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RESEARCH ARTICLE

Evaluation of *in vitro* antiprotozoal activity of *Ajuga laxmannii* and its secondary metabolites

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ABSTRACT

Context Some *Ajuga* L. (Lamiaceae) species are traditionally used for the treatment of malaria, as well as fever, which is a common symptom of many parasitic diseases.

Objective In the continuation of our studies on the identification of antiprotozoal secondary metabolites of Turkish Lamiaceae species, we have investigated the aerial parts of *Ajuga laxmannii*.

Materials and methods The aerial parts of *A. laxmannii* were extracted with MeOH. The H_2O subextract was subjected to polyamide, C_{18}-MPLC and SiO_2 CCs to yield eight metabolites. The structures of the isolates were elucidated by NMR spectroscopy and MS analyses. The extract, subextracts as well as the isolates were tested for their *in vitro* antiprotozoal activities against *Plasmodium falciparum*, *Trypanasoma brucei rhodesiense, T. cruzi* and *Leishmania donovani* at concentrations of 90–0.123 μg/mL.

Results Two iridoid glycosides harpagide (**1**) and 8-*O*-acetylharpagide (**2**), three *o*-coumaric acid derivatives *cis*-melilotoside (**3**), *trans*-melilotoside (**4**) and dihydromelilotoside (**5**), two phenylethanoid glycosides verbascoside (**6**) and galactosylmartynoside (**7**) and a flavone-*C*-glycoside, isoorientin (**8**) were isolated. Many compounds showed moderate to good antiparasitic activity, with isoorientin (**8**) displaying the most significant antimalarial potential (an IC_{50} value of 9.7 μg/mL).

Discussion and conclusion This is the first report on the antiprotozoal evaluation of *A. laxmannii* extracts and isolates. Furthermore, isoorientin and dihydromelilotoside are being reported for the first time from the genus *Ajuga*.

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Introduction

The majority of vector-borne parasitic infectious diseases, such as malaria, African or American trypanosomiasis and leismaniasis are generally categorized as neglected tropical diseases (NTDs) due to their low priority in western world as well as lack of interest of pharmaceutical companies. NTDs principally affect more than one billion people in 149 countries in the world's poorest regions (Feasey et al. 2010; WHO 2015a). Malaria is caused by the protozoan parasite *Plasmodium*, with *P. falciparum* being the deadliest. It is estimated that 584 000 malaria deaths occurred in 2013 worldwide and 453 000 of these deaths were children under 5 years of age (WHO 2014). Human African trypanosomiasis (sleeping sickness) is caused by *Trypanasoma brucei rhodesiense*, a parasite which enters the body through the bites of tsetse flies. An estimated 70 million people are at risk (Feasey et al. 2010). Chagas' disease (*American trypanasomiasis*) that is caused by *Trypanasoma cruzi* affects around 18 million people in Latin America. According to World Health Organization (WHO), 7–8 million individuals are infected worldwide (WHO 2015b). Visceral leishmaniasis, which is also known as Kala-azar, is caused by *Leishmania donovani* (Marinho et al. 2015). It is estimated that 200 000–400 000 new cases occur worldwide each year (WHO 2015c). Although there are some available chemotherapeutic drugs for the treatment of these infectious parasitic diseases, the occurrence of widespread drug-resistant strains as well as the serious adverse effects of the currently used drugs points out the necessity of new, safe and effective antiprotozoal agents. The use of medicinal plants as a promising source for the development of new

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natural antiprotozoal drugs has long been a wise approach.

