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Novel perspectives on two *Digitalis* species: Phenolic profile, bioactivity, enzyme inhibition, and toxicological evaluation

J. Katanić [a], R. Ceylan [b], S. Matić [c], T. Boroja [a], G. Zengin [b,*], A. Aktumsek [b], V. Mihailović [a], S. Stanić [c]

[a] Department of Chemistry, Faculty of Science, University of Kragujevac, Radoja Domanovića 12, 34000 Kragujevac, Serbia
[b] Department of Biology, Science Faculty, Selcuk University, 42075 Konya, Turkey
[c] Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Radoja Domanovića 12, 34000 Kragujevac, Serbia

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ABSTRACT

The present study outlines the phenolic composition of methanolic extracts of *Digitalis ferruginea* and *Digitalis lamarckii* aerial parts and evaluates their biological activities. The quantification of phenolic compounds was carried out by HPLC–DAD analysis. Antioxidant properties were assessed using different assays. Enzyme inhibition was tested on AChE, BChE, α-amylase, α-glucosidase, and tyrosinase. The antimicrobial properties of the extracts were determined using the microdilution method. Genotoxicity of the extracts was assessed on *Drosophila melanogaster* by comet assay. *D. lamarcki* extract was richer in phenolic compounds than *D. ferruginea*, with chlorogenic acid as dominant compound in both *D. lamarckii* and *D. ferruginea* extracts (2.99 and 1.30 mg/g, respectively). Both extracts showed similar values in terms of antioxidant activity. The extracts showed enzyme inhibitory activity on above-mentioned enzymes and moderate antimicrobial activity (MIC range 0.625–10 mg/mL). *D. ferruginea* extract (20 mg/mL) indicated the absence of genotoxicity, while all concentrations of *D. lamarckii* extract showed significant DNA damage. Antigenotoxic effect was more evident in the group treated with *D. lamarckii* extract, 80 mg/mL (%R = 80.3). The results emphasized the biological potential of examined plants and encourage further studies for their potential use as phytopharmaceuticals.

1. Introduction

Medicinal plants have been used since pre-historic ages in the treatment of various diseases. In spite of the great advances observed in contemporary medicine in the last decades, plants still have a significant influence in the therapeutic arsenal (Cragg et al., 1997; Shu, 1998; Gao et al., 2012). Thus, over the past decade, the use of medicinal plants has progressively increased together with the number of investigations of their biological effects (Newman and Cragg, 2007). It is known that medicinal plants contain several classes of phytochemical compounds, especially secondary metabolites, with different biological effects such as antioxidant, antimicrobial, antimutagenic, anticarcinogenic, antigenotoxic, anti-inflammatory effects, and enzyme inhibitory properties (including global health problems like Alzheimer disease, diabetes mellitus, etc.) (Leopoldini et al., 2011; Gyawali and Ibrahim, 2014; Velderrain-Rodríguez et al., 2014; Zengin et al., 2014a,b). This knowledge stimulated an increased interest for natural products from medicinal plants which could be used as new and promising therapeutical agents.

The genus *Digitalis* (Plantaginaceae) consists of about 36 species, of which nine grow wild in Turkish flora (Davis, 1978). *Digitalis* species, especially *Digitalis purpurea* and *D. lanata* are pharmacologically important plants as they contain cardiac glycosides (digitoxin, gitoxin, digoxin, etc.) that strengthen cardiac diffusion and regulate heart rhythms (Baytop, 1999). Steroidal saponins, pregnane glycosides, and phenylethanoid glycosides are the main compounds occurring in the chemical composition of the genus *Digitalis*, in addition to cardiac glycosides (Kırmızıbekmez et al., 2009; Perrone et al., 2012; Kirmizibekmez et al., 2014). Among the other compounds occurring in *Digitalis* species, flavones, anthraquinones, and organic acids have also been detected (Usai et al., 2007). Besides very important role of *Digitalis* cardiac glycosides in modern medicine, some *Digitalis* species are used in Turkish traditional medicine as diuretics, stimulants, and tonics (Benli et al., 2009). Based on the reported traditional uses of *Digitalis* species, *Digitalis ferruginea subsp. ferruginea* and *Digitalis lamarckii* may be considered as valuable sources of bioactive agents.

