store, Shiraz, Iran in April 2015. It was then dried and stored in the freezer at -18°C. The sample was powdered in a coffee grinder prior to the experiment.

Preparation of crude extracts
To prepare crude extracts of *K. odoratissima*, 1000 g of dried leaves was powdered. The extraction was performed by the percolation method using 95% ethanol with a flow rate of 1 mL/minute for 10 days. After filtration, the solvent was removed using a rotary evaporator. The remaining semi-solid material was then freeze-dried and placed into a sterile glass and stored at -20°C for further use (Dong et al., 2015).

Chlorophyll and oil elimination
The removal of chlorophyll and oil from the total extract was carried out using medium pressure liquid chromatography (MPLC) system, bypassing the sample dissolved in 65% ethanol through a reverse-phase column (RP-18) (Reid and Sarker, 2012). Subsequently, the total extract was concentrated by a rotary evaporator.

Fractionation of the total extract
As a downstream process, the prepared extract was fractionated based on the polarity of its components. Due to high polarity, proteins and carbohydrates were dissolved in the aqueous phase. Moderately polarity flavonoid, glycosides, and saponins dissolved in the butanol phase, and the low polarity terpenoids, phenolic compounds and ketones remained in the ethyl acetate phase. Accordingly, three fractions, namely aqueous, ethyl acetate, and butanol fractions were prepared from the total extract. Fractionation was performed using the liquid-liquid extraction (LLE) method (Tang et al., 2012). Ethyl acetate (100 mL) and distilled water (100 mL) were equally added to 20 g of the total extract. After stirring, the mixture was transferred to a decanter in which two phases formed and the ethyl acetate was separated from the aqueous phase. Once again, 100 mL of ethyl acetate was added to the aqueous phase and then separated. In the next step, 100 mL of butanol was added to the remaining aqueous phase in the decanter. The mixture was well-stirred and the butanol phase separated from the aqueous phase. The last process was repeated as well. Finally, the three extracts were dried and refrigerated until use (Tang et al., 2012).

Parasite and cell culture
The standard strain of *Leishmania major* promastigotes (MRHO/IR/75/ER) was provided by the Department of Parasitology, Isfahan University of Medical Sciences, Isfahan, Iran. The parasite was maintained *in vitro* by repeated subculture in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin, 100 IU/mL penicillin at 24°C. Murine macrophage cells (J774-A1) were obtained from Pasteur Institute, Tehran, Iran. Cells were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 10% FCS, 100 μg/mL streptomycin, and 100 IU/mL penicillin at 37°C under 5% CO_2 (Gupta et al., 2005).

In vitro Anti-*Leishmania* Activity
Anti-Promastigote Assay
Approximately 1×10^6 promastigotes, which were in their early stationary phase, were incubated at 24°C. A separate stock solution was provided for each of the three fractions: aqueous, butanol and ethyl acetate. These extracts were diluted with dimethyl sulfoxide (DMSO) and transferred to Eppendorf tubes to reach final concentrations of 40, 80, 160, 320, 640, 1000 and 1280 μg/mL. Moreover, the following groups were considered for each fraction: a positive control with amphotericin Band the parasite at a concentration of 1 μg/mL, a negative control (culture medium with promastigotes and DMSO), and a blank (culture medium). After 24, 48, 72 hours of incubation, promastigotes detached from each tube and the final number of viable parasites (with 0.4% of viable trypan blue considered) was counted with a hemocytometer. The experiments were repeated trice and performed twice for each experiment.

Anti-Amastigote Assay

For this purpose, at first 2 cm^2 cover slips were placed in the wells of 6-chamber slides (Lab-Tek, Nalge Nunc International, NY, USA). In the final step, 200 µL of macrophage cells (J774-A1) (10^5/mL) were added to each well and incubated at 37°C in 5% CO_2 for 2 hours. Then, 200 µL (10^6/mL) of promastigotes were added to the murine macrophages (10:1) and incubated for 24 hours. Free parasites were removed by washing the wells with RPMI-1640 medium and the infected macrophages treated with 50 µL of various concentrations (40-1280 µg/mL) of aqueous extract at 37°C in 5% CO_2 for 48 hours (Gupta et al. 2005). Finally, Giemsa stained methanol-fixed dried slides were studied under a light microscope (Olympus BX41TF, New York, USA). Moreover, macrophages containing amastigotes without extracts and parasites and macrophages with extracts and parasites were considered as positive and negative controls, respectively. Macrophages were infected with the amastigote cells. The infected macrophages were treated with different concentrations of each fraction and incubated for 48 hours. The inhibitory activity was investigated and compared with the control. Anti-*Leishmania* activity of the three extracts were measured by the amastigote count in every 100 macrophages examined and comparing them with those obtained in the positive control. Each experiment was performed triplicate, with two replicates for each. Moreover, two other extract fractions (ethyl acetate and butanol) of *K. odoratissima M.* were used for this study according to the method mentioned above.