The genus *Ajuga* L. (Lamiaceae) comprises around 300 species worldwide. Some *Ajuga* species, such as *A. remota,* is traditionally utilized as an herbal remedy for the treatment of fever, infections, malaria as well as tuberculosis (Israili and Lyoussi 2009; Cocquyt et al. 2011). Hypoglycaemic, anti-arthritic, antioxidant, antimicrobial, anti-fungal and anti-inflammatory effects of *Ajuga* species have also been reported in previous studies (Kariba 2001; El-Hilaly and Lyoussi 2002; El-Hilaly et al. 2006; Kaithwas et al. 2012; Singh et al. 2012; Ben Mansour et al. 2013; Hsieh et al. 2014). The genus *Ajuga* is represented by 11 species in the flora of Turkey (Davis 1982). Some of these species are consumed for the treatment of haemorrhoids and as a wound-healing agent in Anatolian folk medicine (Sezik et al. 1992; Yesilada et al. 1995). Previous phytochemical studies on *Ajuga* species have shown the presence of diterpenes, phytoecdysteroids, iridoids, sterol glycosides, flavonoids and phenylethanoid glycosides as the secondary metabolites of the genus (De la Torre et al. 1997; Takasaki et al. 1998; Akbay et al. 2002, 2003; Sadati et al. 2012; Inomata et al. 2013). There is only one report on *A. laxmannii* (L.) Bentham collected from Bulgaria stating the isolation of a diterpene, a coumarin, a phytoecdysteroid, two iridoid glycosides and an *o*-coumaric acid derivative (Malakov et al. 1998).

In the continuation of our studies on the isolation and structure elucidation of antiprotozoal metabolites from the plants of the family Lamiaceae found in Turkish flora, now we have selected *A. laxmannii* for the identification of its antiprotozoal secondary metabolites. Our rationale for the selection of this genus was based on the use of some *Ajuga* species for the treatment of malaria, as well as fever, which is a common symptom of all parasitic diseases of interest herein. We were further encouraged by the reports on the *in vivo* and *in vitro* antiplasmodial effects of the water extracts of some *Ajug*a species, e.g. *A. remota* which is traditionally used against fever and infections in Kenya (Kuria et al. 2001; Gitua et al. 2012). Herein we describe the isolation and structure elucidation of eight glycosides from *A. laxmannii* as well as their *in vitro* antiprotozoal activities. To our knowledge, this is the first study revealing the *in vitro* antiprotozoal potential of *A. laxmannii* and its secondary metabolites.

Materials and methods

General

NMR spectra were recorded on a Bruker AVANCE 500 MHz Bruker spectrometer (Bruker Corporation, New Orleans, LA). MS analyses were performed on a Micromass Q-TOF Ultima Global Tandem Mass Spectrometer (Bruker Corporation, New Orleans, LA) using various concentrations of ACN in water and 0.1% formic acid. Kieselgel 60 (0.063–0.200 mm; Merck, Darmstadt, Germany) and polyamide (Fluka, Newport News, VA) were used for column chromatography (CC). Thin-layer chromatography (TLC) analyses were conducted on precoated Kieselgel 60 F_{254} (Merck, Darmstadt, Germany) plates. For medium-pressure liquid chromatography (MPLC), CombiFlash Companion (Teledyne Isco, Lincoln, NE) apparatus equipped with RediSep columns (C_{18}, 130 and 43 g; Teledyne Isco, Lincoln, NE) was used.

Plant material

The aerial parts of *A. laxmannii* were collected from Vize, Kırklareli, Turkey, in May 2008. The plant material was identified by Prof. Dr. Galip Akaydin (Department of Biology Education, Faculty of Education, University of Hacettepe, Beytepe, Ankara, Turkey). A voucher specimen (Akaydin 11862) has been deposited at the Herbarium of the Faculty of Education, Hacettepe University, Ankara, Turkey.

Extraction and isolation

The air-dried and powdered aerial parts of *A. laxmannii* (300 g) were extracted with methanol (MeOH) (2.0 L) by macerating for 3 d at room temperature. The extract was filtered through a filter paper and evaporated to dryness under reduced pressure to give the crude MeOH extract (AjuLaxCRMeOH; 48,1 g, yield: 16.0%). The MeOH extract was then dispersed in 75 mL of MeOH–H_2O (40:35) mixture and extracted with *n*-hexane (4 × 75 mL) in order to remove lipids and waxes. The aqueous MeOH phase was concentrated under reduced pressure and 40 mL H_2O was added before partitioning against $CHCl_3$ (4 × 75 mL). The H_2O subextract (AjuLax–H_2O; 30 g) was applied on a polyamide (110 g) column eluting with H_2O (300 mL) and a stepwise gradient of MeOH in H_2O (10–100% in steps of 10%, each 200 mL) to yield 12 fractions (Fr A–L). Fraction D (610 mg) was separated by C_{18}-MPLC (130 g, 5–70% MeOH in H_2O) and afforded Fr. D_5, **3** (14 mg) and D_7. Compound **1** (8 mg) was purified from Fr. D_5 (20 mg) by SiO_2 (4 g) column chromatography [CC] eluting with mixtures of $CHCl_3$–MeOH–H_2O (85:15:1, 80:20:2 and 70:30:3). Likewise, Fr. D_7 (52 mg) yielded compounds **2** (7 mg) and **5** (10 mg) by CC over SiO_2 (9 g) and eluting with mobile systems of $CHCl_3$–MeOH–H_2O mixtures (95:5:0.5, 85:15:1 and 80:20:2). Fraction F (802 mg) was applied to C_{18}-medium pressure

