Research Journal of Pharmacognosy (RJP) 1(4), 2014: 27-33
Received: June 2014
Accepted: July 2014

Original article

Chemical composition and leishmanicidal activity of *Pulicaria gnaphalodes* essential oil

G. Asghari[1*], F. Zahabi[2], A. Eskandarian[3], H. Yousefi[3], M. Asghari[4]

[1]*Department of Pharmacognosy, Faculty of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran.*
[2]*Isfahan Pharmaceutical Sciences Research Center, Isfahan University of Medical Sciences, Isfahan, Iran.*
[3]*Department of Parasitology, faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.*
[4]*Department of Biotechnology, College of Agriculture & Natural Resources,University of Tehran, Tehran, Iran.*

Abstract

Background and objectives: Several natural compounds have been identified for the treatment of leishmaniasis. Due to a few safe drugs and the side effects caused by available chemotherapy, some new drugs for treatment of leishmaniasis are requested. The genus *Pulicaria* (Asteraceae*)* is represented in the flora of Iran by five species. Phytochemical studies on *Pulicaria* species have revealed some flavonoids and terpenoids with leishmanicidal activity. In the present investigation chemical composition and leishmanicidal activity of *Pulicaria gnaphalodes* essential oil have been studied. **Methods**: The essential oil of the aerial parts of the plant was obtained by Clevenger apparatus and was analyzed by GC/MS. Antileishmanil activity was assessed against promastigoes of *Leishmania major*. **Results**: The major components from *P. gnaphalodes* essential oil have been reported to be geraniol, 1,8-cineole, chrysanthenone, α-pinene, chrystanthenone, α-terpineol and filifolone. The alcohol monoterpenes with contribution of 25.04% constituted the major portion of the essential oil, while hydrocarbon monoterpenes and hydrocarbon sesquiterpenes with contribution of 7.08% and 2.38%, respectively occupied the next rates. In the present experiment the essential oil of *P. gnaphalodes* progressively inhibited *Leishmania major* growth in concentrations ranging from 0.125 to 50 µL/mL (parasite culture) in 24 h. The essential oil at 50 µL/mL eliminated the promastigotes at the beginning of treatment. It showed antileishmanial activity in concentration of 1.06 µL/mL and destroyed all parasits in 24 h. **Conclusion**: *Pulicaria gnaphalodes* antileishmanial activity, could suggest the species and constituents as possible lead structures for antileishmanial drug discovery.

Keywords*:* essential oil, GC/MS, *Leishmania major, Pulicaria gnaphalodes*

Introduction

The family Asteraceae (Compositae) includes about 200 genera and 2000 species, out of which, the genus *Pulicaria* is widely distributed in Asia, Europe, and Africa [1]. Five species of the genus grow in Iran which includes *P. dysenterica* (L.) Bernh, *P. arabica* L. Cass, *P. gnaphalodes*

[*]*Corresponding author: asghari@pharm.mui.ac.ir,* Tel/Fax: +9831-37922644

(Vent.) Boiss, *P. salvifolia* Bunge, and *P. vulgaris* Gaertn [2]. *P. gnaphalodes* is found in dry places and rocks. It has an erect stem which might be leafy throughout the whole length or only at the top with many branches [3]. *P.gnaphalodes* has been investigated extensively and phytochemical studies have indicated the presence of hardwickiic acid derivative [4], terpenes and terpene derivatives, new cadinene and bisabolane derivatives [5]. Several biological activities such as antioxidant [6] and insecticide properties [7] have been reported for *P. gnaphalodes*. Moreover, it has been traditionally used as anti-inflammatory and to treat severe heatstroke and diarrhea [6]. It has been found that the species has no anti-bacterial activity [8].

Leishmaniasis is considered as a major health problem, causing significant morbidity and mortality in Africa, Asia and the Latin America, with an estimated number of 500,000 new cases each year. Visceral leishmaniasis is still considered as one of the most severe infections by the World Health Organization [9].

The drugs recommended for the treatment of leishmaniasis include the pentavalent antimonials sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantime®), which constitute the first-line treatment. Pentavalent antimonials are potentially toxic and sometimes ineffective against leishmaniasis, and the second-line drugs, such as amphotericin B and pentamidine, may be even more toxic [10].

