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ORIGINAL PAPER

Assessment of the antiprotozoal activity of *Pulicaria inuloides* extracts, an Algerian medicinal plant: leishmanicidal bioguided fractionation

Hamza Fadel[1] · Ines Sifaoui[2,3] · Atteneri López-Arencibia[2] · María Reyes-Batlle[2] · Soumaya Hajaji[4] · Olfa Chiboub[2,3] · Ignacio A. Jiménez[5] · Isabel L. Bazzocchi[5] · Jacob Lorenzo-Morales[2] · Samir Benayache[1] · José E. Piñero[2]

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Abstract

The lack of an effective chemotherapy for treatment of protozoan disease urges a wide investigation for active compounds, and plant-derived compounds continue to provide key leads for therapeutic agents. The current study reports the in vitro antiprotozoal evaluation of the Algerian medicinal plant *Pulicaria inuloides* against *Leishmania amazonensis*, *Trypanosoma cruzi*, and *Acanthamoeba castellanii str.* Neff. All the extracts from the aerial part showed to be present a higher leishmanicidal activity than anti-*Acanthamoeba* or *Trypanosoma*. Therefore, bioguided fractionation of the active $CHCl_3$ extract led to the isolation and characterization of the flavonol, quercetagetin-3,5,7,3′-tetramethyl ether (*1*) as the main component. The structure of compound *1* was established by extensive 1D and 2D NMR spectroscopic analysis (COSY, HSQC, HMBC, and ROESY experiments), chemical transformation (derivatives *2* and *3*), and comparison with data in the literature. Compound *1* and derivatives *2* and *3* were further evaluated against the promastigote and amastigote stage of *L. amazonensis*. Compounds *1–3* exhibited moderate leishmanicidal activity with IC_{50} values ranging from 0.234 to 0.484 mM and from 0.006 to 0.017 mM for the promastigote and amastigote forms, respectively, as well as low toxicity levels on macrophages (CC_{50} ranging from 0.365 to 0.664 mM). This study represents the first report of the antiprotozoal evaluation of *Pulicaria inuloides*, and the results highlight this species as a promising source of leishmanicidal agents.

Keywords *Pulicaria inuloides* · *Leishmania* · *Trypanosoma* · *Acanthamoeba* · Bioguided fractionation

Introduction

Parasitic diseases are serious problems for public health in the world, especially in tropical and subtropical regions (WHO 2015). Thus, leishmaniasis affects 98 countries with approximately 1.7 million cases worldwide each year, and 350 million people are at risk of becoming infected (Alvar et al. 2012). Furthermore, trypanosomiasis, it is a major health concern in many African countries where about 50 million people are at risk of infection (Févre et al. 2006). The present treatments for this illness are old, expensive, complicated to administer, low effectiveness, causing several side effects, and inducing drug resistance parasites (Hotez et al. 2007). Regarding, several species of free-living amoebae belonging to the genus *Acanthamoeba* present a serious risk to human health. In fact, they act as causative agents of serious infections involving the human brain, lung, skin, and eyes (Walochnik et al. 2000). Due to the presence of cysts, until present, no effective treatments are commercially available. This situation underlines the urgent need for the search of new, effective, cheap, and

✉ Jacob Lorenzo-Morales
JMLORENZ@ULL.EDU.ES

[1] Unité de recherche Valorisation des Ressources Naturelles, Molécules Bioactives et Analyses Physicochimique et Biologiques, Université Constantine-1, Route d'Ain El Bey, 25 000 Constantine, Algeria

[2] University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, Avda. Astrofisico Fco. Sanchez, S/N, 38203 La Laguna, Tenerife, Canary Islands, Spain

[3] Laboratoire Materiaux-Molecules et Applications, IPEST, University of Carthage, La Marsa, Tunisia

[4] Laboratoire de Parasitologie, École Nationale de Médecine Vétérinaire de Sidi Thabet, Université de la Manouba, 2020 Sidi Thabet, Tunisia

[5] Instituto Universitario de Bio-Orgánica Antonio González, Departamento de Química Orgánica, Universidad de La Laguna, Avenida Astrofisico Francisco Sánchez 2, 38206 La Laguna, Tenerife, Canary Islands, Spain

safe drugs for the treatment of these parasitic diseases, and plants used in traditional medicine have been proved to be an extraordinary source of new leads (Hertweck 2015).

