Antiprotozoal activity of *Melampyrum arvense* and its metabolites

Hasan Kirmizibekmez, Irem Atay, Marcel Kaiser, Reto Brun, Michelle Cartagena, Néstor Carballeira, Erdem Yesilada, Deniz Tasdemir

► To cite this version:

Hasan Kirmizibekmez, Irem Atay, Marcel Kaiser, Reto Brun, Michelle Cartagena, et al.. Antiprotozoal activity of *Melampyrum arvense* and its metabolites. *Phytotherapy Research*, 2010, 25 (1), pp.142. 10.1002/ptr.3233 . hal-00599832

HAL Id: hal-00599832

<https://hal.science/hal-00599832>

Submitted on 11 Jun 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Antiprotozoal activity of *Melampyrum arvense* and its metabolites

Journal:	<i>Phytotherapy Research</i>
Manuscript ID:	PTR-10-0093.R2
Wiley - Manuscript type:	Short Communication
Date Submitted by the Author:	23-Apr-2010
Complete List of Authors:	Kirmizibekmez, Hasan; Yeditepe University, Faculty of Pharmacy, Department of Pharmacognosy Atay, Irem; Yeditepe University, Faculty of Pharmacy,, Department of Pharmacognosy Kaiser, Marcel; Swiss Tropical Institute, Department of Medical Parasitology and Infection Biology Brun, Reto; Swiss Tropical Institute, Department of Medical Parasitology and Infection Biology Cartagena, Michelle; University of Puerto Rico, Faculty of Natural Sciences, Department of Chemistry Carballeira, Néstor; University of Puerto Rico, Faculty of Natural Sciences, Department of Chemistry Yesilada, Erdem; Yeditepe University, Faculty of Pharmacy,, Department of Pharmacognosy Tasdemir, Deniz; University of London, School of Pharmacy, Centre for Pharmacognosy and Phytotherapy
Keyword:	Melampyrum arvense, iridoid glucosides, flavonoids, Trypanosoma, Leishmania, Plasmodium

Antiprotozoal activity of *Melampyrum arvense* and its metabolites

Hasan Kirmizibekmez¹, Irem Atay¹, Marcel Kaiser², Reto Brun², Michelle M. Cartagena³, Néstor M. Carballeira³, Erdem Yesilada¹ and Deniz Tasdemir^{4,*}

¹ Department of Pharmacognosy, Faculty of Pharmacy, University of Yeditepe, 34755 Kayisdagi, Istanbul, Turkey

² Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, 4002 Basel, Switzerland

³ Department of Chemistry, University of Puerto Rico, Faculty of Natural Sciences, PO Box 70377, San Juan, Puerto Rico 00936-8377, United States of America

⁴ Centre for Pharmacognosy and Phytotherapy, School of Pharmacy, University of London, London WC1N 1AX, United Kingdom

Running head: **Antiprotozoal compounds of *Melampyrum arvense***

Correspondence

Dr. Deniz Tasdemir

Centre for Pharmacognosy and Phytotherapy, School of Pharmacy

University of London

29-39 Brunswick Square, London WC1N 1AX, United Kingdom

Phone: +44-20-7753 5845

Fax: +44-20-7753 5909

E-mail: deniz.tasdemir@pharmacy.ac.uk

Abstract

An activity guided isolation of the H₂O subextract of the crude extract of *Melampyrum arvense* L. afforded iridoid glucosides aucubin (**1**), melampyroside (**2**), mussaenoside (**3**), mussaenosidic acid (**4**), 8-*epi*-loganin (**5**); flavonoids, apigenin (**6**), luteolin (**7**), luteolin 7-*O*- β -glucopyranoside (**8**); a lignan glycoside dehydrodiconiferyl alcohol 9-*O*- β -glucopyranoside (**9**); and benzoic acid (**10**). β -Sitosterol (**11**) and a fatty acid mixture (**12**) were identified as the active principles of the CHCl₃ subextract. The structures of the isolates were elucidated by spectroscopic methods, while the composition of **12** was identified by GC-MS after methylation. Luteolin (**7**) appeared as the most active compound against *Trypanosoma brucei rhodesiense* and *Leishmania donovani* (IC₅₀ values 3.8 and 3.0 μ g/ml). Luteolin 7-*O*- β -glucopyranoside (**8**) displayed the best antiplasmodial activity against *Plasmodium falciparum* (IC₅₀ value 2.9 μ g/ml). This is the first detailed phytochemical study on Turkish *M. arvense* and the first report of the antiprotozoal effect of *Melampyrum* species and its constituents.