liquid chromatography (C_{18}-MPLC, 130 g) and eluted with MeOH/H_2O mixtures (10–100%) to obtain F_7 (78 mg) which was further chromatographed over silica gel CC (14 g) eluting with $CHCl_3$–MeOH–H_2O mixtures (95:5, 90:10:1, 85:15:1, 80:20:1, 75:25:1, 70:30:3 and 61:32:7) to afford **7** (7 mg). Fraction H (230 mg) was subjected to C_{18}-MPLC (43 g) eluting with MeOH/H_2O mixtures (10–100%) to obtain **4** (5 mg) and Fr. H_3 (59 mg). The latter was further purified by SiO_2 (9 g) CC eluting with $CHCl_3$–MeOH–H_2O mixtures (95:5:0.5, 90:10:1, 85:15:1, 80:20:2 and 75:25:1) to yield compound **6** (20 mg). Fraction J (900 mg) was similarly chromatographed by C_{18}-MPLC (130 g) using MeOH/H_2O mixtures (20 to 90%) as an eluent to yield compound **8** (513 mg).

In vitro assay for Plasmodium falciparum

Activity against erythrocytic stages of *P. falciparum* was determined by an *in vitro* modified [^{3}H]-hypoxanthine incorporation assay, using the chloroquine- and pyrimethamine-resistant K1 strain. Briefly, parasite cultures were incubated in RPMI 1640 medium with 5% Albumax (without hypoxanthine). A series of drug dilutions were applied in microtitre plates and incubated for 48 h at 37 °C in a reduced oxygen atmosphere. After incubation, 0.5 µCi ^{3}H-hypoxanthine was added to each well. Cultures were incubated for a further 24 h before they were harvested onto glass–fibre filters and washed with distilled water. The radioactivity was counted using a Betaplate^{TM} 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. IC_{50} values were calculated from the sigmoidal inhibition curves using Microsoft Excel. Chloroquine was used as a standard drug.

In vitro assay for Trypanosoma brucei rhodesiense

Trypanosoma brucei rhodesiense STIB 900 strain which was isolated in 1982 from a human patient in Tanzania and after several mouse passages cloned and adapted to axenic culture conditions was used in this assay. Minimum essential medium (50 µL) supplemented with 25 mM HEPES, 1 g/L additional glucose, 1% MEM non-essential amino acids (100×), 0.2 mM 2-mercaptoethanol, 1 mM Na-pyruvate and 15% heat inactivated horse serum was added to each well of a 96-well microtitre plate. Serial drug dilutions of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/mL were prepared. Then 10^4 blood stream forms of *T. b. rhodesiense* STIB 900 in 50 µL were added to each well and the plate was incubated at 37 °C under a 5% CO_2 atmosphere for 72 h. Alamar Blue (resazurin, 12.5 mg in 100 mL double-distilled water) (10 µL) was then added to each well and incubated for a further 2–4 h. At the end of the assay, the plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA) 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). Melarsoprol was used as standard drug for the assay.

In vitro assay for Trypanosoma cruzi

Rat skeletal myoblasts (L-6 cells) were seeded in 96-well microtitre plates (2000 cells/well) in 100 µL RPMI 1640 medium with 10% FBS and 2 mM L-glutamine for 24 h. After that, the medium was removed and replaced by 100 µL per well containing 5000 trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the β-galactosidase (*Lac Z*) gene. The medium was removed from the wells after 48 h, and replaced by 100 µL fresh medium with or without a serial drug dilution of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/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/Non-idet (50 µL) was added to all wells. A colour reaction developed within 2–6 h and could be read photometrically at 540 nm. Data were transferred into the graphic program Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA) and the IC_{50} values were calculated. Benznidazole was used as standard drug in this assay.

