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Exploring the therapeutic potential and phenolic composition of two Turkish ethnomedicinal plants – *Ajuga orientalis* L. and *Arnebia densiflora* (Nordm.) Ledeb.

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ABSTRACT

Ajuga orientalis L. and *Arnebia densiflora* (Nordm.) Ledeb. are plants traditionally used in Turkish ethnomedicine to manage common ailments. Nonetheless, there is currently a dearth of investigations geared towards highlighting the inhibitory properties of *A. orientalis* and *A. densiflora* against key carbohydrate hydrolyzing enzymes (α-amylase and α-glucosidase), tyrosinase, and cholinesterases (acetylcholinesterase and butyrylcholinesterase) involved in diabetes, post inflammatory hyperpigmentation, and neurodegenerative diseases, respectively. We aimed to shed light on the antioxidant potential and various enzymatic inhibitory properties of tested methanolic extracts of these plants. *In vitro* antioxidant activity, DNA protective effects, antimicrobial activity (against 8 bacteria, 10 fungal strains and 1 yeast), as well as phenolic compounds determination and HPLC (high performance liquid chromatography) analysis were performed. 17 phenolic components were quantified in tested extracts. Major phenolic compounds present in *A. orientalis* extract were chlorogenic acid, benzoic acid, and luteolin. *A. densiflora* extract revealed the presence of gallic acid, rutin, rosmarinic acid, quercetin, luteolin, and apigenin. *A. densiflora* showed a stronger antioxidant capacity, higher total phenolic (33.72 mg GAE/g), and total flavonoid contents (55.93 mgRE/g), as well as protection of DNA against hydroxyl radical compared to *A. orientalis* extract. The tested extracts showed similar antimicrobial activity with low effect against the bacterial and fungal strains. The tested extracts showed a slightly higher potential to inhibit tyrosinase activity, while inhibition of the activity of the other examined enzymes by the extracts was weak. The observed biological activities and presence of bioactive phytochemicals can open new perspectives to develop novel dietetic supplements.

1. Introduction

The *Ajuga* genus belongs to Lamiaceae family and is composed of 301 species (Göger et al., 2015). In Turkey, *Ajuga* species are traditionally used in primary health care as diuretic, antipyretic, tonic, diaphoretic, and astringent (Baytop, 1999). On the other hand, *Ajuga* species are used worldwide in the treatment/management of gout, rheumatism, malaria, asthma, and gastrointestinal diseases. Reported biological properties include antibacterial, antitumor, neuroprotective effects, anti-inflammatory, and antioxidant activity (Israili and Lyoussi, 2009; Qing et al., 2017; Toiu et al., 2018). This genus showed the presence of various phytochemicals such as anthocyanins, diterpenoids, sterols, ionones, iridoids, phenylethanol, and flavonoid glycosides (Göger et al., 2015). Panoply of biologically active compounds has been isolated from this genus. These phytocompounds have showed antibacterial activities against *Staphylococcus aureus*, cancer chemopreventive, hypoglycemic properties, vasoconstrictor and antiarthritic effects in acute and chronic models, anti-inflammatory, cytotoxicity against Jurkat cells, anti-proliferation against tumor cells *in vitro*, neuroprotective potential against MPP^+ and antimalarial activity

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(Ebrahimabadi et al., 2013). The main components of the essential oil of *Ajuga orientalis* were found to be germacrene D (24.7%), β- cubaben (18.3%), and β- caryophyllene (16.9%) (Göger et al., 2015).

Arnebia densiflora belongs to the Boraginaceae family (Akkol et al., 2009). This species is used as folk medicine in Turkey. The pigmented root and/or root bark of the plant is locally termed as 'Eyilik'. Eyilik is usually kept in olive oil and applied on wounds, cuts, and scratches for quick recovery since ancient times in Anatolia. In Malatya district, a wound healing ointment is prepared using the roots of the plant which is mixed with melted butter. The roots are then removed, the butter is combined with bee wax and the ointment is used against open wounds (Akkol et al., 2009). *A. densiflora* root bark is used against vulnerary diseases and burns (Altundag and Ozturk, 2011). Phytochemicals present in the roots of some genera of the Boraginaceae family include naphthoquinones, shikonin (R configuration), alkanin (S-configuration), and their derivatives show a widespread range of biological activities such as wound healing, antibacterial, antifungal, antiviral, antiamoebic, anti-inflammatory, topoisomerase I and II inhibitory activities, and anticancer properties (Akkol et al., 2009). In this sense, these two plants have been widely used in folk medicine and recently received much attention of the scientific community for their medicinal properties. Nonetheless, there is still a paucity of studies to highlight the potential of extracts prepared from these plants as other pharmacological targets.