Statistical analysis

The IC$_{50}$ was calculated and the graphs plotted using SigmaPlot™13 (Systat Software Inc, CA, USA). The percentage of growth inhibition (% GI) used for the calculated IC$_{50}$, was obtained with respect to growth control as follows (Tunc et al., 2015).

Growth inhibition (%) = [1-(Growth $_{treatment}$ / Growth $_{control}$)] × 100

RESULTS AND DISCUSSION

The anti-*Leishmania* activity of 3 fractions including aqueous, ethyl acetate and butanol of *K. odoratissima* against *L. major* promastigote and amastigote was determined. It was observed that the 3 fractions significantly inhibited the growth of *L. major* in a concentration and time-dependent manner (P<0.001). Growth inhibition of 94% and 69% of promastigotes was inhibited when cells were treated with the fractions for 72 hours, respectively. The butanol fraction of *K. odoratissima* showed the highest anti-*Leishmania* effects against *L. major* promastigotes (figure 1C), compared to the aqueous fraction (figure 1A) and the ethyl acetate fraction (Figure 1B). Growth inhibition of 94% and 69% of promastigotes was found to be inhibited when cells were treated with the 1,280 µg/mL butanol and ethyl acetate fractions for 72 hours, respectively. However, the aqueous fraction slightly suppressed the growth of *L. major*.
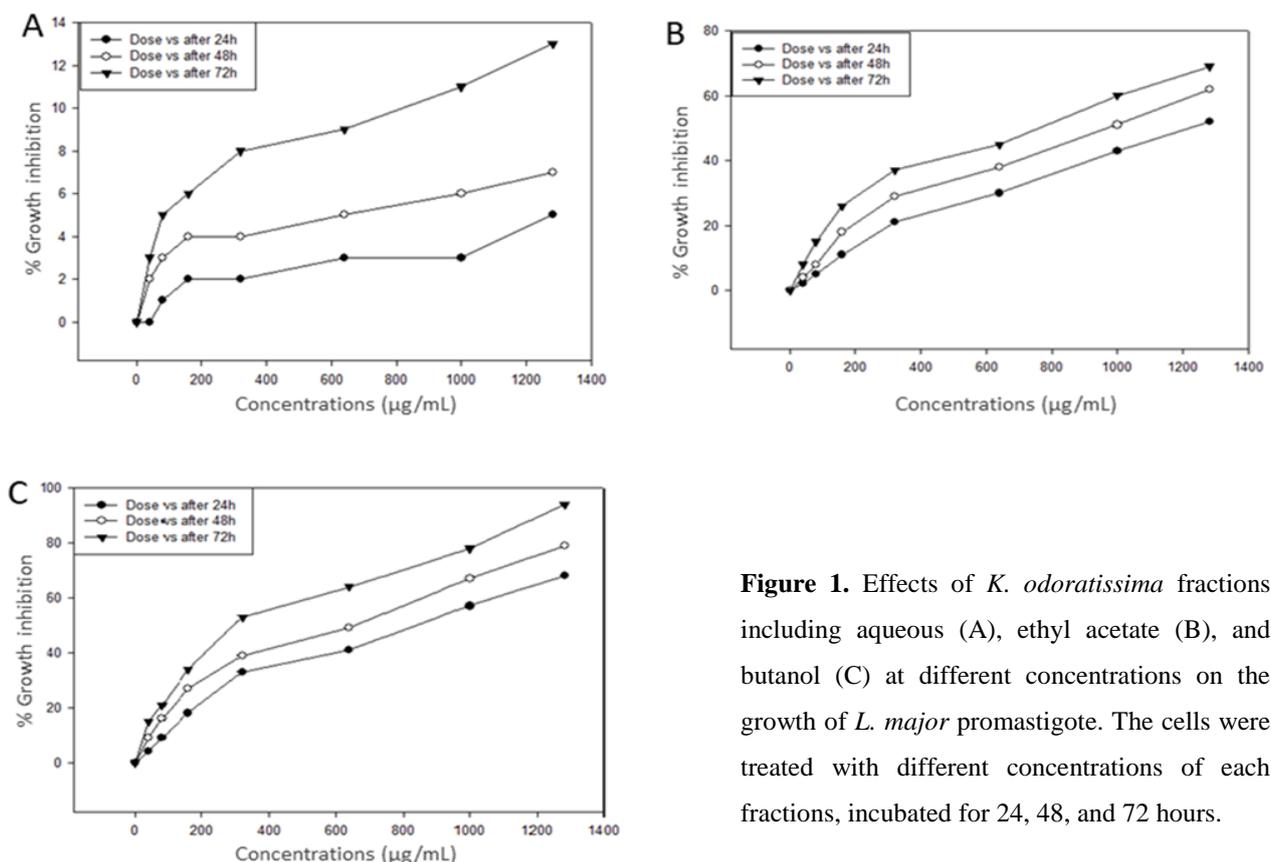

