Food Chemistry Advances 3 (2023) 100395

Contents lists available at ScienceDirect

Food Chemistry Advances

journal homepage: www.elsevier.com/locate/focha

Pumpkin pulp extracts from a Serbian *Cucurbita maxima* Breeding Collection: Phenol profile and in vitro bioactivity

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ARTICLE INFO

Keywords:
Pumpkin
Phenolics
Antiproliferative activity
Antimicrobial activity
Functional food

ABSTRACT

Methanolic pulp extracts from the four selected Serbian accessions of *Cucurbita maxima* were evaluated for phenol profile cytotoxic effects and antimicrobial activity. The results revealed that quinic acid and amentoflavone were the most abundant phenols. The extracts increased the viability of HTR-8 SV/Neo, JEG-3, JAR cells, with the most pronounced increase in the treatment with MAX 113 extract. Furthermore, in HeLa cells, the extracts showed a modest cytotoxic effect. The antimicrobial effects evaluation showed that out of four pumpkin extracts, MAX 117 could moderately suppress the growth of *Staphylococcus aureus* and *Staphylococcus epidermidis* (MIC=1000 μg/mL). The observed biological effects indicate the potential medicinal properties of these pumpkin extracts and contribute to the varietal selection of the most suitable accessions in national breeding programs as candidates for improving human health.

Introduction

Balanced nutrition and food rich in antioxidants is known to have beneficial impact on human health and well-being. Consumption of food abundant with polyphenols, carotenoids, tocopherols, sterols, minerals, vitamins, bioactive proteins positively affects physiological processses and metabolism. Recent research on functional foods is increasingly being focused on pumpkins as one of the richest source of bioactive molecules (Sharma et al., 2020). Pumpkins belong to the genus *Curcubita*, with the species *C. moschata, C. maxima, C. pepo* being the most studied and important in terms of chemical composition and biological activity. Different parts of the pumkin plant (pulp, seeds, flowers, leaves, shoots, roots) are consumed in everyday diet all over the world, but also in the pharmaceutical and cosmetics industries (Kulczynski & Gramza-Michałowska, 2019a). Scientific research has so far focused mainly on pumpkin seeds, while only a few reports can be found on the fruit (Patel & Rauf, 2017; Salehi et al., 2019).

The main factors that classify this plant species as a functional food belong to the group of terpenoids and polyphenols (Kulczynski & Gramza-Michałowska, 2019a). The most important terpenoids in pumpkins are carotenoids, well-known compounds with plenty of human health benefits (Bohn et al., 2021; Johnson, 2002; Wimalasiri et al., 2017) among which β-carotene, α-carotene, neoxanthin, violaxanthin and lutein stand out (Bemfeito et al., 2020).

Besides these, triterpenoid compounds curcubitacins, attracted significant attention, especially Curcubitacin B and E that possess various pharmacological activities such as antioxidant, antiinflammatory and anticancer activity (Chan et al., 2010; Duncan et al., 1996; Jayaprakasam et al., n.d.). Large number of epidemiological studies reported a strong association of intake of bioactive molecules, such as those found in pumpkins, with the reduced risks of various types of cancers, prevention of osteoporosis and hypertension, antidiabetic activity etc. (Black et al., 2020; Ceclu et al., 2020,; Lemus-Mondacaet al., 2019). There are many studies that mostly indicate the presence and biological activity of carotenoids of pumpkins fruits, however, only a few data can be found on the polyphenols present in this plant species (Mokhtar et al., 2015; Peiretti et al., 2017; Zdunić et al., 2016). Therapeutic potential of pumpkins lies in their high content of secondary metabolites such as polyphenols and good antioxidant activity coupled to their low-caloric nutritional value (El Khatib & Muhieddine, 2020; Stevenson et al., 2007). The reaserch on antidiabetic- and antihypertension-relevant

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https://doi.org/10.1016/j.focha.2023.100395
Received 8 January 2023; Received in revised form 24 June 2023; Accepted 20 July 2023
Available online 1 August 2023