The genus *Pulicaria* belongs to the tribe *Inuleae*, *Asteraceae* family (Dubaie and El-Khulaidi 1993; Anderberg 1991), and consist of more than 100 species widespread all over the world, particularly, around the Mediterranean (Williams et al. 2003). Several species within this genus are used as traditional medicine to treat inflammation, intestinal disorders as diuretic, antihelmintic, antimicrobial, and antimalarial, among others. Phytochemical studies have reported phenolic derivatives, monoterpenes, sesquiterpenes, diterpenes, flavonoids, triterpenes, stroides, and essential oils as constituents of *Pulicaria* species (Liu et al. 2010).

Pulicaria inuloides (Poir.) DC. is a perennial herb which flowers and leaves are used as spice and in herbal tea (Al-Hajj et al. 2014a). Only few reports on the chemical constituents of *P. inuloides* have been reported, including the major components of the essential oil (Al-Hajj et al. 2014b), and *ent*-kaurane diterpernoids from the aerial part of the plant (Galala et al. 2016).

As a part of our containing search for new antiparasitic compounds from plants used in traditional medicine, the present study investigates the in vitro antiprotozoal activity of extracts from the aerial part of *Pulicaria inuloides* against *Leishmania*, *Trypanosoma*, and *Acanthamoeba*. The bioassay-guided fractionation of the active $CHCl_3$ extract led to the isolation and characterization of quercetagetin-3,5,7,3′-tetramethyl ether as the main component.

Materials and methods

General experimental procedure ^1H (600 MHz) and ^{13}C (150 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 600 spectrometer; the chemical shifts are given in δ (ppm) with residual $CDCl_3$ (δ_H 7.26, δ_C 77.0) and CD_3OD (δ_H 3.34, δ_C 49.86) as internal reference and coupling constants in hertz; the experiments were carried out with the pulse sequences given by Bruker. COSY, ROESY (spin lock field 2500 Hz), HSQC, and HMBC (optimized for $J = 7.7$ Hz) experiments were conducted on a Bruker Avance 600 spectrometer at 600 MHz with the pulse sequences given by Bruker. EIMS and HREIMS were collected with a Micromass Autospec spectrometer. Silica gel 60 (particle size 15–40 and 63–200 μm, Macherey-Nagel) was used for column chromatography, while silica gel 60 F_{254} (Macherey-Nagel) was used for analytical or preparative TLC. The spots were visualized by UV light and heating silica gel plates sprayed with H_2O-H_2SO_4-AcOH (1:4:20). All solvents used were purchased from Sigma-Aldrich.

Plant material *Pulicaria inuloides* (Poir.) DC. was collected by Professor Samir Benayache at flowering stage in 2013 in Bechar Dam Djorf Ettorba (80 km south west from Bechar). A voucher specimen (PU/105/VAR/05-15) was identified by Ben Abd El-Hakem Mohamed, Head of the Protection Plants Service in Algeria, and was deposited in the Herbarium of the Varenbiomol Research Unit, Université des Frères Mentouri, Constantine, Algeria.