Many plants considered as current remedies have supplied modern medicine with some effective pharmaceutical agents to treat protozoan diseases [11]. It has been reported that *P. gnaphalodes* extract has a good leishmanicidal effect and it seems that the leishmanicidal activity has been mostly related to the terpenoid constituents of the plant [12]. The main object of the present study was to determine the chemical composition and the leishmanicidal activity of the essential oil of *P. gnaphalodes* against *Leishmania major* promastigotes.

Experimental

Plant material

Pulicaria gnaphalodes was collected from Tabas region, South Khorasan province, center of Iran in May 2012. The plant was identified by Majid Zargaran (botanist) and was deposited int the Herbarium of School of Pharmacy, Isfahan University of Medical Sciences, Isfahan, Iran (No: 2750).

Preparation of essential oil

Plant aerial parts including leaves, stems, and flowers were air-dried for 3 days in shade at 23-27 °C, then powdered mechanically using a blender (mesh No:10). One hundred and fifty gram of the plant aerial part powder was mixed with water (1:10) and was distillated for four hours. The essential oil was obtained using a Clevenger apparatus and was collected in screwed glass tubes and was stored in at 4 °C.

GC/MS analysis of the essential oils

The essential oils of *P. gnaphalodes* were analyzed by GC and GC/MS. Gas chromatography analysis was carried out on a Perkin-Elmer 8500 gas chromatograph with FID detector and a BP-1 capillary column (30 m × 0.25 mm; film thickness 0.25 μm). The carrier gas was helium with a flow rate of 2 mL/min, the oven temperature for the first 4 min was kept at 60 °C then it was increased at a rate of 4 °C/min until the temperature reached 280 °C. The injector and detector temperatures were set to 280 °C.

The mass spectra were recorded on a Hewlett Packard 6890 MS detector coupled with Hewlett Packard 6890 gas chromatograph equipped with HP-5MS capillary column (30 m × 0.25 mm; film thickness 0.25 μm). The gas chromatography condition was as mentioned previously. Mass spectrometer condition was as follows: ionized potential 70 eV and source temperature 200 °C. Identification was based on retention data and computer matching with the WILEY275.L library as well as comparison of

the electron-impact-mass spectra (EI-MS) with those relevant reference samples and the literature [13,14].

Parasite culture

The *in vitro* cultured form (promastigotes) of *Leishmania major* (MRHO/IR/75/ER) was cultured in Novy-MacNeal-Nicolle medium (NNN) and was then transferred to the enriched Roswell Park Memorial Institute medium (RPMI1640), (Sigma Alderich Germany) for mass reproduction. The parasites were kept in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin G (150 IU/mL) and streptomycin (150 µg/mL). A suspension of 10^6/mL *L. major* promastigotes in RPMI1640 was incubated at 22 °C and was sub-cultured after 72 h [15].

Evaluation of antipromastigote activity

There are different methods to evaluate the leishmanicidal activity of potential drugs. Most of them are based on viability, metabolic activity or proliferation assessment in promastigotes by microscopic observation [16].

The stock solution of the essential oil was diluted with culture medium to obtain serial dilutions as follow: 0.125, 0.25, 0.5, 1.06, 3.12, 6.25, 12.5, 25 and 50 µL/mL. For calculating The IC_{50} values (the concentration that is required to inhibit the growth of *L. major* promastigotes by 50%), 100 µL of the serial dilutions of the essential oil and 100 µL of promastigote suspension were added to 800 mL of RPMI-1640 medium and the final concentration of the essential oil was adjusted as mentioned above. The leishmanicidal effect of each essential oil concentration was determined by counting the number of dead parasites in a total number of 100 promastigotes (dead+alive) and then a measure was defined as the Death Rate as follow:

Death Rate= number of killed [promastigotes/Total (killed+Alive) promastigotes] × 100 [17]. The mortality of parasites was determined by counting with a Neubauer chamber at the beginning of the determined course time. For each bioassay, different concentrations were assessed in triplicate.

DMSO in RPMI 1.4% without essential oil was used as the negative control while amphotericin B (Cipla, India, 50 µg/mL) was used as the positive control.

The IC_{50} value was calculated using Graph analysis. The mean and standard deviation (SD) at nine independent groups were determined. Statistical analysis for comparison of different concentrations was performed with one-way ANOVA and $p \leq 0.05$ was considered as significant.