To the best of our knowledge, there is a little scientific evidence on chemical profiles and biological activity of these two plants so far. Regarding the aforementioned facts, this study focused on potential usefulness of these *Digitalis* species based on bioactivity evaluation of methanolic extracts from their aerial parts by antioxidant activity assays, determination of anti-cholinesterase, anti-amylase, anti-glucosidase and anti-tyrosinase effects, and antimicrobial activity. To have a precise evaluation of toxic effects the assay for genotoxic and antigenotoxic activity was employed. Moreover, HPLC-DAD analysis

* Corresponding author.
E-mail address: gokhanzengin@selcuk.edu.tr (G. Zengin).

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was used in order to have the accurate insight into the phenolic profile of these two *Digitalis* species.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used for analyses of total phenolic content, antioxidant and enzyme inhibitory activities were of analytical grade and purchased from Sigma-Aldrich, St. Louis, MO, USA. Nutrient agar (NA), Sabouraud dextrose agar (SDA), Mueller–Hinton broth (MHB), and Sabouraud dextrose broth (SDB) were purchased from Torlak Institute of Virology, Vaccines and Sera (Belgrade, Serbia). For antigenotoxic analysis the phosphate-buffered saline (PBS) without calcium and magnesium, agarose for DNA electrophoresis, low-melting point agarose (LMA), and collagenase were obtained from Alfatrade Enterprise D.O.O. (Serva Electrophoresis GmbH, Heidelberg, Germany); methyl 4-hydroxybenzoate and ethyl methanesulphonate (EMS) were purchased from Sigma-Aldrich, St. Louis, MO, USA. All other chemicals were obtained locally and were of analytical reagent grade.

2.2. Plant material and preparation of the extracts

Aerial parts of *D. ferruginea* subsp. *ferruginea* and *D. lamarckii* were collected at flowering stage (June and July, 2013) in Turkey. Taxonomical information and localities of *Digitalis* species studied are given below. Identification of the plant materials was confirmed by the senior taxonomist Dr. Murad Aydin Sanda, in the Department of Biology at Selcuk University, Konya, Turkey, and the voucher specimen was deposited at the University's herbarium.

1. *Digitalis ferruginea subsp. ferruginea*: Afyon, between Suhut and Sandikli, 38° 28′ 31″ N, 30° 24′ 35″ E, 1550 m.
2. *Digitalis lamarckii*: Ankara, between Cubuk and Kizilcahamam, 40° 27′ 57″ N, 32° 49′ 27″ E, 1473 m (endemic).

The samples (10 g) were macerated with methanol (100 mL) at room temperature for 24 h. Extracts were filtered through a filter paper. Solvent from the samples was removed using a rotary evaporator to obtain the dry extracts in the yield of 21.2% and 26.1% (w/w), respectively.

2.3. Quantification of phenolics content by RP-HPLC

Phenolic compounds were evaluated by RP-HPLC (Shimadzu Scientific Instruments, Tokyo, Japan). Detection and quantification were carried out with LC-10ADvp pump, a Diode Array Detector, a CTO-10Avp column heater, SCL-10Avp system controller, DGU-14A degasser and SIL-10ADvp auto sampler (Shimadzu Scientific Instruments, Columbia, MD). Separations were carried out at 30 °C on Agilent® Eclipse XDB C-18 reversed-phase column (250 mm × 4.6 mm length, 5 μm particle size). The eluates were detected at 280 nm.

Phenolic compositions of the extracts were determined by a modified method of Sarikurkcu et al. (2014). Gallic acid, (+)-catechin, *p*-hydroxybenzoic acid, *p*-coumaric acid, chlorogenic acid, caffeic acid, (−)-epicatechin, ferulic acid, benzoic acid, rutin, rosmarinic acid, quercetin and apigenin were used as standards. The phenolic compounds were characterized according to the UV spectra, retention times, and comparison with authentic standards. The identified phenolic acids were quantified by comparison of the area of their peaks recorded at 280 nm (for phenolic acids) and 360 nm (for flavonoids) with calibration curves obtained from commercial standards of each compound. The results were expressed in mg per g of dry extract.