Abbreviation

DMSO	Dimethyl sulfoxide
LC-MS/MS	liquid chromatography tandem mass spectrometry
SRM	selected reactions monitoring
ESI-MS	Electrospray Ionization Mass Spectrometer
N2	nitrogen
NI	Negative Ionization
MRM	Multiple reaction monitoring
ver.	version
MTT	thiazolyl blue tetrazolium bromide
DMEM/F12	Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham
FCS	Fetal Calf Serum
EDTA	Ethylenediaminetetraacetic Acid
SDS	Sodium dodecyl sulfate
CFU	colony-forming unit
MHB	Mueller-Hinton broth
TTC	triphenyltetrazolium chloride
MIC	minimal inhibitory

potentials of phenolic phytochemicals from traditional plant foods identified pumpkin with the best overall potential among the investigated food sources, and showed correlation between pumpkin total soluble phenolic content and potential to reduce cellular oxidation stress (Kwon et al., 2007). Therefore pumpkins as phenolic antioxidant-enriched sources could be a valuable tool in health promoting and disease preventing strategies. Previous research of the antioxidant potential of curcubita fuits revealed significant differentiation among the tested culticars.(Kostecka-Gugała et al., 2020). In our recently published study, we conducted research on 20 pumpkins accessions from the breeding collection of the Institute of the Republic of Serbia, Novi Sad (Serbia). The investigations have inculded carotenoid qunatification, analysing the lipidomic profile as well as assessment of antioxidant capacity. The aim of the study is to continue the research of our previously published study (Miljić et al., 2021).Therefore, for the present study, the varieties that showed the best antioxidant activity and that had the highest plant of bioactive compounds were selected.

In this regard, we were able to gather important data that will enable us to carry out more research on these samples with the aim to expand our understanding of their chemical composition and biological activity in order to consider their potential applications in the food and pharmaceutical industries in order to improve human health.

Thereby the aim of this study was to expand the research on these samples by selecting those species with the best antioxidant capacity. The investigations were reflected in the characterization of the phenolic profile, antiproliferative and antimicrobial potential of *C. Maxima* varieties. The cytotoxic potential of selected extracts was examined in a concentration range (10, 100, 1000 μg/mL) using four different cell lines: two malignant choriocarcinoma cell lines JEG-3 and JAR, HeLa human cervical carcinoma cell line and HTR-8/SV Neoas non-malignant control cell line to determine if normal cells respond to treatment differently from the cells of malignant phenotype. To the best of our knowledge, this is the first time to evaluate the cytotoxic effects of the extracts of pumpkin fruit against human reproductive system cancer cell lines, as well as in human trophoblast cells. Further, antimicrobial potential of the selected extracts was examined against Gram-positive and Gram-negative bacteria, and yeast in relation to their phenol content.

Material and methods

Chemicals: The phenolic chemical reference standards were obtained from Sigma-Aldrich Chem (Steinheim, Germany), Fluka Chemie GmbH (Buchs, Switzerland) and ChromaDex

(Santa Ana, USA). HPLC gradient grade methanol was purchased from J. T. Baker (Deventer, The Netherlands), p.a. formic acid was obtaind from Merck (Darmstadt, Germany).

Plant material: Four pumpkin varieties are selected according their great biological activity which they showed in our previous study (Miljic et al., 2021). MAX113, MAX118–1, MAX117 and MAX1 are assigned collection designations for the samples obtained from the Institute of Field and Vegetable Crops collection, Bački Petrovac, North Serbia where they grown and harvested. Serbian production standards were followed when growing pumpkins, with the exception of the 5 m space between each row. There was no irrigation necessary because the land had ample moisture, and no pesticides were used because there were no serious diseases. Further, mineral fertilizer was added to the soil. To prevent and control weeds, inter-row cultivation was conducted twice within the rows, and weeds were manually removed. Fruits were collected in 2018 from late September to early October after sowing took place in early May. Pumpkin pulp samples were collected from 5 to 8 different fruits within the same plot. Fresh pumpkin samples (50 g each) were subsequently freeze-dried for 48 h at −80 C using a Christ Alpha 1–2 LD Freeze Dryer (Switzerland).

