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Short communication

Brine shrimp cytotoxic activity of methanolic extracts of *Juniperus communis* L. berries

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Abstract

Methanolic extracts from *Juniperus communis* L. berries collected from five different localities in the Republic of Macedonia were evaluated for their cytotoxicity by Brine shrimp lethality assay. The obtained cytotoxic activity is descending as follows: Pelister (128 μg/mL) > Jakupica (221 μg/mL) > Prilep (662 μg/mL) > Demir Hisar (863 μg/mL) > Makedonski Brod (969 μg/mL). Berries collected from mountain areas (Pelister and Jakupica) demonstrated prominent cytotoxic effects, while berries collected from localities near urban areas exhibited lower cytotoxicity. Variations in their bioactivity are probably due to their complex phytochemical composition, which may vary with different ecological and geographical conditions.

Keywords: *Juniperus communis* L. berries, methanolic extracts, brine shrimp lethality assay, probit analysis, LC_{50}

Introduction

Juniperus communis L. (common juniper) is an evergreen shrub belonging to the family Cupresaceae and is widely distributed throughout the Northern Hemisphere, from cool temperate to polar regions. Juniper trees are adapted for optimal growth on a wide range of soils and soil pH, and are drought tolerant (Lim, 2012). Common juniper shrubs grow natively in all parts of the Republic of Macedonia (Micevski, 1998). Ripe berries and needles have long been used for the treatment of gastrointestinal conditions, dyspepsia, flatulence, egzema (Charles, 2013). Indigenous people from the East were using the juniper berries as herbal tea to treat urinary tract infections and as tonic against flu, muscle aches and kidney problems, while the needles were mainly used as remedy for acute and chronic cystitis, albuminuria, renal suppression, amennorhoea (Bais et al., 2014). Despite the wide usage in traditional medicine, the biological activity of the *Juniperus communis* berries still remains insufficiently examined. Previous studies on the biological activity of the berries demonstrated their antibacterial, fungicidal (Pepeljnjak et al., 2005), antioxidant (Höferl et al., 2014), anticholinesterase (Orhan et al., 2011) and antiinflamatory (Han and Parker, 2017) activity. However, limited data are reported on their cytotoxic properties. Therefore, the aim of this study was *in vivo* determination of the cytotoxic potential of *Juniperus communis* L. berries collected from several localities of the Republic of Macedonia.

Materials and methods

Plant materials

The plant material was harvested in the year 2010 and 2011 from five different localities: Pelister (JCP), Jakupica

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(JCJ), Prilep (JCPr), Demir Hisar (JCH) and Makedonski Brod (JCB). Dried plant material was milled to fine powder and stored in airtight containers until use.

Preparation of extracts

Plant samples were extracted with methanol in ultrasonic bath. Obtained filtrates were evaporated until dry and later freeze dried and preserved at -18 °C in a dark place until use. Lyophilizates were reconstituted with DMSO (dimethyl sulfoxide).

Brine shrimp lethality assay

The cytotoxic potential of the extracts was evaluated by Brine Shrimp Lethality Assay *in vivo* (Meyer et al., 1982) based on the number of dead *Artemia salina* nauplii after 24 hours exposure to the plant samples. $K_2Cr_2O_7$ (potassium dichromate) was used as a positive control in a concentration range from 0.01 to 10 mg/mL. Final results were expressed as LC_{50} values using probit regression analysis (Finney, 1952). LC_{50} values represent the concentration of each extract needed to elicit mortality in 50% of the tested population of brine shrimps.

Toxicity criteria and classification of extracts

Based on the obtained LC_{50} values, plant extracts were classified according two scales of toxicity: Meyer's scale and Clarkson's scale (Meyer et al., 1982; Clarkson et al., 2004). Both scales classify extracts as toxic if their LC_{50} values were below 1000 μg/mL, while Clarkson's scale additionally categorizes them as extracts with high (0 – 100 μg/mL), moderate (100 – 500 μg/mL) and low (500 – 1000 μg/mL) toxicity.

Results and discussion

According the obtained data, it was established that all tested samples possess cytotoxic potential in different extent (Table 1). Their cytotoxic bioactivities diminish in the following order: JCP (128 μg/mL) > JCJ (221 μg/mL) > JCPr (662 μg/mL) > JCH (863 μg/mL) > JCB (969 μg/mL). Most prominent cytotoxic effect was observed for the berries collected from Pelister (128 μg/mL) and the lowest cytotoxic activity was demonstrated by the berries collected from Makedonski Brod (969 μg/mL).