Keywords: *Melampyrum arvense*; iridoid glucosides; flavonoids; *Trypanosoma*; *Leishmania*; *Plasmodium*.

INTRODUCTION

An estimated one billion people – one seventh of the world’s population – are infected with one or more neglected tropical diseases (WHO, 2006). There are an estimated 70 million people in 16 countries in sub-Saharan Africa who suffer from Human African trypanosomiasis (sleeping sickness). One of the two forms of *Trypanosoma*, *T. brucei rhodesiense*, is responsible for this acute illness usually occurring in residents of eastern and southern Africa. The WHO estimates that American trypanosomiasis (Chagas disease), which is caused by *T. cruzi*, affects about 20 million people, and causes 45.000-70.000 deaths in South America annually (Bonomo and Salata, 2007). Leishmaniasis currently threatens approximately 350 million people in 88 countries around the world, with about 2 million affected annually (Mishra *et al.*, 2009). *Leishmania donovani* is the causative agent of visceral leishmaniasis (Kala-azar), which is considered as the most severe form of leishmaniasis (Rocha *et al.*, 2005). *Plasmodium falciparum* is the predominant species causing malaria in Africa, Haiti, New Guinea, Southeast Asia, South America and Oceania (Krause, 2007), and kills over one million people annually (WHO, 2006). Current chemotherapeutics for these diseases have some drawbacks such as serious adverse effects, requirement of long treatment regimes as well as the emergence of drug resistant parasites. Thus, discovery of new, safe and effective antiprotozoal drugs is becoming a pressing need.

The genus *Melampyrum* (formerly Scrophulariaceae *sensu lato*, now Orobanchaceae) consists of annual semi-parasitic plants and is represented by two species, *M. arvense* and *M. pratense* in the flora of Turkey (Hedge, 1978). Iridoid glucosides were reported to be the chief chemical constituents of *M. arvense*. (Damtoft

1
2
3
4
5 *et al.*, 1984) Recent studies have revealed antioxidant (Stajner *et al.*, 2009) and protein
6
7 kinase C inhibitory (Galkin *et al.*, 2009) effects of *M. barbatum* and *M. pratense*. There
8
9 is however no report on antiprotozoal effect of any member of the *Melampyrum* genus.
10
11 In the continuation of our efforts to find natural antiprotozoal lead compounds from
12
13 Turkish plants (Kirmizibekmez *et al.*, 2004; Tasdemir *et al.*, 2005) we now report *in*
14
15 *vitro* antimalarial, trypanocidal, leishmanicidal and cytotoxic activities of the crude
16
17 methanolic extract and its subextracts of *M. arvense*, as well as the constituents of those
18
19 extracts.
20
21
22
23
24
25

26 MATERIAL AND METHODS

27
28 **General experimental procedures.** NMR spectra were recorded on a Bruker
29
30 AVANCE 500 MHz spectrometer. Chemical shift values (δ) were reported in parts per
31
32 million (ppm) relative to appropriate internal solvent standard, and coupling constants
33
34 (*J* values) are given in hertz (Hz). Nano-electrospray mass spectrometry (nano-ES-MS)
35
36 analyses were performed on a LCQ^{duo}, ion trap instrument (Thermo Fisher Scientific,
37
38 San Jose, CA, USA) fitted with a nano-electrospray ionisation (nano-ES) source. TLC
39
40 analyses were carried out on silica gel 60 F₂₅₄ precoated plates (Merck, Darmstadt),
41
42 detection by 1 % vanillin/H₂SO₄. For medium-pressure liquid chromatographic (MPLC)
43
44 separations, Combi Flash Companion (Isco), were used with Redi step columns packed
45
46 with LiChroprep C₁₈ (130 g and 43 g, Teledyne Isco). Silica gel 60 (0.063-0.200 mm;
47
48 Merck, Darmstadt), polyamide (Fluka) and Sephadex LH-20 (Fluka) were utilized for
49
50 open column chromatography (CC). All solvents were HPLC quality.
51
52
53
54
55
56
57
58
59
60