Extraction and isolation The air dried and powdered aerial part (1.5 kg) of *P. inuloides* was repeatedly extracted with 70% aqueous MeOH (3 × 6 L and 3 × 2 L, respectively) at room temperature during 72 h. The extracts were concentrated under reduced pressure until dryness. The result extracts were dissolved into H_2O (0.6 L) and submitted to liquid-liquid extraction with chloroform (3 × 300 mL), ethyl acetate (3 × 300 mL), and *n*-butanol (5 × 300 mL). The organic solvents were removed using a rotavapor under reduced pressure. The extraction yields were 5 g ($CHCl_3$), 6 g (EtOAc), and 50 g (*n*-BuOH). Bioguided fractionation of the active chloroform extract was performed against the promastigote stage of *L. amazonensis*. Thus, the $CHCl_3$ extract (5 g) was fractionated by column chromatography on silica gel (column dimensions 90 × 5 cm) using a mixture of $CHCl_3$-MeOH of increasing polarity to obtain 26 fractions which were combined based on their TLC profile in 9 fractions (F1–F9). The active fraction F5 (278 mg) was chromatographed on a silica gel column using mixtures of hexanes-ethyl acetate of increasing polarity (0–100%; 50 mL at each polarity step) to obtain 15 fractions which were combined based on their TLC profile to 5 sub-fractions (F5A–F5E). Fraction F5D yield compound *1* ($R_f = 0.22$, hexanes-EtOAc, 3:7) as a colorless yellow amorphous solid (77 mg). Compound *1* was identified by spectrometric and spectroscopic data as 6,4′-dihydroxy-3,5,7,3′-tetramethoxyflavone, also named as quercetagetin 3,5,7,3′-tetramethyl ether (Ahmed et al. 1994; El-Negoumy et al. 1982).

6,4′-Dihydroxyl-3,5,7,3′-tetramethoxyflavone (*1*). Yellow amorphous solid; EIMS *m/z* (%) 375 $[M+H]^+$ (100), 374 $[M]^+$ (93), 360 (45), 356 (43), 342 (16), 327 (13), 313 (11), 270 (6); HREIMS *m/z* 374.0987 $[M]^+$ (calcd for $C_{19}H_{18}O_8$, 374.1002); ^1H and ^{13}C NMR (600 MHz, CD_3OD) data, see Table 3.

Acetylation of compound 1 To a solution of compound *1* (7.0 mg) and a catalytic amount of 4-dimethylaminopyridine (DMAP) in 1 mL of CH_2Cl_2 were added triethylamine (2 drops) and acetic anhydride (1 drop). The reaction was stirred for 24 h at room temperature until TLC showed complete conversion and after, the mixture was quenched with MeOH (4 drops) and stirred for 30 min. The mixture was evaporated to dryness and the residue was purified by preparative TLC on silica gel, using CH_2Cl_2-EtOAc (9:1) as eluent to yield derivatives *2* (1.7 mg) and *3* (3.3 mg).

6-Acetoxy-4'-hydroxyl-3,5,7,3'-tetramethoxyflavone (*2*). Yellow amorphous solid; EIMS *m/z* (%) 416 $[M]^+$ (92), 401 (21), 373 (100), 359 (71), 355 (51), 341 (29), 313 (9); HREIMS *m/z* 416.1107 $[M]^+$ (calcd for $C_{21}H_{20}O_9$, 416.1115); ^1H and ^{13}C NMR (600 MHz, $CDCl_3$) data, see Table 3.

6,4'-Diacetoxy-3,5,7,3'-tetramethoxyflavone (*3*). Yellow amorphous solid; EIMS *m/z* (%) 458 $[M]^+$ (88), 416 (79), 401 (31), 373 (100), 359 (70), 355 (53), 341 (30); HREIMS *m/z* 458.1213 $[M]^+$ (calcd for $C_{23}H_{22}O_{10}$, 458.1229); ^1H and ^{13}C NMR (600 MHz, $CDCl_3$) data, see Table 3.

Parasite strains The collected material was evaluated against promastigotes and amastigotes of *Leishmania amazonensis* (MHOM/BR/77/LTB0016), epimastigotes of *Trypanosoma cruzi* Y, and trophozoite stage of *Acanthamoeba castellanii* Neff (ATCC 30010).

