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Antiprotozoal activity of *Melampyrum arvense* and its metabolites

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Antiprotozoal activity of *Melampyrum arvense* and its metabolites

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

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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

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

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

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4 to obtain **6** (2 mg) and **7** (3 mg). The CHCl₃ extract (3.25 g) was fractionated over a
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6 SiO₂ (90 g) column (*n*-hexane-acetone, 90:10 to 10:90 with increasing 10% of acetone,
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8 each 200 mL) to give nine fractions, 1-9. Fraction 3 (164 mg) was applied onto a SiO₂
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10 (20 g) column (*n*-hexane-acetone, 90:10 and 85:15, each 100 mL) to yield **11** (3 mg).
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12 The fatty acid mixture (**12**, 4 mg) was obtained from fraction 4 (77 mg) by using
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14 Sephadex LH-20 (20 g) CC (MeOH, 200 mL).
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21 **GC-MS analysis of 12 and the CHCl₃ subextract.** In order to analyze the FA
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23 composition of **12** and the CHCl₃ extract, the total FAs were converted to fatty acid
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25 methyl esters (FAME) with methanolic HCl followed by column chromatography on Si
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27 gel eluting with hexane/ether (9:1). The total FAMES were analyzed qualitatively and
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29 quantitatively by GC-MS by comparing the mass spectra of the FA methyl esters with
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31 those in the literature (NIST/EPA/NIH Mass Spectral Library) and comparing their
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33 equivalent chain length (ECL) values with known commercial standards (Sigma). The
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35 double-bonds and methyl-branching in these compounds were determined by
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37 pyrrolidide derivatization following the preparation procedure. The FAME were
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39 analyzed by electron ionization using GC-MS (Agilent 5975C MS ChemStation
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41 coupled to an Agilent 7890A GC) at 70 eV equipped with a 30 m x 0.25 mm special
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43 performance capillary column (HP-5MS). The GC temperature program was: 130 °C for
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45 1 min, increased at a rate of 3 °C/min to 270°C, and maintained for 30 min at 270 °C.
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54 ***In vitro* assay for Plasmodium falciparum.** *In vitro* activity against erythrocytic stages
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56 of *P. falciparum* was determined by a modified [³H]-hypoxanthine incorporation assay,
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58 using the chloroquine- and pyrimethamine-resistant K1 strain and the standard drug
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4 chloroquine. Briefly, parasite cultures incubated in RPMI 1640 medium with 5%
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6 Albumax (without hypoxanthine) were exposed to serial drug dilutions in microtiter
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8 plates. After 48 h of incubation at 37°C in a reduced oxygen atmosphere, 0.5 µCi ³H-
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10 hypoxanthine was added to each well. Cultures were incubated for a further 24 h before
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12 they were harvested onto glass-fiber filters and washed with distilled water. The
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14 radioactivity was counted using a BetaplateTM liquid scintillation counter (Wallac,
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16 Zurich, Switzerland). The results were recorded as counts per minute (CPM) per well at
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18 each drug concentration and expressed as percentage of the untreated controls. IC₅₀
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20 values were calculated from the sigmoidal inhibition curves using Microsoft Excel.
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28 ***In vitro* assay for *Trypanosoma brucei rhodesiense*.** *T. b. rhodesiense*, STIB 900
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30 strain, and the standard drug, melarsoprol, were used for the assay. This stock was
31
32 isolated in 1982 from a human patient in Tanzania and after several mouse passages
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34 cloned and adapted to axenic culture conditions. Minimum Essential Medium (50 µl)
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36 supplemented with 25 mM HEPES, 1g/l additional glucose, 1% MEM non-essential
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38 amino acids (100x), 0.2 mM 2-mercaptoethanol, 1mM Na-pyruvate and 15% heat
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40 inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug
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42 dilutions of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/ml were
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44 prepared. Then 10⁴ bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 µl was
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46 added to each well and the plate incubated at 37 °C under a 5 % CO₂ atmosphere for 72
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48 h. 10 µl Alamar Blue (resazurin, 12.5 mg in 100 ml double-distilled water) was then
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50 added to each well and incubation continued for a further 2-4 h. Then the plates were