Plant material. The aerial parts of *M. arvensis* L. were collected from Kastamonu, Turkey in 2007. The plant material was identified by Dr. Galip Akaydin. The voucher specimens (Akaydin 11546) of this plant were deposited at the Herbarium of the Faculty of Education, Hacettepe University, Ankara, Turkey.

Extraction and isolation. The air-dried and powdered aerial parts of *M. arvensis* (110 g) were extracted twice with MeOH (600 ml) at 45 °C for 4 h. The combined methanolic extracts were evaporated to a residue (13.9 g; yield 12.6%). The extract was suspended in H₂O (25 ml), and extracted with equal volumes of CHCl₃ (25 ml x 6). The H₂O-soluble portion (8 g) was loaded onto a polyamide (40 g) column eluting with H₂O (200 mL) and a stepwise gradient of MeOH in H₂O (10-100% in steps of 10%, each 100 mL) to yield nine fractions, A-I. Fraction B (4.5 g) was subjected to a SiO₂ (140 g) column using CH₂Cl₂-MeOH-H₂O (90:10:1, 85:15:1, 80:20:2, 70:30:3 and 50:40:10; each 250 mL) to give subfraction B₇ (150 mg) which was further purified by C₁₈-medium pressure liquid chromatography (C₁₈-MPLC, 43 g) eluting with stepwise H₂O-MeOH gradient (10 to 60 % MeOH with increasing 5%, each 100 mL) to obtain **5** (15 mg) and **3** (23 mg). Fraction C (811 mg) was separated by C₁₈-MPLC (130 g, 0-65 % MeOH/H₂O, with increasing 5%, each 150 mL) to afford **4** (12 mg), **1** (30 mg) and **3** (40 mg). Compound **2** (5 mg) was purified from fr. D (43 mg) by SiO₂ (10 g) CC (CHCl₃-MeOH-H₂O, 95:5:0.5, 90:10:1 and 85:15:1, each 100 mL). Fr. F (72 mg) was chromatographed by C₁₈-MPLC (43 g, 5-60 % MeOH/H₂O, with increasing 5%, each 100 mL) and yielded compounds **10** (13 mg) and **9** (6 mg). Purification of fraction H (50 mg) by Sephadex LH-20 (20 g) CC (MeOH, 200 mL) gave **8** (2 mg). Similarly, fraction I (100 mg) was applied on a Sephadex LH-20 (20 g) column (MeOH, 200 mL)

to obtain **6** (2 mg) and **7** (3 mg). The CHCl₃ extract (3.25 g) was fractionated over a SiO₂ (90 g) column (*n*-hexane-acetone, 90:10 to 10:90 with increasing 10% of acetone, each 200 mL) to give nine fractions, 1-9. Fraction 3 (164 mg) was applied onto a SiO₂ (20 g) column (*n*-hexane-acetone, 90:10 and 85:15, each 100 mL) to yield **11** (3 mg). The fatty acid mixture (**12**, 4 mg) was obtained from fraction 4 (77 mg) by using Sephadex LH-20 (20 g) CC (MeOH, 200 mL).