In vitro effect on the promastigote and amastigote forms of *L. amazonensis* The activity of the crude extracts, fractions, and compounds *1–3* against *L. amazonensis* were determined by the modified Alamar Blue® assay as previously described by Cabrera-Serra et al. (2007). For the in vitro studies, samples were dissolved in dimethyl sulfoxide (DMSO) and further dilutions were made in RPMI 1640 medium. Promastigotes of *L. amazonensis* were adapted for growth at 26 °C in RPMI 1640 modified medium (Gibco) and supplemented with 10% heat-inactivated fetal bovine serum. Logarithmic phase cultures were used for experimental purposes, and the in vitro susceptibility assay was performed in sterilized 96-well microtiter plates (Corning™). To these wells were added 10^6/well parasites, and the samples at the concentration to be tested. The final volume was 200 μL in each well. After an incubation of 72 h, the analysis of the plate was carried out visually using an inverted microscope. Subsequently, the plates were analyzed for 72 h on an EnSpire multimode plate reader (PerkinElmer, MA, USA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Percentages of inhibition, 50% inhibitory concentrations (IC_{50}) for active samples, were calculated by linear regression analysis with 95% confidence limits. All experiments were performed three times each in duplicate and the mean values were also calculated. A paired two-tailed *t* test was used for analysis of the data. Values of $p < 0.05$ were considered significant. The obtained inhibition curves statistical analysis was undertaken using the Sigma Plot 12.0 software program (Systat Software Inc.). Activity assays against intracellular amastigotes was performed as previously described in our laboratory (López-Arencibia et al. 2017).

Screening assay on the epimastigote stage of *Trypanosoma cruzi* The activity of the extracts was tested in vitro against the epimastigote stage of *T. cruzi*. Briefly, extracts were serially diluted in 100 μL LIT medium and supplemented with 10% heat-inactivated fetal bovine serum in 96-well plates. After that, parasites in log-phase growth were counted, diluted (10^5/well) and were also added to these wells. Finally, the plates were observed under an inverted microscope after 72 h of incubation at 27 °C.

Screening assay on the trophozoite stage of *Acanthamoeba castellanii* Neff The anti-*Acanthamoeba* activity of the extracts was determined as previously described (Martín-Navarro et al. 2008). Briefly, *Acanthamoeba* strains were seeded on a 96-well micro titer plate with 50 μL from a stock solution of 10^4 cells mL^{-1}. Amoebae were allowed to adhere for 15 min process. After that, parasites in log-phase growth were counted and diluted and were also added to these wells. Finally, the plates were observed under the inverted microscope after 96 h of incubation at 27 °C.

Cytotoxicity for host macrophages Cytotoxicity was evaluated after 24 h incubation of macrophage, the J 774 cell line, with different concentrations of compounds *1–3*. The viability of the macrophages was determined with the Alamar Blue assay. Dose response curves were plotted and the CC_{50} was obtained. The analyses were performed in triplicate (Lorenzo-Morales et al. 2010).

Results and discussion

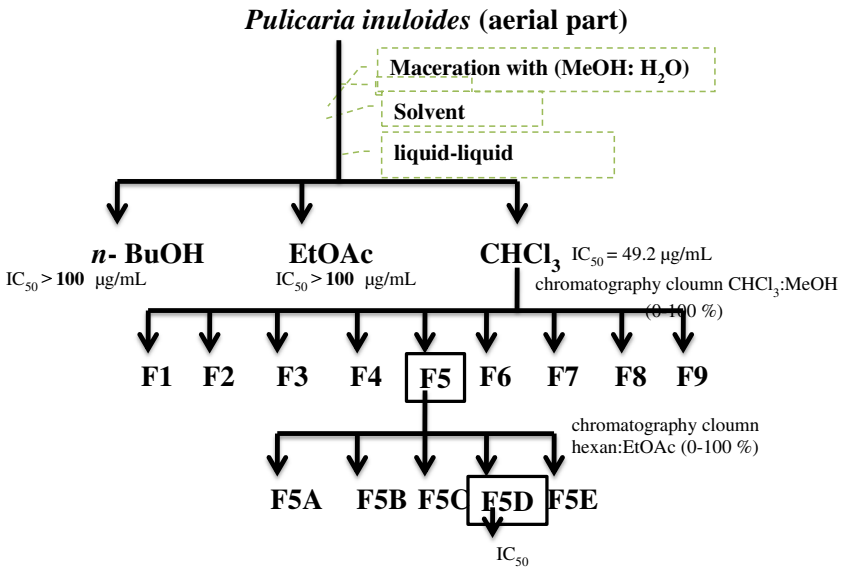
Preparation of extracts is one of the possible processes to extract bioactive molecules from plant. However, no single solvent may extract all the secondary metabolites due to their varying solubility and polarity (Mohamed and Khan 2013). In the present study, *P. inuloides* aerial parts were macerated using three organic solvents with a wide range of polarity index: $CHCl_3$, EtOAC, and *n*-BuOH. As observed in Table 1, results showed that the highest extraction yield was obtained with *n*-BuOH with yield of 3.33%.