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52 read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices
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54 Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an
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4 emission wavelength of 588 nm. Data were analyzed using the microplate reader
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6 software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA).
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11 ***In vitro* assay for *Trypanosoma cruzi*.** Rat skeletal myoblasts (L-6 cells) were seeded in
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13 96-well microtitre plates at 2000 cells/well in 100 μ L RPMI 1640 medium with 10%
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15 FBS and 2 mM l-glutamine. After 24 h the medium was removed and replaced by 100 μ l
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17 per well containing 5000 trypomastigote forms of *T. cruzi* Tulahuen strain C2C4
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19 containing the β -galactosidase (Lac Z) gene. After 48 h, the medium was removed from
20
21 the wells and replaced by 100 μ l fresh medium with or without a serial drug dilution of
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23 seven 3-fold dilution steps covering a range from 90 to 0.123 μ g/ml. After 96 h of
24
25 incubation the plates were inspected under an inverted microscope to assure growth of
26
27 the controls and sterility. Then the substrate CPRG/Nonidet (50 μ l) was added to all
28
29 wells. A color reaction developed within 2-6 h and could be read photometrically at 540
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31 nm. Data were transferred into the graphic programme Softmax Pro (Molecular Devices),
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33 which calculated IC₅₀ values. Benznidazole was the standard drug used.
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42 ***In vitro* assay for *Leishmania donovani*.** Amastigotes of *L. donovani* strain
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44 MHOM/ET/67/L82 were grown in axenic culture at 37 °C in SM medium at pH 5.4
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46 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5%
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48 CO₂ in air. One hundred microlitres of culture medium with 10⁵ amastigotes from
49
50 axenic culture with or without a serial drug dilution were seeded in 96-well microtitre
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52 plates. Serial drug dilutions covering a range from 90 to 0.123 μ g/ml were prepared.
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54 After 72 h of incubation the plates were inspected under an inverted microscope to
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56 assure growth of the controls and sterile conditions. Alamar Blue (10 μ l; 12.5 mg
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4 resazurin dissolved in 100 ml distilled water) were then added to each well and the
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6 plates incubated for another 2 h. Then the plates were read with a Spectramax Gemini
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8 XS microplate fluorometer using an excitation wavelength of 536 nm and an emission
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10 wavelength of 588 nm. Data were analyzed using the software Softmax Pro (Molecular
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12 Devices Cooperation, Sunnyvale, CA, USA). Decrease of fluorescence (= inhibition)
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14 was expressed as percentage of the fluorescence of control cultures and plotted against
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16 the drug concentrations. From the sigmoidal inhibition curves the IC₅₀ values were
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18 calculated. Miltefosine was used as a reference drug.
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26 ***In vitro* assay for cytotoxicity.** Assays were performed in 96-well microtiter plates,
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28 each well containing 100 µl of RPMI 1640 medium supplemented with 1% L-glutamine
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30 (200 mM) and 10% fetal bovine serum, and 4×10^4 L⁻⁶ cells (a primary cell line derived
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32 from rat skeletal myoblasts Serial drug dilutions of seven 3-fold dilution steps covering
33
34 a range from 90 to 0.123 µg/ml were prepared. After 72 h of incubation the plates were
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36 inspected under an inverted microscope to assure growth of the controls and sterile
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38 conditions. Alamar Blue (10 µl, 12.5 mg resazurin dissolved in 100 ml double-distilled
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40 water) was then added to each well and the plates incubated for another 2 h. Then the
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42 plates were read with a Spectramax Gemini XS microplate fluorometer using an
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44 excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were
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46 analysed using the microplate reader software Softmax Pro. Podophyllotoxin was the
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48 standard drug used.
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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 (IC₅₀ values >90 µg/ml) and was selected for in-depth chemical investigations. The crude MeOH extract was partitioned between CHCl₃ and H₂O, 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 H₂O 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 [α]_D) with those published in the literature. As displayed in Table 1, the CHCl₃ subextract was more active against all parasitic protozoa than the H₂O extract. The highest bioactivity was tracked to fractions 3 and 4 obtained by a SiO₂ column chromatography of the CHCl₃ 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