GC-MS analysis of 12 and the CHCl₃ subextract. In order to analyze the FA composition of **12** and the CHCl₃ extract, the total FAs were converted to fatty acid methyl esters (FAME) with methanolic HCl followed by column chromatography on Si gel eluting with hexane/ether (9:1). The total FAMES were analyzed qualitatively and quantitatively by GC-MS by comparing the mass spectra of the FA methyl esters with those in the literature (NIST/EPA/NIH Mass Spectral Library) and comparing their equivalent chain length (ECL) values with known commercial standards (Sigma). The double-bonds and methyl-branching in these compounds were determined by pyrrolidide derivatization following the preparation procedure. The FAME were analyzed by electron ionization using GC-MS (Agilent 5975C MS ChemStation coupled to an Agilent 7890A GC) at 70 eV equipped with a 30 m x 0.25 mm special performance capillary column (HP-5MS). The GC temperature program was: 130 °C for 1 min, increased at a rate of 3 °C/min to 270°C, and maintained for 30 min at 270 °C.

***In vitro* assay for *Plasmodium falciparum*.** *In vitro* activity against erythrocytic stages of *P. falciparum* was determined by a modified [³H]-hypoxanthine incorporation assay, using the chloroquine- and pyrimethamine-resistant K1 strain and the standard drug

chloroquine. Briefly, parasite cultures incubated in RPMI 1640 medium with 5% Albumax (without hypoxanthine) were exposed to serial drug dilutions in microtiter plates. After 48 h of incubation at 37°C in a reduced oxygen atmosphere, 0.5 µCi ³H-hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a BetaplateTM liquid scintillation counter (Wallac, Zurich, Switzerland). The results were recorded as counts per minute (CPM) per well at each drug concentration and expressed as percentage of the untreated controls. IC₅₀ values were calculated from the sigmoidal inhibition curves using Microsoft Excel.

***In vitro* assay for *Trypanosoma brucei rhodesiense*.** *T. b. rhodesiense*, STIB 900 strain, and the standard drug, melarsoprol, were used for the assay. This stock was isolated in 1982 from a human patient in Tanzania and after several mouse passages cloned and adapted to axenic culture conditions. Minimum Essential Medium (50 µl) supplemented with 25 mM HEPES, 1g/l additional glucose, 1% MEM non-essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 1mM Na-pyruvate and 15% heat inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/ml were prepared. Then 10⁴ bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 µl was added to each well and the plate incubated at 37 °C under a 5 % CO₂ atmosphere for 72 h. 10 µl Alamar Blue (resazurin, 12.5 mg in 100 ml double-distilled water) was then added to each well and incubation continued for a further 2-4 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an

emission wavelength of 588 nm. Data were analyzed using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA).

***In vitro* assay for *Trypanosoma cruzi*.** Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtitre plates at 2000 cells/well in 100 μ L RPMI 1640 medium with 10% FBS and 2 mM l-glutamine. After 24 h the medium was removed and replaced by 100 μ L per well containing 5000 trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the β -galactosidase (Lac Z) gene. After 48 h, the medium was removed from the wells and replaced by 100 μ L fresh medium with or without a serial drug dilution of seven 3-fold dilution steps covering a range from 90 to 0.123 μ g/ml. After 96 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterility. Then the substrate CPRG/Nonidet (50 μ L) was added to all wells. A color reaction developed within 2-6 h and could be read photometrically at 540 nm. Data were transferred into the graphic programme Softmax Pro (Molecular Devices), which calculated IC₅₀ values. Benznidazole was the standard drug used.

***In vitro* assay for *Leishmania donovani*.** Amastigotes of *L. donovani* strain MHOM/ET/67/L82 were grown in axenic culture at 37 °C in SM medium at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5% CO₂ in air. One hundred microlitres of culture medium with 10⁵ amastigotes from axenic culture with or without a serial drug dilution were seeded in 96-well microtitre plates. Serial drug dilutions covering a range from 90 to 0.123 μ g/ml were prepared. After 72 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Alamar Blue (10 μ L; 12.5 mg

resazurin dissolved in 100 ml distilled water) were then added to each well and the plates incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA). Decrease of fluorescence (= inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves the IC₅₀ values were calculated. Miltefosine was used as a reference drug.