In this study, the inhibitory properties of the extracts prepared from *A. orientalis* and *A. densiflora* were assessed against key carbohydrate hydrolyzing enzymes involved in diabetes (α-amylase, and α-glucosidase), postinflammatory hyperpigmentation (tyrosinase), and cholinesterases involved in neurodegenerative diseases (acetylcholinesterase and butyrylcholinesterase). In addition, the *in vitro* DNA protective effects of the two plants were studied against hydroxyl radical-induced DNA damage. Moreover, the antimicrobial effects of the studied extracts were also assessed. Therefore, this study was set out to investigate enzymatic inhibitory, antimicrobial, DNA protective effects, and antioxidant activities of the *A. orientalis* and *A. densiflora* methanolic extracts. Total flavonoid and phenolic contents were also profiled using HPLC and spectrophotometrical analysis to compare any observed biological activity.

2. Material and methods

2.1. Plant materials collection and extraction

Different samples of the aerial parts of *Ajuga orientalis* L. (between Adana and Pozantı, Turkey) and *Arnebia densiflora* (Nordm.) Ledeb. (between Konya and Beysehir, Turkey) were collected during flowering season (summer) in 2014 and air-dried at room temperature until constant weight. Botanical confirmation was done by Dr. Murad Aydın Sanda, a senior taxonomist of the Department of Biology, Selcuk University, Turkey.

In order to prepare samples extracts, the air-dried samples (5 g) were subjected to exhaustive maceration with 100 mL of methanol at room temperature for 24 h. Further, the solvents were removed under vacuum at 40 °C by using a rotary evaporator. All samples were then stored at + 4 °C in the dark until further use.

2.2. Profile of bioactive compounds

Phenolic and flavonoid compounds of the extracts were totally noted, as previously reported (Slinkard and Singleton, 1977; Zengin et al., 2016) by using well-established procedures such as Folin-Ciocalteu and $AlCl_3$ tests, respectively. Their contents were expressed as gallic acid (mg GAEs/g extract) and rutin equivalents (mg REs/g extract), respectively.

Several phenolic compounds in the methanol extracts were also analyzed using RP-HPLC-DAD (Shimadzu Scientific Instruments, Kyoto, Japan). Separation procedure was achieved at 30 °C on Eclipse XDB C-18 reversed-phase column (250 mm × 4.6 mm length, 5 µm particle size, Agilent, Santa Clara, CA, USA) under optimized experimental conditions (Movahhedin et al., 2016). Each phenolic compound was characterized and quantitated (as µg/g dry extract) after comparison of retention times, UV–Vis spectra, and chromatographic profile with commercial standard compounds.

2.3. Antioxidant and enzyme inhibition assays

The antioxidant and enzyme inhibitory effects of the methanol extracts were determined. Among the well-known antioxidant assays, metal chelating, phosphomolybdenum, FRAP, CUPRAC, ABTS and DPPH tests were performed and compared. The antioxidative activities were reported as trolox equivalents, whereas EDTA was used as a reference compound for metal chelating assay. The enzyme inhibitory activity was detected against a panel of important enzymes such as cholinesterases (AChE and BChE), tyrosinase, α-amylase and α-glucosidase comparing the results with those obtained with specific standard drugs (galantamine, kojic acid, and acarbose). All experimental procedures were in agreement with those reported by Grochowski et al. (2017).

2.4. In vitro DNA protective effect against hydroxyl radical

The experiments were performed with Herring sperm DNA (Carl Roth GmbH, Karlsruhe, Germany) according to standard procedure that was previously described (Lin et al., 2008; Matić et al., 2015). Extracts were dissolved in methanol at 1 mg/mL and various concentrations (25, 50, 100, 200, and 400 µg/mL) were separately taken into eppendorf tubes. The blank was the Herring sperm DNA solution, while quercetin (50 µM) was used as a reference compound (Poorna et al., 2013). Each mixture (5 µL) was loaded onto a 1% agarose gel in 1xTAE buffer containing ethidium bromide (10 mg/mL) and were run at 100 V in an electrophoresis system (BlueMarine™ 100, SERVA Electrophoresis GmbH, Heidelberg, Germany). The gels were visualized (UV transilluminator, Vilber Lourmat at 365 nm), photographed and DNA bands intensity were quantified using ImageJ software (version 1.48 for Windows).

2.5. Antimicrobial assays

The antimicrobial activity was evaluated using microdilution method of Sarker et al. (2007), against a panel of eight bacterial and ten fungal species of medical interest, along with yeast *Candida albicans,* as already experimentally described in our previous study (Katanić et al., 2017). All these microbial strains were kindly provided from the Faculty of Chemistry, University of Belgrade and Laboratory for Microbiology, Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia. The bacteria and fungi cultures were stored at + 4 °C and subcultured once a month. Erythromycin was used a positive control for evaluating antibacterial activity, and nystatin and ketoconazole were selected as standard antifungal compounds.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) (with Tukey's assay) was employed to detect differences among the extracts ($p < 0.05$). This statistical analysis was calculated by SPSS v. 17.0 program.

3. Results and discussion

During normal cell metabolism, several reactive toxic molecules can be produced. These toxic molecules are highly reactive and are mainly reactive oxygen species. Free radicals such as singlet oxygen, superoxide ion, hydroxyl ion, and hydrogen peroxide can induce tissue injury