Figure 1. Effects of *K. odoratissima* fractions including aqueous (A), ethyl acetate (B), and butanol (C) at different concentrations on the growth of *L. major* promastigote. The cells were treated with different concentrations of each fractions, incubated for 24, 48, and 72 hours.

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At 72 hours, half-maximal inhibitory concentration (IC_{50}) of the ethyl acetate and butanol fractions on the promastigotes was 341.56 and 264.1 µg/mL (table 1), respectively. However, the IC_{50} of aqueous extract against promastigotes could not be calculated due to the percentage inhibition of growth that was remarkably low for 72 hours incubation.

The butanol fraction of plant species demonstrated the highest anti-*Leishmania* effects against *L. major* amastigotes, followed by ethyl acetate and aqueous fraction. At 48 hours, a 100% inhibition of amastigote cell growth was detected after the treatment with the 1,280 µg/mL butanol compared to the control (Figure 2). In addition, 89% and 51% inhibition of amastigote growth were inhibited when cells were treated with ethyl acetate and aqueous fractions at 1280 µg/mL for 48 hours, respectively. As shown in table 2, the IC_{50} of the aqueous, ethyl acetate, and the butanol fractions on the amastigotes were 5.5.8, 221.7, and 154.1 µg/mL, respectively.

Table 1. IC_{50} of different *K. odoratissima* fractions against *L. major* promastigote

Time	IC_{50} (µg/mL) with 95% confidence		
	Aqueous	Ethyl acetate	Butanol
After 24 hours	-	530.68	449.75
After 48 hours	-	444.33	418.94
After 72 hours	-	341.56	264.1

Table 2. IC_{50} of different *K. odoratissima* fractions against *L. major* amastigote

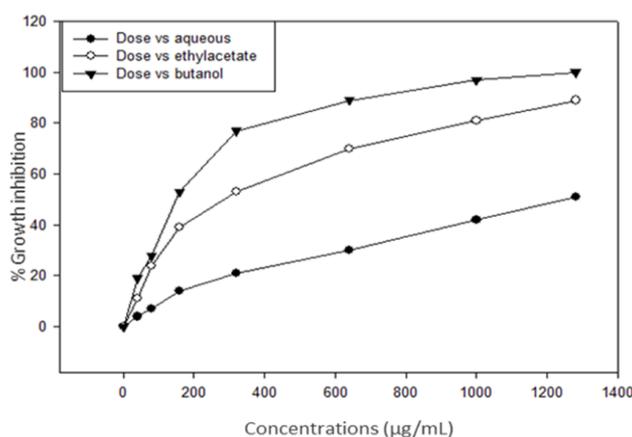| Time	IC_{50} (µg/mL) with 95% confidence		
	Aqueous	Ethyl acetate	Butanol
After 48 hours	515.8	221.7	154.1

Figure 2. Effects of *K. odoratissima* fractions including aqueous, ethyl acetate, and butanol at different concentrations on the growth of *L. major* amastigote.