Table 1 Percentage yields and antiparasitic screening of extracts from the aerial part of *Pulicaria inuloides* against the different parasites

Extracts	Yield (%)	*T. cruzi*	*L. amazonensis*	*A. castellanii*
$CHCl_3$	0.33	–	+	+
EtOAc	0.40	–	+	–
n-BuOH	3.33	–	+	–

Key: (−) Inactive, (+) moderate activity, all the extracts were assayed at 400 μg/mL

The percentage yield of the organic extracts of the aerial part were calculated based on dry weight as: Yield (%) = ($W_1 \times 100$) 100, where W_1 = weight of extract after solvent evaporation, W_2 = weight of the grinded mushroom powder



Scheme 1 Diagram for extraction and leishmanicidal bioguided fractionation of the chloroform extract of aerial part from *P. inuloides*

In vitro antiparasitic screening of *P. inuloides* extracts The $CHCl_3$, EtOAc, and *n*-BuOH extracts from the aerial parts of *P. inuloides* were screened for their antiparasitic activity against *L. amazonensis*, *T. cruzi*, and *A. castellanii* (Table 1). All the assayed extracts showed moderate leishmanicidal activity at a concentration of 400 μg/mL. Moreover, epimastigotes of *T. cruzi* appeared less sensitive to the extracts than promastigotes of *L. amazonensis* at the same assayed concentration, since all the extracts were inactive. Regarding the anti-amoebic activity, the $CHCl_3$ extract showed a moderate activity, while the others extracts showed to be inactive.

Bioassay-guided fractionation The chloroform extract from the aerial part of *P. inuloides* was bioguide fractionated by its activity against *Leishmania amazonensis* (Scheme 1). Thus, the crude extract was fractionated following the methodology described in the "General experimental procedure" section, and after combining the resulting fractions based of their TLC profile, nine fractions (F1–F9) were collected. These fractions were further evaluated for leishmanicidal activity at a final concentration of 400 μg/mL, and the plates observed under an inverted microscope after 72 h of incubation. The most active fraction, F5, was further subjected to silica gel flash chromatography, and the collected sub-fractions (F5A–F5E) were further evaluated at a concentration of 400 μg/mL. Sub-fraction F5D exhibited the highest leishmanicidal activity with an IC_{50} value of 0.484 mM (Table 2). Sub-fraction F5D revealed to be a pure compound by TLC (Fig. 1). Therefore, its structure was elucidated by means of spectrometric and spectroscopic studies, including ^{1}H and ^{13}C NMR (Table 3), and homonuclear (COSY and ROESY) and heteronuclear correlations (HSQC and HMBC) as follows.