In vitro assay for Leishmania donovani

Amastigotes of *L. donovani* (strain MHOM/ET/67/L82) were grown in axenic culture at 37 °C in the SM medium at pH 5.4 supplemented with 10% heat-inactivated FBS under an atmosphere of 5% CO_2. Culture medium (100 µL) with 10^5 amastigotes from axenic culture with or without a serial drug dilution was seeded in 96-well microtitre plates. Serial drug was prepared covering a range of dilutions from 90 to 0.123 µg/mL. 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) was added to each well and the plates were incubated for another 2 h. The plates were then read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analysed using the software Softmax Pro (Molecular Devices Cooperation,

Sunnyvale, CA). A 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 IC_{50} values were calculated. Miltefosine was used as a reference drug.

In vitro assay for cytotoxicity on mammalian cells

Assays were performed in 96-well microtitre plates ($4 \times 10^4\,L^{-6}$ cells/well), each well containing 100 µl of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% FBS. L-6 cells are 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) 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 (Molecular Devices Cooperation, Sunnyvale, CA) 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). Podophyllotoxin was the positive control.

Results

The *in vitro* antiprotozoal activity assays and cytotoxicity test against mammalian L6 cells were performed as described by Kirmizibekmez et al. (2011). As shown in Table 1, the crude MeOH extract (AjuLaxCRMeOH) of the plant showed moderate antiprotozoal activity against *T. b. rhodesiense* and *L. donovani* (IC_{50} values of 10.8 and 30.1 µg/mL, respectively) without cytotoxicity towards mammalian L6 cells at the highest test concentrations. After partition against *n*-hexane and $CHCl_3$, the water subextract (AjuLax–H_2O) of the crude extract was selected for in depth chemical studies, as the water infusion of the *Ajuga* species are traditionally used for antimalarial or fever-relieving effects (Kuria et al. 2001). The H_2O subextract exerted trypanocidal activity against *T. b. rhodesiense* and *T. cruzi* (IC_{50} values of 17.5 and 37.4 µg/mL, respectively), and was subjected to a gravity-driven column chromatography on polyamide material. Further chromatographic separations of the polyamide fractions by a combination of RP-MPLC and gravity-driven silica CC led to the isolation of eight known secondary metabolites (**1**–**8**) (Figure 1).

The chemical structures of the compounds were identified as harpagide (**1**), 8-*O*-acetylharpagide (**2**) (Calis et al. 1992), *cis*-melilotoside (**3**), *trans*-melilotoside (**4**) (Vasänge et al. 1997), dihydromelilotoside (**5**) (Ranarivalo et al. 1990) verbascoside (**6**) (Sticher and Lahloub 1982), galactosylmartynoside (**7**) (Takasaki et al. 1998) and isoorientin (**8**) (Calis et al. 2006) by comparison of their 1D and 2D NMR and MS data with those published previously.

Table 1 displays the antiprotozoal activities of the isolated compounds from *A. laxmannii*. Since some of the isolates (**1**, **2** and **6**) had been isolated and evaluated for their *in vitro* antiprotozoal activity in our previous studies using the same panel of parasitic protozoa and experimental methods (Kirmizibekmez et al. 2004; Tasdemir et al. 2008), they were not tested again to avoid duplication, however, their activity results are included in Table 1 to allow comparison. It has to be noted that these compounds have shown quite remarkable potential, against *T. b. rhodesiense* and *L. donovani*. The most significant activity was exhibited by the iridoid glucoside 8-*O*-acetylharpagide (**2**) against *L. donovani* with an IC_{50} value of 2.0 µg/mL. Their trypanocidal activity was lower (IC_{50} values from 14.2 to 26.9 µg/mL). The remaining

Table 1. *In vitro* antiprotozoal and cytotoxic activities of crude MeOH extract (AjuLaxCRMeOH), subextracts (AjuLax–Hex, AjuLax–$CHCl_3$ and AjuLax–H_2O) and compounds **1**–**8** from *Ajuga laxmannii* (IC_{50} in µg/mL).