2.4. Total phenolic and flavonoids content

Total phenolic content was determined using the Folin–Ciocalteu colorimetric method with slight modification and expressed as gallic acid equivalents (GAEs/g extract), and total flavonoid content was determined by previously reported method (Zengin et al., 2014a) with slight modification and expressed as rutin equivalents (REs/g extract).

2.5. Antioxidant activity

The DPPH radical and ABTS radical cation scavenging activities were determined using the previously described method (Zengin et al., 2014b), and results were expressed as trolox equivalents (TEs/g extract).

The reducing power was measured according to the method reported by, using cupric ion reducing antioxidant power (CUPRAC) and ferric ion reducing antioxidant power, and results were expressed as trolox equivalents (TEs/g extract).

Total antioxidant capacity was determined using phosphomolybdenum method. The β-carotene/linoleic acid bleaching method was also employed (Zengin et al., 2014b). Metal chelating activity on ferrous ions, determined by the method described by Zengin et al. (2014b), was expressed as EDTA equivalents (EDTAEs/g extract).

2.6. Enzyme inhibitory activity

Enzyme inhibitory activities (acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), α-amylase, α-glucosidase, and tyrosinase inhibition) of the extracts were determined. In cholinesterase inhibitory assays, acetyl thiocholine iodide (ATCl) or butyryl thiocholine chloride (BTCl) were used as substrates and the results were expressed as milligrams of galanthamine equivalents (mg GALAE/g extract). Tyrosinase inhibitory activity was measured using a modified dopachrome method with L-DOPA as substrate and the results were expressed as kojic acid equivalents (mg KAE/g). α-Amylase and α-glucosidase inhibitory activities were determined with iodine/potassium iodide and chromogenic PNPG methods, respectively. The results of α-amylase and α-glucosidase inhibitory activities were expressed as standard drug equivalents (acarbose) (mmol ACE/g extract). The details of the enzyme inhibitory methods are given in our previous study (Zengin et al., 2014a).

2.7. Antimicrobial activity

2.7.1. Test microorganisms

The tests for evaluation of antimicrobial activity of *D. ferruginea* subsp. *ferruginea* and *D. lamarckii* extracts were performed on the selected ATCC cultures and clinically isolated strains of bacteria and fungi (FSB). For antibacterial activity measurements the following strains were used: *Klebsiella pneumoniae* (ATCC 70063), *Micrococcus lysodeikticus* (ATCC 4698), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 10145), *Pseudomonas fluorescens* (FSB 28), *Enterococcus faecalis* (FSB 24), *Agrobacterium tumefaciens* (FSB 11), and *Azobacter chroococum* (FSB 14). For the antifungal activity evaluation 11 fungal strains were assessed: *Candida albicans* (ATCC 10259), *Trichoderma harzianum* (FSB 12), *Trichoderma longibrachiatum* (FSB 13), *Penicillium cyclopium* (FSB 23), *Penicillium canescens* (FSB 24), *Aspergillus niger* (FSB 31), *Aspergillus glaucus* (FSB 32), *Fusarium oxysporum* (FSB 91), *Alternaria alternata* (FSB 51), *Doratomyces stemonitis* (FSB 41), and *Phialophora fastigiata* (FSB 81). All tested microbial strains were obtained from the Faculty of Chemistry, University of Belgrade, Serbia and Laboratory for Microbiology, Department of Biology, Faculty of Science, University of Kragujevac, Serbia. The bacteria and fungi cultures were stored at +4 °C and subcultured once a month. Bacterial strains were cultured overnight at 37 °C on nutrient agar (NA) and fungi were cultured on Sabouraud dextrose agar (SDA) and potato glucose agar (PDA) at 28 °C for 3 days.