The trend of mortality rate for the *Artemia* nauplii after their 24-hour exposure to each sample and to the positive control are demonstrated on Figure 1. A notable increase in toxicity was observed for the samples collected from Pelister (40% to 100%) and Jakupica (50% to 100%) at concentrations 1 mg/mL and 3 mg/mL, and for the positive control $K_2Cr_2O_7$ (100% mortality rate at 1 mg/mL). Moreover, a maximal mortality rate of 100% at the highest concentration (10 mg/mL) was observed for all samples except for the berries collected from Makedonski Brod.

J. communis berries collected from the localities of Pelister and Jakupica were classified as moderately toxic, while samples collected from locations near the cities of Prilep, Demir Hisar and Makedonski Brod were classified as extracts with low toxicity. The amount of certain bioactive compounds present in the berries may significantly vary as a result of differences in temperature, soil chemistry, soil acidity (Ložienė and Labokas, 2012), light quality (Artemkina et al., 2016), rainfall levels, season (Peñuelas et al., 2002) and altitude of the habitat (Martz et al., 2009). Ecological conditions of plant growth may significantly change the phytochemical composition of the plants upon transition from the base to the top of the mountains (Artemkina et al., 2016). In the current research, a moderate cytotoxicity was obtained for the samples collected from mountain areas (Pelister and Jakupica), while the berries collected from the localities near urban area with an altitude range from 550 m to 650 m (Prilep, Demir Hisar and Makedonski Brod) demonstrated low cytotoxicity. The variations in their cytotoxic activity are probably related to their complex phytochemical composition. This observation is supported by previous research on the variability in concentration of bioactive compounds present in *Juniperus* berries as a result of different latitude and altitude of the

Table 1. Cytotoxic potential of methanolic extracts of *Juniperus communis* berries collected from 5 different localities and a positive control according the Brine Shrimp Lethality Assay

Location	Abbr.	Year	LC_{50} (μg/mL)	Meyer's scale	Clarkson's scale
Pelister	JCP	2010	128	toxic	moderately toxic
Jakupica	JCJ	2010	221	toxic	moderately toxic
Prilep	JCPr	2011	662	toxic	low toxic
Demir Hisar	JCH	2011	863	toxic	low toxic
Makedonski Brod	JCB	2010	969	toxic	low toxic
Positive control			LC_{50} (μg/mL)		
$K_2Cr_2O_7$			41	toxic	highly toxic