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4 CHCl₃ subextract as well. The CHCl₃ extract was almost a 1:1 mixture of saturated
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6 (45.1-48.6%) and unsaturated (54.9-51.7%) fatty acids.
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10 Table 1 shows the antiprotozoal activity and cytotoxic potential of all
11 *Melampyrum arvense* extracts, subextracts and the metabolites isolated from them. All
12 purified compounds, but **3** (mussaenoside), which was inactive in all assays, exhibited
13 moderate trypanocidal activities against *T. b. rhodesiense*. Luteolin (**7**) appeared to be
14 most potent trypanocidal compound with an IC₅₀ value of 3.8 µg/ml and was followed
15 by apigenin (**6**, IC₅₀ of 14.0 µg/ml). Luteolin 7-*O*-β-glucopyranoside (**8**) had some
16 trypanocidal activity (IC₅₀ of 24.1 µg/ml) as well. Among the non-flavonoidal
17 compounds, 8-*epi*-loganin (**5**) and the fatty acid mixture (**12**) exhibited the highest
18 activity (IC₅₀s 17.1 and 22.1 µg/ml). Only the two non-glycosidic flavones, apigenin (**6**)
19 and luteolin (**7**), as well as β-sitosterol (**11**) and **12** exhibited some weak growth
20 inhibitory effect against the American trypanosome, *T. cruzi* (IC₅₀ values of 31.9, 17.0,
21 35.4 and 24.2 µg/ml, respectively). With the exception of the iridoid glycosides **1** and **3**,
22 all compounds also exerted leishmanicidal effects. The flavonoids showed the highest
23 activity with luteolin (**7**) being the most potent (IC₅₀ of 3.0 µg/ml). Apigenin (**6**) and
24 luteolin 7-*O*-β-glucopyranoside (**8**) appeared to be equipotent (IC₅₀ values of 7.5 and
25 7.0 µg/ml). The components of the CHCl₃ extract, i.e. the fatty acid mixture (**12**) and
26 phytosterol (**11**) also showed weak antileishmanial activities (IC₅₀ values of 10 and 27.8
27 µg/ml). All iridoids (**1-5**), plus the compounds **9** and **10** failed to arrest the growth of
28 drug-resistant *P. falciparum* cultures at the highest test concentrations (20 µg/ml). The
29 flavonoids **6-8** and both **11-12** however showed significant antiplasmodial effects (IC₅₀
30 values of 15.5, 4.2, 2.9, 3.6 and 4.9 µg/ml), with luteolin 7-*O*-β-glucopyranoside (**8**)
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4 being the most active one. Among all compounds investigated, only compounds **6-7** and
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7 **11-12** displayed some moderate cytotoxicity towards mammalian cells (IC₅₀ values of
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9 57.5, 16.2, 49.8 and 40.9 µg/ml, respectively).
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13 In conclusion, this is the first detailed phytochemical study on Turkish *M.*
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15 *arvense* and the first report of the antiprotozoal effect of a member of the genus
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17 *Melampyrum* and its constituents. In comparison to the reference compounds used in the
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19 assays, the biological activity of the *Melampyrum* isolates are low. However, their low
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21 toxicity may still render them promising lead compounds for future studies. Among the
22
23 isolated compounds, the lignan glycoside dehydrodiconiferyl alcohol 9-*O*-β-
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25 glucopyranoside (**9**), benzoic acid (**10**) and the common phytosterol, β-sitosterol (**11**)
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27 are being reported for the first time from the genus *Melampyrum*.
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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