Extract/compound	*T. b. rhodesiense*	*T. cruzi*	*L. donovani*	*P. falciparum*	Cytotoxicity (L6 cells)
AjuLaxCRMeOH	10.8	>90	30.1	>50	>90
AjuLax–Hex	8.4	88.7	7.8	7.5	65.4
AjuLax–$CHCl_3$	12.1	35.9	7.4	3.2	36.9
AjuLax–H_2O	17.5	37.4	>90	>50	9.01
Standard	0.003[a]	0.38[b]	0.201[c]	0.12[d]	0.008[e]
1[f]	21.0	>90	2.0	>50	>90
2[f]	26.9	>90	6.9	>50	>90
3	>100	78.2	>100	>50	84.1
4	58.9	58.0	>100	48.7	63.6
5	>100	56.3	>100	>50	76.2
6[g]	14.2	>90	8.7	>50	37.1
7	31.6	57.4	>100	>50	61.0
8	52.8	57.2	>100	9.7	71.6

Reference drugs: [a]Melarsoprol, [b]Benznidazole, [c]Miltefosine,[d]Chloroquine, [e]Phodophllotoxin. [f]These results are from our earlier studies (Tasdemir et al. 2008). [g]These results are from our earlier studies (Kirmizibekmez et al. 2004).

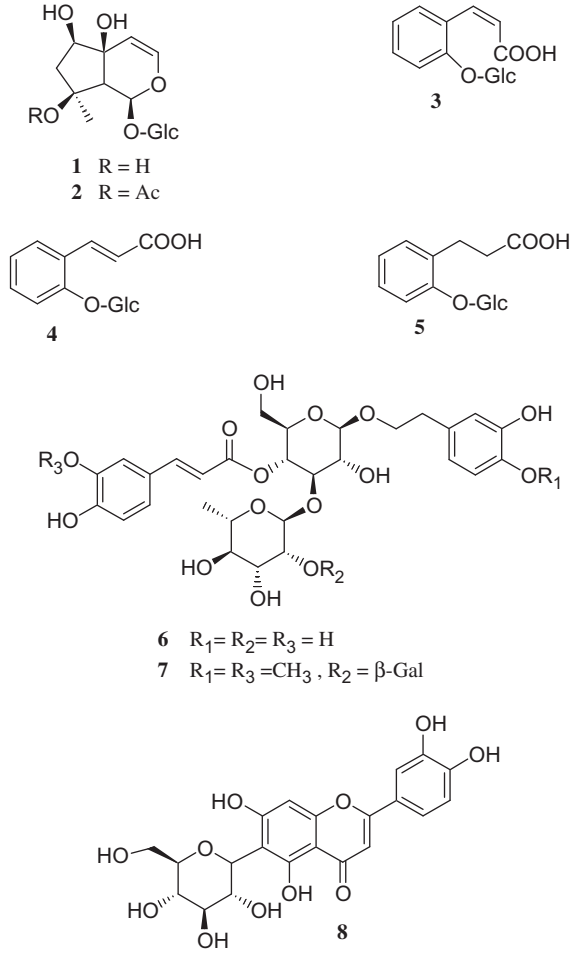

Figure 1. Structures of the isolated compounds (**1**–**8**).

compounds, with the exception of **3** and **5**, exerted moderate activity against *T. b. rhodesiense* with IC_{50} values ranging from 31.6 to 58.9 µg/mL (Table 1). All compounds except for **1**, **2** and **6** moderately arrested the growth of American trypanosome *T. cruzi* with IC_{50} values ranging from 56.3 to 78.2 µg/mL. Although the water subextract was inactive against *P. falciparum,* two compounds, *trans*-melilotoside (**4**) and isoorientin (**8**) displayed antimalarial effect, with isoorientin (**8**) appearing the most potent one (an IC_{50} value of 9.7 µg/mL). This is probably due to masking effect of other, more major compounds found in the H_2O subextract. Cytotoxicity assay towards mammalian (rat) L6 cells revealed that most of the compounds possess low cytotoxicity, at least at their active concentrations.