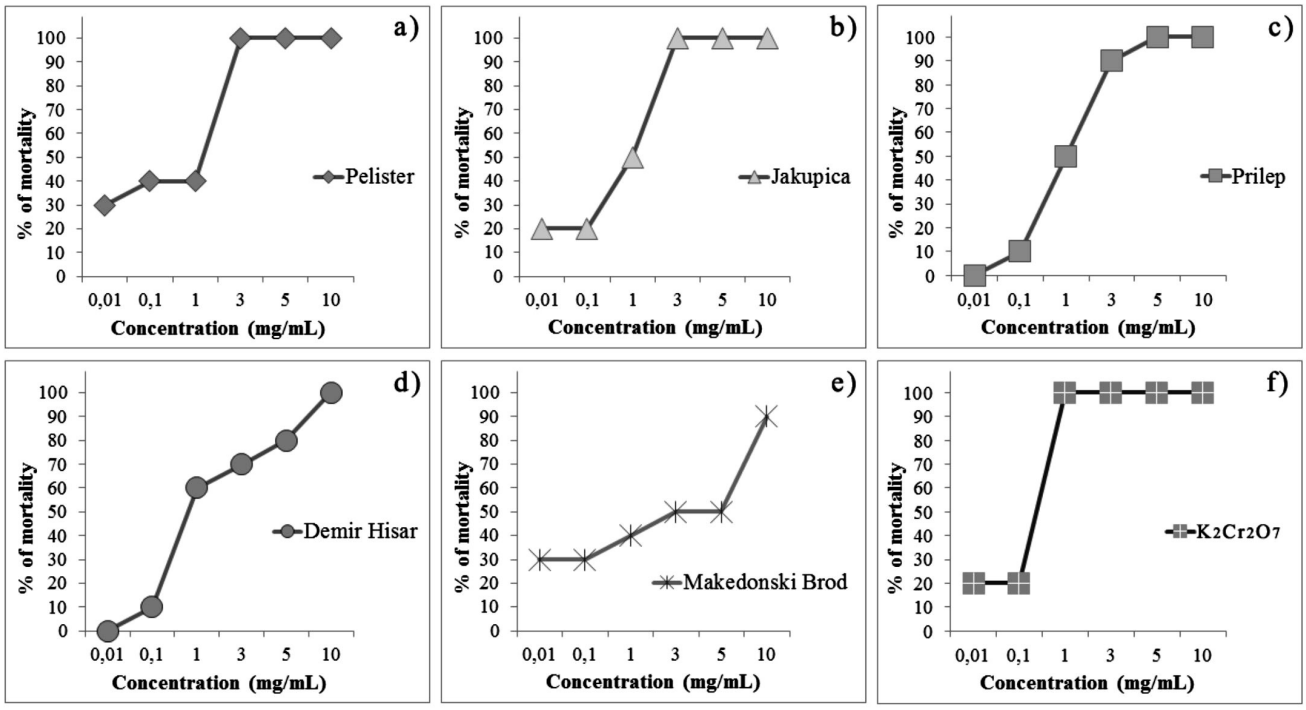

Fig. 1. Trend of mortality for *A. salina* against concentration for berries collected from a) Pelister; b) Jakupica; c) Prilep; d) Demir Hisar; e) Makedonski Brod; and the positive control f) Potassium dichromate.

habitat. The proportion of limonene, sabinene, 3-carene, myrcene, β-pinene and terpinolene, the most abundant compounds in the juniper essential oil was significantly higher in species collected from higher altitudes compared to lower habitats, as it was demonstrated by Martz et al. (2009). The proportion of α-pinene was affected insignificantly by the geographical factors (Ložienė and Labokas, 2012). Taking into account their complex phytochemical composition, it is highly probable that the bioactive profile of the examined species is due to the synergic action of several phytochemical components with cytotoxic potential.

Additionally, LC_{50} values obtained for the most potent samples in the current research were similar to obtained values for the cytotoxic potential against several *in vitro* cell lines demonstrated in previous studies on the bioactivity of *Juniperus communis* berries collected from different localities. In the study of Ghaly et al. (2016), treatment with methanolic extracts from the berries resulted in a significant cytotoxic activity against human breast cancer cells MCF7 and the human colon cancer cells HCT-116. The aquaeous extract of leaf and berries from *Juniperus communis* L. var. *saxatilis* Pall. demonstrated cytotoxic and antitumor activity against CRPC (Castration-resistant prostate cancer) cells in the study of Eryilmaz et al. (2017). The anticancer activity of *Juniperus communis* berries is also possible via induction of cell cycle arrest which has an important role in cancer prevention. This type of inhibitory activity was observed for isolated compounds from methanolic extract of *J. communis* berries against the cell cycle progression in p53-null human cancer CaLu-6 cells in the study of Marino et al. (2011).

Furthermore, our results are in conjuction with previously reported studies on other species from the *Juniperus* genus. Methanolic extracts from *Juniperus drupacea* Labill. berries demonstrated significant cytotoxic potential against *Artemia* larvae (LC_{50} 489.47 ± 27.8 μg/mL) and a significant decrease in HepG2 cells viability (5–10 μg/mL) (Miceli et al., 2011). Moreover, similar results were also obtained for the hydroalcoholic extract of *Juniperus sabina* berries against HeLa and MDA-MB-468 cells in the study of Jafarian-Dehkordi et al. (2004).

Conclusion

Overall, *Juniperus communis* berries were identified as samples with a certain cytotoxic potential *in vivo*, and the obtained results suggest that the amount of bioactive compounds could be correlated with the altitude of their habitat. Berries collected from Pelister and Jakupica demonstrated moderate cytotoxicity, while the berries collected from localities near Prilep, Demir Hisar and Makedonski Brod were characterized with low cytotoxic

activity. Obtained results in the current research emphasize the need of more intensive natural product screening for new bioactive compounds with cytotoxic properties. Therefore, further studies would be needed to clarify the mechanism of cytotoxicity and to identify the responsible bioactive compounds present in berries from *Juniperus communis* L.

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Резиме

Цитотоксична активност на метанолни екстракти од бобинки на *Juniperus communis* L. врз солени ракчиња (*Artemia salina*)

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Клучни зборови: *Juniperus communis* L. бобинки, метанолни екстракти, тест со леталност на солени ракчиња, пробит анализа, LC_{50}

Цитотоксичниот потенцијал на метанолни екстракти од *Juniperus communis* бобинки собрани од пет локалитети во Република Македонија е евалуиран со примена на Тестот со солени ракчиња. Добиените резултати за цитотоксичноста опаѓаат според следниот редослед: Пелистер (128 μg/mL) > Јакупица (221 μg/mL) > Прилеп (662 μg/mL) > Демир Хисар (863 μg/mL) > Македонски Брод (969 μg/mL). Бобинките собрани од планинскиот предел предизвикаа значајни цитотоксични ефекти кај ракчињата, додека бобинките собрани од локалитети блиску до урбани подрачја се карактеризираат со пониска цитотоксичност. Варијациите во нивната биоактивност најверојатно се должат на комплексниот фитохемиски состав, што може да варира при различни еколошки и географски услови.