***In vitro* assay for cytotoxicity.** Assays were performed in 96-well microtiter plates, each well containing 100 µl of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum, and 4 x 10⁴ L⁻⁶ cells (a primary cell line derived from rat skeletal myoblasts Serial drug dilutions of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/ml were prepared. After 72 h of incubation the plates were inspected under an inverted microscope to assure growth of the controls and sterile conditions. Alamar Blue (10 µl, 12.5 mg resazurin dissolved in 100 ml double-distilled water) was then added to each well and the plates incubated for another 2 h. Then the plates were read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analysed using the microplate reader software Softmax Pro. Podophyllotoxin was the standard drug used.

RESULTS AND DISCUSSION

The crude MeOH extract of *M. arvensis* showed *in vitro* trypanocidal and leishmanicidal activity (Table 1), without any toxicity towards the mammalian cells (IC_{50} values >90 $\mu\text{g/ml}$) and was selected for in-depth chemical investigations. The crude MeOH extract was partitioned between CHCl_3 and H_2O , and each subextract was submitted to the same panel of antiprotozoal and cytotoxic activity screening. As activity was detected in both extracts, each subextract was individually further purified through an activity-guided isolation process. The H_2O subextract yielded ten compounds, whose chemical structures were identified as iridoid glycosides aucubin (**1**), melampyroside (**2**) (Chaudhuri *et al.*, 1981), mussaenoside (**3**) (El-Naggar and Beal, 1980), mussaenosidic acid (**4**), 8-*epi*-loganin (**5**) (Boros and Stermitz 1990), flavonoids apigenin (**6**), luteolin (**7**), luteolin 7-*O*- β -glucopyranoside (**8**) (Markham and Chari, 1982), a lignan glycoside, dehydrodiconiferyl alcohol 9-*O*- β -glucopyranoside (**9**) (Arens *et al.*, 1985), an aromatic acid, benzoic acid (**10**) by comparison of their spectroscopic data (1D and 2D NMR, ESIMS and $[\alpha]_D$) with those published in the literature. As displayed in Table 1, the CHCl_3 subextract was more active against all parasitic protozoa than the H_2O extract. The highest bioactivity was tracked to fractions 3 and 4 obtained by a SiO_2 column chromatography of the CHCl_3 extract. Fraction 3 yielded the common phytosterol, β -sitosterol (**11**) whose structure was elucidated by comparison with published data (Kojima *et al.*, 1990). Fraction 4 yielded a subfraction (**12**), which appeared to be a fatty acid mixture by ^1H NMR, and its exact composition was identified by GC-MS after methylation (Table 2). Only seven fatty acids (FAs) were detected in **12**, with palmitic acid (16:0) and linolenic acid (18:3) being the most major ones (both at 37% relative abundance). These results prompted us to elucidate the fatty acid composition of the

CHCl₃ subextract as well. The CHCl₃ extract was almost a 1:1 mixture of saturated (45.1-48.6%) and unsaturated (54.9-51.7%) fatty acids.

Table 1 shows the antiprotozoal activity and cytotoxic potential of all *Melampyrum arvense* extracts, subextracts and the metabolites isolated from them. All purified compounds, but **3** (mussaenoside), which was inactive in all assays, exhibited moderate trypanocidal activities against *T. b. rhodesiense*. Luteolin (**7**) appeared to be most potent trypanocidal compound with an IC₅₀ value of 3.8 µg/ml and was followed by apigenin (**6**, IC₅₀ of 14.0 µg/ml). Luteolin 7-*O*-β-glucopyranoside (**8**) had some trypanocidal activity (IC₅₀ of 24.1 µg/ml) as well. Among the non-flavonoidal compounds, 8-*epi*-loganin (**5**) and the fatty acid mixture (**12**) exhibited the highest activity (IC₅₀s 17.1 and 22.1 µg/ml). Only the two non-glycosidic flavones, apigenin (**6**) and luteolin (**7**), as well as β-sitosterol (**11**) and **12** exhibited some weak growth inhibitory effect against the American trypanosome, *T. cruzi* (IC₅₀ values of 31.9, 17.0, 35.4 and 24.2 µg/ml, respectively). With the exception of the iridoid glycosides **1** and **3**, all compounds also exerted leishmanicidal effects. The flavonoids showed the highest activity with luteolin (**7**) being the most potent (IC₅₀ of 3.0 µg/ml). Apigenin (**6**) and luteolin 7-*O*-β-glucopyranoside (**8**) appeared to be equipotent (IC₅₀ values of 7.5 and 7.0 µg/ml). The components of the CHCl₃ extract, i.e. the fatty acid mixture (**12**) and phytosterol (**11**) also showed weak antileishmanial activities (IC₅₀ values of 10 and 27.8 µg/ml). All iridoids (**1-5**), plus the compounds **9** and **10** failed to arrest the growth of drug-resistant *P. falciparum* cultures at the highest test concentrations (20 µg/ml). The flavonoids **6-8** and both **11-12** however showed significant antiplasmodial effects (IC₅₀ values of 15.5, 4.2, 2.9, 3.6 and 4.9 µg/ml), with luteolin 7-*O*-β-glucopyranoside (**8**)