Discussion

The genus *Ajuga* has been reported to contain iridoids, phenylethanoid glycosides, flavonoids, anthocyanins and *o*-coumaric acid derivatives (De la Torre et al. 1997; Takasaki et al. 1998; Akbay et al. 2002, 2003; Sadati et al. 2012; Inomata et al. 2013). Among the isolated compounds in this study, harpagide or its acetyl derivative 8-*O*-acetylharpagide were also reported from other genera of Lamiaceae including *Stachys* (Calis et al. 1992), *Lamium* (Alipieva et al. 2003) and *Galeopsis* (Venditti et al. 2013). Harpagide has also been reported from the genus *Scrophularia* (Tasdemir et al. 2005). Iridoids are accepted as significant chemotaxonomic markers in dicotyledonous plants. Thus, the occurrence of harpagide or its 8-*O*-acetyl derivative might imply a chemotaxonomic proximity between the genus *Ajuga* and the other aforementioned genera of Lamiaceae along with the genus *Scrophularia* from the family Scrophulariaceae. Among the isolates, *cis*- and *trans*-mellitoside (**3** and **4**) have previously been reported from only one *Ajuga* species, namely *Ajuga chamaecistus* ssp. *tomentella* (Sadati et al. 2012). This is the second report of the presence of mellitoside from a member of the *Ajuga* genus. Flavone-*C*-glycosides are relatively rare compounds when compared with their *O*-analogues. They were previously reported to be present in few genera of Lamiaceae such as *Scutellaria*, *Stachy* and *Ajuga*. Regarding the flavonoids, the *O*-glycosides of apigenin and luteolin account for the major flavonoidal constituents of the genus *Ajuga,* while *C*-flavonoid glycosides such as isovitexin and orientin were rarely encountered (Israili and Lyoussi 2009; Singh et al. 2012). It is noteworthy that isoorientin was found as one of the major constituents of *A. laxmannii* in this study. Isoorientin (**8**) and dihydromelilotoside (**5**) are being reported for the first time from the genus *Ajuga*. Although this is the second phytochemical study on *A. laxmannii*, six of the isolates (**3**–**8**) are being reported for the first time from the title plant. Moreover, the extracts and isolates were evaluated for their *in vitro* antiprotozoal and cytotoxic potential for the first time. Of note, although the water subextract had no leishmanicidal or antiplasmodial activity at the highest test concentrations, several compounds isolated from this extract showed such activity. The activity is probably masked by the inactive compounds (e.g. sugars) that occur in high concentrations in this subextract.

In a previous study, commercially available *trans*-*m*-coumaric acid, *trans*-*o*-coumaric acid and *trans*-*p*-coumaric acids were examined against *L. major*, but none of them were found to be active (Takahashi et al. 2004). In this study, on one hand, *trans*-melilotoside (**4**), which is the *o*-glycosylated form of trans-*o*-coumaric acid, was also inactive against *L. donovani*, but exhibited almost equipotent trypanocidal and antiplasmodial activities. On the other hand, *cis*-melilotoside (**3**) and dihydromelilotoside (**5**) were only moderately active against *T. cruzi*. This may imply that *trans* configuration

may be necessary for activity against *T. b. rhodesiense* and *P. falciparum*.

In our previous study, glucopyranosyl-(1→G_i-6)-martynoside isolated from another Turkish Lamiaceae plant, *Phlomis brunneogaleata,* was shown to be active against *L. donovani* and *T. b. rhodesiense* (Kirmizibekmez et al. 2004). In the current work, a very structurally related compound, galactosylmartynoside (**7**), was found to be active against both forms of *Trypanosoma*. To our knowledge, it is the first report of the antitrypanosomal activity of galactosylmartynoside.

Isoorientin (**8**) has previously been reported as the antimalarial component of *Hymenocardia acida* from Phyllantaceae (Murakami et al. 2005). In the current study, isoorientin is being reported as the most active antimalarial principle of *A. laxmannii,* which is consistent with the previous study. Furthermore, isoorientin also displayed moderate activity against *T. b. rhodesiense* and *T. cruzi* in our panel of assays.

Conclusion

In conclusion, this is the first report on the *in vitro* antiprotozoal activity testing and antiprotozoal activity-guided isolation of eight secondary metabolites from Turkish *A. laxmannii*. The current study provides a preliminary confirmation of the traditional use of *Ajuga* species in the relief of the symptoms of parasitic diseases. Among the tested metabolites, the flavone-*C*-glycoside, isoorientin (**8**) may deserve further studies such as *in vivo* antimalarial tests. Finally, this study also reveals the chemotaxonomic potential of some of the metabolites for the genus *Ajuga* within the family Lamiaceae.

Declaration of interest

The authors report that they have no conflicts of interest.

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