Leishmaniasis is one of the most dangerous neglected parasitic diseases, not currently known an effective drug or vaccine to ensure effective immunity against this scourge (Ghorbani et al., 2018). Traditional evidence has been indicated the use of natural plant products may be useful for specific medical conditions (Ghorbani et al., 2018). Historically, many oils and plant extracts have been used for control bacterial, fungal and parasitic diseases (Akthar et al., 2014).

In the present study, the anti-*Leishmania* effect of different fractions of *Kelussia odoratissima* Mozaff on *Leishmania major* was investigated. The butanol fraction was found to have the highest anti-*Leishmania* activity against promastigotes and amastigotes, followed by the ethyl acetate fraction. However, no clear anti-*Leishmania* effects of the aqueous fraction on *L. major* promastigote were observed during the incubation periods. It indicates that the aqueous fraction may not be effective as anti-*Leishmania* compounds. Many studies indicate that several metabolites such as flavonoids, saponins, alkaloids, and some other components in plant extracts have potent anti-*Leishmania* effects (Tasdemir et al., 2006; Shah et al., 2014). Flavonoid compounds have been reported to have antibacterial, antiviral, -larval, -inflammatory, -toxin, -oxidant, and -stress properties. These compounds have also been reported on the treatment properties of diabetes mellitus, ulcerative colitis, cancer, hyperlipidemia, and tranquilizer (Ahmadi et al., 2007). Anti-bacterial and antiviral activities of *K. odoratissima* mediated by flavonoids and phthalides in this medicinal plant were reported (Surai, 2014).

In a study, 105 flavonoid components reported a strong anti-*Leishmania* (Tasdemir et al., 2006). While, another study has shown that Propolis has an inhibitory effect on parasite proliferation (Duran et al., 2008), and this property may be due to the presence of large amounts of flavonoids (Silva et al., 2008). Flavonoid is considered the main chemical compound of the plant (Ahmadi et al., 2007) and its anti-*Leishmania* effect may be related to the flavonoid compound. In the literature review, it can be inferred that many plants have moderate to strong activity against *Leishmania* spp. by different mechanisms of action (Rodrigues et al., 2014). Another study also revealed that plant

extracts can also be used as adjuvants in vaccination against *Leishmania* spp. (Kaur et al., 2014). Recently, secondary metabolites from *Cyathus cf. crassimurus* have been reported anti-*Leishmania* activity against both promastigote and amastigote cells of *L. mexicana* (Mbekeani et al., 2019). Interestingly, *Urtica dioica* extract demonstrated *in vivo* anti-*Leishmania* activity against zoonotic cutaneous leishmaniasis in BALB/c mice. It has been reported that Th1 cells were activated throughout *U. dioica* treatment resulting in cytokine activation (Alireza et al., 2020). Therefore, the anti-*Leishmania* effect of this plant, together with its tissue repair effect, made it a promising candidate for the treatment of cutaneous leishmaniasis caused by *L. major*.

CONCLUSION

The present study shows the potent anti-*Leishmania* effects of *K. odoratissima* on *L. major* promastigote and amastigote. The butanol fraction of *K. odoratissima* showed the highest anti-*Leishmania* effects against both *L. major* promastigotes and amastigotes. The present findings highlight that 100% inhibition of *L. major* amastigotes was detected after treatment with the butanol fraction. Hence, it suggests the potential medicinal benefits of *K. odoratissima* butanol fraction as an alternative treatment of leishmaniasis caused by *L. major* infections. The *in vitro* examinations are in the preliminary step to prove the efficacy and safety of medicinal plants and a comprehensive study involving HPLC, metabolomics, and nanotechnology is recommended to further validate these potent compounds for application in the treatment of some diseases such as leishmaniasis.

DECLARATIONS

Competing interests
The authors have not declared any conflict of interest.

Author`s contribution

Farzaneh Mirzaei, Mohammadali Nilforoushzadeh, and Amir Maleksabet designed the study. Mohsen Hosseini supervised the laboratory procedures. Farzaneh Mirzaei and Sayed Hossein Hejazi performed the experiments. Manuscript preparation and the statistical analysis were conducted by Roghayeh Norouzi, Abolghasem Siyadatpanah, Watcharapong Mitsuwan, Veeranoot Nissapatorn and Maria de Lourdes Pereira. All authors have read and approved the final manuscript.

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