Compound *1* was isolated as a yellow amorphous solid and showed a molecular formula of $C_{19}H_{18}O_8$ by HREIMS. Its ^{1}H and ^{13}C NMR data (Table 3) indicated it to be a 5,6,7,3′,4′-pentasubstituted-flavanol with one additional hydroxy group and four methoxy groups. 2D NMR experiments (HMBC and ROESY) allowed to locate one hydroxy group at C-4′, and three methoxy groups at C-5, C-7, and C-3′. However, the spectroscopic data were unable to establish the position of the remaining two functional groups. Thus, derivatives *2* and *3* were prepared by acetylating of compound *1* according to standard methods (see "General experimental procedure" section) to establish its structure unambiguously (Tasdemir et al. 2006). The structure of 6-acetyl and 6,4′-diacetyl derivatives (*2* and *3*) by means of NMR spectroscopic data is presented in Table 3, and they have not been previously reported. The most

Table 2 In vitro leishmanicidal (promastigote and amastigote forms), cytotoxicity activities, and selectivity index (SI) of compounds 1–3, IC_{50} and CC_{50} values are expressed in mM

Compound	*L. amazonensis* IC_{50} ± SD (mM)	*L. amazonensis amastigotes* IC_{50} (μM)	Cytotoxicity CC_{50} (mM)	SI
1	0.483 ± 0.021 (< 5%)	0.017	0.524 ± 0.055 (< 11%)	29.49
2	0.239 ± 0.003 (< 2%)	0.013	0.664 ± 0.193 (< 29%)	50.37
3	0.479 ± 0.010 (< 3%)	0.006	0.3655 ± 0.080 (< 22%)	57.11
Miltefosine	6.4×10^{-3}	–	–	–

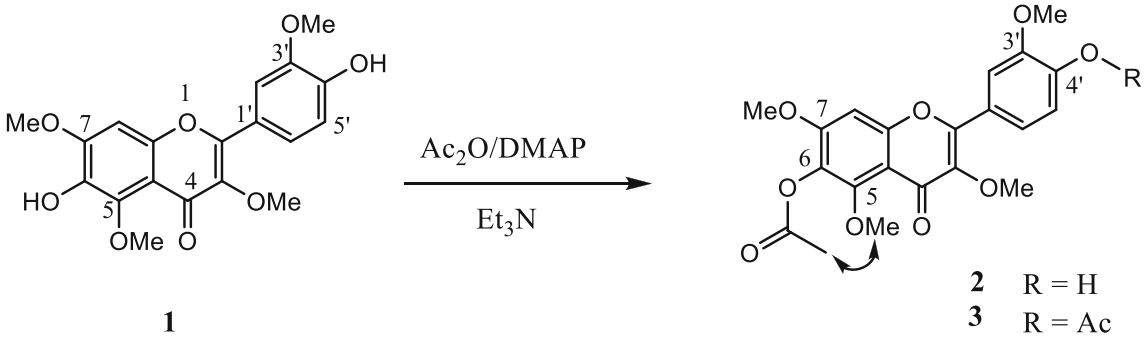


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Fig. 1 Preparation of derivatives *2* and 3 from the natural compound *1*

relevant spectroscopic data was the cross peak from the signals at δ_H 3.96 (OMe-5) and δ_H 0.93 (OMe-7) to the signal at δ_H 2.38 (OAc) observed in the NOESY experiment of compound *3*. This correlation located the acetate group at C-6 position on the flavonol skeleton. Therefore, the structure of compound *1* was identifying as (6,4′-dihydroxy-,3,5,7,3′-tetramethoxyflavone), and its spectral data were totally superposed upon those reported for queretagetin-3,5,5,7,3′-ether (Ahmed et al. 1994; El-Negoumy et al. 1982).