Results and Discussion

Twenty seven compounds identified in the essential oil. The retention time and their relative proportions have been listed in table 1. The main components from *P. gnaphalodes* essential oil were found to be geraniol, 1,8-cineole, chrysanthenone, α-pinene, chrystanthenone, α-terpineol and filifolone. The results of the leishmanicidal activity are shown in figure 1.

The essential oil of the aerial parts of *P. gnaphalodes* inhibited the growth of *L. major* promastigotes with IC_{50} values 1.37 and 1.47 µL/mL in 2 and 4 h treatment, respectively. Finally after 24 h, IC_{50} was 0.27 µL/mL.

The concentrations less than 1.06 µL/mL revealed significant inhibitory effects on promastigotes ($p \leq 0.05$) compared to other treatments. Amphotricin B as the positive control inhibited the growth of *L. major* promastigotes with IC_{50} of 2 µL/mL at 2 h. The negative control was performed using DMSO (1.4%) at the same concentration which was used in treatments. Tested concentration of DMSO did not affect the growth of parasites. Chemical analysis of the *P. gnaphalodes* essential oil revealed the constituents presented in table 1.

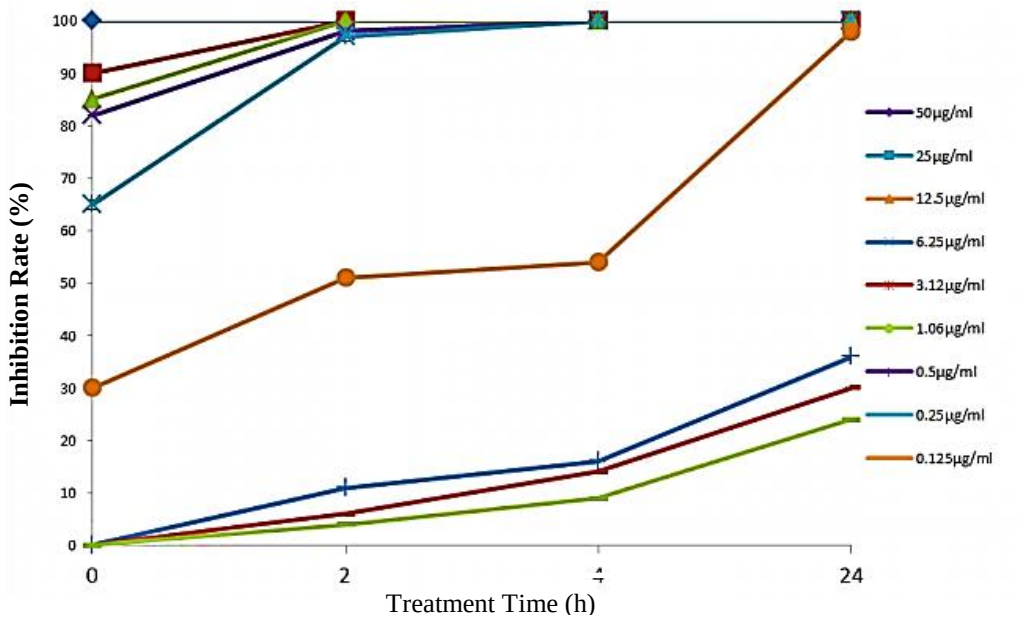

Figure 1. Leishmanicidal activity of *P.gnaphalodes* essential oils

The alcohol monoterpenes with contribution of 25.04%, constituted the major portion of the essential oil, then the more represented compound were hydrocarbon monoterpenes and hydrocarbon sesquiterpenes with contribution of 7.08% and 2.38%, respectively.