being the most active one. Among all compounds investigated, only compounds **6-7** and **11-12** displayed some moderate cytotoxicity towards mammalian cells (IC₅₀ values of 57.5, 16.2, 49.8 and 40.9 µg/ml, respectively).

In conclusion, this is the first detailed phytochemical study on Turkish *M. arvensis* and the first report of the antiprotozoal effect of a member of the genus *Melampyrum* and its constituents. In comparison to the reference compounds used in the assays, the biological activity of the *Melampyrum* isolates are low. However, their low toxicity may still render them promising lead compounds for future studies. Among the isolated compounds, the lignan glycoside dehydrodiconiferyl alcohol 9-*O*-β-glucopyranoside (**9**), benzoic acid (**10**) and the common phytosterol, β-sitosterol (**11**) are being reported for the first time from the genus *Melampyrum*.

Acknowledgements

The authors thank Dr. Galip Akaydin (Hacettepe University, Department of Biology Education, Ankara, Turkey) for authentication of the plant material. M. Cartagena thanks the NIH-RISE program for a graduate fellowship.

REFERENCES

- Arens H, Fischer H, Leyck S, Römer A, Ulbrich B. 1985. Antiinflammatory compounds from *Plagiorhegma dubium* cell culture. *Planta Med* **51**: 52-56.
- Bonomo RA, Salata RA. 2007. American trypanosomiasis (Chagas disease; *Trypanosoma cruzi*). In *Nelson Textbook of Pediatrics*, Kleigman RM, Behrman RE, Jensen HB, Stanton BF, (eds). Elsevier Inc: Philadelphia, 1474-1477.

- Boros CA, Stermitz FR. 1990. Iridoids. An updated review Part I. *J Nat Prod* **53**: 1055-1147.
- Chaudhuri RK, Salama O, Sticher O. 1981. Iridoid and aryl glucosides from *Globularia nudicalus* and *Globularia nana*. *Helv Chim Acta* **64**: 2401-2404.
- Damtoft S, Hansen SB, Jacobsen B, Jensen SR, Nielsen BJ. 1984. Iridoid glucosides from *Melampyrum*. *Phytochemistry* **23**: 2387-2389.
- El-Naggar LJ, Beal JL. 1980. Iridoids. A review. *J Nat Prod* **43**: 649-776.
- Galkin A, Jokela J, Wahlsten M, Tammela P, Sivonen K, Vuorela P. 2009. Discovering protein kinase C active plants growing in Finland utilizing automated bioassay combined to LC/MS. *Nat Prod Commun* **4**: 139-142.
- Hedge IC. 1978. *Melampyrum* L. In *Flora of Turkey and East Aegean Islands*, Volume 6, Davis PH (ed). University Press: Edinburgh, 755-756.
- Kirmizibekmez H, Perozzo R, Brun R, Linden A, Rüedi P, Dönmez AA, Çalış I, Tasdemir D. 2004. Inhibiting activities of the secondary metabolites of *Phlomis brunneogaleata* against parasitic protozoa and plasmodial enoyl-ACP reductase, acrucial enzyme in fatty acid biosynthesis. *Planta Med* **70**: 711-717.
- Kojima H, Sato N, Hatano A, Ogura H. 1990. Sterol glucosides from *Prunella vulgaris*. *Phytochemistry* **29**: 2351-2355.
- Krause PJ. 2007. Malaria (*Plasmodium*). In *Nelson Textbook of Pediatrics*, Kleigman RM, Behrman RE, Jensen HB, Stanton BF, (eds). Elsevier Inc: Philadelphia, 1477-1485.
- Markham KR, Chari VM. 1982. Carbon-13 NMR Spectroscopy of Flavonoids. In *The Flavonoids: Advances in Research*, Harborne JB, Mabry TJ. (eds). Chapman and Hall: London, 19-132.