Derivatives *2* and *3* were further evaluated for their leishmanicidal activity (Table 2). Compound *2* was found to be the most active among the assayed compounds with an IC_{50} value of 0.240 mM, and also the less toxic against murine macrophages (CC_{50} = 0.664 mM), suggesting that an acetate group at C-6 enhances slightly the cytotoxic profile of the parent compound *1*. Moreover, compound *1* and its derivatives showed a low toxicity to the host cells, and important property to be considered. In general, the natural compound *1*

Table 3 ^{1}H and ^{13}C NMR ($CDCl_3$) chemical shift δ and *J* (Hz) values (in parentheses) of compounds 1–3

Position	Compound *1*		Compound *2*		Compound *3*	
	δ_H[a]	δ_C[a,b]	δ_H	δ_C[b]	δ_H	δ_C[b]
2		156.9 s		153.6 s		152.8 s
3		142.4 s		140.8 s		141.5 s[c]
4		176.6 s		173.3 s		173.3 s
5		146.1 s		151.9 s		151.9 s
6		139.9 s		131.0 s		131.1 s
7		156.3 s		156.1 s		156.3 s
8	7.06 s	98.0 d	6.78 s	96.1 d	6.78 s	96.1 d
9		153.2 s		155.3 s		155.3 s
10		114.2 s		113.0 s		113.1 s
1′		124.0 s		122.6 s		129.3 s
2′	7.76 d (1.8)	113.6 d	7.72 d (1.8)	111.0 d	7.77 d (2.0)	112.6 d
3'		149.8 s		146.3 s		151.1 s
4'		151.6 s		148.0 s		141.5 s[c]
5'	6.96 d (8.5)	117.3 d	7.04 d (8.5)	122.2 d[c]	7.17 d (8.4)	122.9 d
6'	7.69 dd (8.5, 1.8)	124.3 d	7.63 dd (8.5, 1.8)	122.2 d[c]	7.65 dd (8.4, 2.0)	121.0 d
3-OMe	3.80 s	61.1 q	3.85 s	59.9 q	3.87 s	60.1 q
5-OMe	3.92 s	63.1 q	3.96 s	56.5 q	3.96 s	62.5 q
7-OMe	4.03 s	57.9 q	3.93 s	62.6 q	3.93 s	56.5 q
3'-OMe	3.97 s	57.4 q	3.98 s	56.1 q	3.92 s	56.0 q
4'-OAc					2.36 s	20.7 q
6-OAc			2.38 s	20.4 q	2.38 s	20.3 q
4'-OAc						168.7 s[c]
6-OAc				168.9 s		168.7 s[c]

[a] Spectra were reported in CD_3OD

[b] Data based on HSQC and HMBC experiments

[c] Overlapping signals

and analogues *2* and *3* showed a moderate leishmanicidal activity against *L. amazonensis*, suggesting that some synergic effect could be involved.

These results are in line with previously works reporting the leishmanicidal activity of *Pulicaria crispa* (El-On et al. 2009), which support that species for the genus *Pulicaria* could be a source of new leishmanicidal agents.

Conclusions

The in vitro antiprotozoal evaluation of *Pulicaria inuloides* extracts against *L. amazonensis*, *T. cruzi*, *and A. castellanii* revealed that the $CHCl_3$ extract possesses a moderate leishmanicidal and low anti-*Acanthamoeba* activities, respectively. Furthermore, the leishmanicidal bioassay-guided fractionation of the active extract led to the isolation and characterization of quercetagetin-3,5,7,3′-tetramethyl ether (*1*) as the main component.

Compound *1* and its 6-acetyl (*2*) and 6,4′-diacetyl (*3*) derivatives showed a moderate activity against promastigote forms of *L. amazonensis* with IC_{50} values ranging from 0.240 to 0.484 mM and 0.006 and 0.017 mM for the promastigote and amastigote stages, respectively, and a low toxicity on macrophages (CC_{50} 0.37–0.66 mM).

Up to date, only a few studies have been conducted to explore chemical or pharmacological properties of *P. inuloides.* The present study reports for the first time the leishmanicidal properties of the Algerian medicinal plant *Pulicaria inuloides* contributing to the phytochemical and pharmacological knowledge of this species. Moreover, the species for the genus *Pulicaria* could be a source of new leishmanicidal agents, and deepen studies on *P. inuloides* deserve further investigation.

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