In previous analyses that were reported about *P. gnaphalodes* essential oil by Peter Weyersthal and Rustaiyan, some cadinene derivatives were reported [18]. Also other researchers have demonstrated a dimmer of sesquiterpene (gnapholide), along with another known dimmer of the same structure [19]. In addition, literature survey has shown the presence of flavonoids, sesquiterpenoid, diterpenoids and sesquiterpenoid lactones which have been biologically active [3]. A study about the composition of *P. gnaphalodes* essential oil has introduced chrysanthenyl acetate (22.38%), 2L-4L-dihydroxy eicosane (18.5%), verbenol (16.59%), dehydroaromadendrene (12.54%), β-pinen (6.43%), and 1,8-cineol (5.6%) as the major constituents [20]. It seems that the site of plant collection and different geographical locations might influence the composition of the essential oil. As presented in figure 1, the results of the present study have revealed the biological activity of *P. gnaphalodes* against *L. major* which has killed all promastigotes in the time of facing promptly which is highlighted in the text. This could be due to the presence of components such as alpha-pinene, borneol, 1,8-cineole, p-cymene, geraniol and thymol in the essential oils which have already exhibited lishmanicidal activity. It might be concluded that many chemicals available in the essential oil of the species could be partially responsible for leishmanicidal actvity.

It has been previously reported that *Thymus capitellatus* containing alpha-pinene, borneol, 1,8-cineole, *p*-cymene, geraniol and thymol has exhibited anti-leishmania activity [21]. Also chemical compositions like eugenol obtained from *Ocimum gratissimum* have shown activity against *Leishmania* [22]. In another study on leishmaniacidal activity of *Thymus vulgaris* and *Melaleuca alternifolia* essential oil, similar mono- and sesquiterpenes have been found in *P. gnaphalodes* which were responsible for *Leishmania major* mortality [23]. These findings are in accordance with our results about the

Table 1. Chemical composition of the essential oil of *P. gnaphalodes*.

Compounds	Rt (min)	KI	[%]
Hydrocarbon monoterpenes			
thujene	3.68	0930	0.13
α-pinene	3.79	0939	3.81
β-pinene	4.62	0979	0.21
α-terpinene	5.46	1017	0.91
p-cymene	5.65	1026	0.45
δ- terpinen	6.54	1060	1.51
Bicyclic hydrocarbon keton			
Filifolone	7.76	1082	2.38
Monoterpene keton			
Chrysanthenone	7.98	1128	3.41
alcoholic monoterpene			
1,8-cineole	5.83	1031	9.45
1-terpineol	8.78	1134	0.48
terpinen-4-ol	9.99	1177	3.66
α-terpineol	10.42	1189	3.63
myrtenol	10.58	1196	1.9
trans-carveol	11.25	1217	0.46
trans-geraniol	11.61	1230	3.53
geraniol	12.43	1253	1.93
Aldehyde monoterpene			
neral	11.94	1238	1.13
geranial	12.88	1267	1.32
Phenyl propan			
Thymol	13.57	1290	1.64
Ester monoterpene			
neryl-acetate	15.76	1362	0.42
geranyl-acetate	16.35	1381	0.21
oxygenated monoterpene			
cis-sabinen–hydrate	6.76	1070	0.16
methyl eugenol	17.00	1404	0.46
hydrocarbon sesquiterpene			
α-amorphen	19.10	1485	0.28
δ-cadinene	19.60	1512	1.22
Δ-cadinene	20.51	1523	1.23
α-cadinene	24.77	1539	1.87
calamenene	25.22	1540	0.24

lishmanicidal activity of *P. gnaphalodes.*

The low density of the plant essential oil and the rapid diffusion across cell membranes could be enhanced by targeting the active components of the oils to endoparasites [24], therefore it seems that essential oil of *P. gnaphalodes* has the potential for topical application in patients with cutaneus leishmaniasis. Figure 1 shows the output that indicates the concentrations less than 1.06 µL/mL revealed significant inhibitory effects on promastigotes ($p \leq 0.05$) compared to other treatments.

The presented results could provide new perspectives on drug development against leishmaniasis, since the extract of *P. gnaphalodes* has shown to be a potent leishmanicidal agent. Most studies about detection of plant secondary metabolites with leishmanicidal activity have been done using the promastigote form of the parasite because of its easier maintenance under *in vitro* conditions. However, since the promastigote is not the infective form of the parasite in hosts, these evaluations have only a suggestive value of the possible leishmanicidal activity of the compound tested [25]. Further studies would therefore be needed to evaluate the *in vivo* and clinical responses and also the associated toxicities, however, the species can be a potential source of antiprotozoa compounds for pharmaceutical researches.

Acknowledgements

The authors would like to acknowledge the financial support by the Isfahan University of Medical Sciences.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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