- 1
2
3
4 Mishra BB, Kale RR, Singh RK, Tiwari, VK. 2009. Alkaloids: Future prospective to
5
6 combat leishmaniasis. *Fitoterapia* **80**: 81-90.
7
8
9 Rocha LG, Almeida JRGS, Macedo RO, Barbosa-Filho JM. 2005. A review of natural
10
11 products with antileishmanial activity. *Phytomedicine* **12**: 514-535.
12
13
14 Stajner D, Popovic BM, Boza P, Kapor A. 2009. Antioxidant capacity of *Melampyrum*
15
16 *barbatum* - weed and medicinal plant. *Phytother Res* **23**: 1006-1010.
17
18
19 Tasdemir D, Güner ND, Perozzo R, Brun R, Dönmez AA, Çalış I, Rüedi P. 2005.
20
21 Antiprotozoal and plasmodial FabI enzyme inhibiting metabolites of
22
23 *Scrophularia lepidota* roots. *Phytochemistry* **66**: 355-362.
24
25
26 World Health Organisation. 2006. Neglected tropical diseases, hidden successes,
27
28 emerging opportunities web page. Available at
29
30 http://whqlibdoc.who.int/hq/2006/WHO_CDS_NTD_2006.2_eng.pdf . 2006.
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Table 1. Table 1: Trypanocidal, leishmanicidal, antiplasmodial and cytotoxic activities of the extracts of *M. arvensis* and its metabolites.

Extract/ Compound	<i>T. b.</i> <i>rhodesiense</i>	<i>T.</i> <i>cruzi</i>	<i>L.</i> <i>donovani</i>	<i>P.</i> <i>falciparum</i>	Cytotoxicity (L6 cells)
Standard	0.003 ^a	0.359 ^b	0.2 ^c	0.056 ^d	0.004 ^e
MeOH extract	8.8	>90	41.4	>20	>90
CHCl ₃ extract	3.0	67.5	16.3	8.3	89.7
H ₂ O extract	29.5	>90	50.9	>20	>90
1	66.6	>90	>90	>20	>90
2	56.6	>90	52.7	>20	>90
3	>90	>90	>90	>20	>90
4	58.3	>90	88.1	>20	>90
5	17.1	>90	41.1	>20	>90
6	14.0	31.9	7.5	15.5	57.5
7	3.8	17.0	3.0	4.2	16.2
8	24.1	>90	7.0	2.9	>90
9	60.8	>90	68.8	>20	>90
10	60.8	>90	71.5	>20	>90
11	86.1	35.4	27.8	3.6	49.8
12	22.1	24.2	10.0	4.9	40.9

The *in vitro* IC₅₀ values represent the average of at least two independent assays (the variation is max. 20%). All IC₅₀ values are in µg/ml. Standard compounds: ^amelarsoprol, ^bbenznidazole, ^cmiltefosine, ^dchloroquine, ^epodophyllotoxin.

Table 2 Fatty acid composition of the fatty acid mixture (**12**) and the CHCl₃ extract

Fatty acid	12 Weight percent (%)	CHCl ₃ extract Weight percent (%)
n-14:0	3.8	2.1
n-16:0	37.0	37.4
n-17:0	0.4	1.0
n-18:0	3.5	6.8
18:2(n-6)	17.9	13.3
18:3(n-3)	37.0	38.4
n-20:0	0.4	1.0