Extract preparation

The extraction was performed at room temperature according to the procedure developed by Haminiuk et al. (2014) with slighter modifications. 1 g of lyophilized pumpkin sample was dissolved in 20 mL of methanol. The samples were mixed for 10 min on a vortex and centrifuged at 4000 g for 20 min on 20 °C. The plant material was removed by filtration and the supernatant is evaporated by dryer at 50 °C. To determine the content of phenolic compounds, evaporated samples was redissolved in methanol to the final concentration of 2 mg/ mL.

For the cytotoxic evaluation, the evaporated samples were redissolved in DMSO at the final concentration of 50 mg/mL from which the working solutions were prepared in growth mediums.

Phenol content evaluation

LC–MS/MS Analysis of fenolic compounds:

The quantification of the selected phenolic compounds was carried out using the LC–MS/MS method by Orcic et al., 2014. For the mixes of 45 compounds, 15 working standards, ranging from 1.53 ng/mL to $25.0 \cdot 10^3$ ng/mL, were prepared by serial dilutions in methanol. To obtain the high selectivity and sensitivity, the selected reactions monitoring (SRM) acquisition mode was used since only ions specific to the targeted analytes were monitored. The both samples were analyzed using Agilent Technologies 1200 Series high-performance liquid chromatography coupled with Agilent Technologies 6410A Triple Quad tandem mass spectrometer with electrospray ion source. Five microlitres of the sample injected into the system. The phenols were separated on Zorbax Eclipse XDB-C18 (50 mm × 4.6 mm, 1.8 m) rapid resolution column held at 50 °C. Mobile phase was delivered at flow rate of instead of 1 mL/min in gradient mode (0 min 30% B, 12 min 70% B, 18 min 100% B, 24 min 100% B, re-equilibration time 0 min 30% B, 6 min 70% B, 9 min 100% B, 12 min 100% B, re-equilibration time 3 min). Eluted compounds were detected by ESI-MS, using the ion source parameters as follows: nebulization gas (N2) pressure 40 psi, drying gas (N2) flow 9 L/min and temperature 350 °C, capillary voltage 4 kV, in negative polarity (negative ionization mode, NI). Data were acquired in dynamic MRM mode-retention time, precursor ion, product ion, fragmentor voltage, collision voltage. For all the compounds, peak areas were determined using Agilent MassHunter Workstation software Qualitative Analysis (ver. B.03.01). Calibration curves were plotted (the function peak area logarithm depending on the standard concentration logarithm, $\log(A) = f \log(C)$), and concentrations of samples calculated using the OriginLabs Origin Pro (ver. 9.0) software.

Cytotoxicity evaluation

Antioxidant and anti-inflammatory properties of natural phytochemical are known to influence anti-cancer effects (Surh, 2003). Since we previously evaluated antioxidative potential in the Serbian accesions of *Cucurbita maxima*, and selected these four extracts according to the most pronounced antioxidative potential (Miljić et al., 2021), this research was the next step towards investigating the potential biological activity of these extracts by determining their cytotoxic potential in healthy and malignant trophoblast cells, of which so far, there is no available reports. The cytotoxicity of 4 selected pumpkin extracts was evaluated by using the 3-[4,5-*dimethylthiazole*-2-*yl*]−2,5-diphenyltetrazolium bromide (MTT) assay in HTR-8/SVNeotrophoblast cells, malingnant choriocarcinoma JAR and JEG-3 cells, and in HeLa human cervical carcinoma cells.

The HTR-8/SVNeocell line was obtained as a kind gift of Dr. Charles H. Graham, Queen's, Kingston, Canada, and originated from the human first-trimester placenta explant cultures immortalized by SV40 large T antigen. HTR-8/SVNeocells and JAR choriocarcinoma cell line (American Type Culture Collection, Virginia, USA) were cultured in a complete medium containing RPMI 1640 (Gibco, Waltham, MA, USA), 10% fetal calf serum (FCS, Gibco, Waltham, MA, USA), and 1% antibiotic–antimycotic solution (Capricorn Scientific GmbH, Ebsdorfergrund Germany). JEG-3 choriocarcinoma cell line (ECACC, Salisbury, UK) and Human Cervical Adenocarcinoma (HeLa, ATCC® CCL-2™, American Type Culture Collection, Virginia, USA) cell line were cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) (PAN-Biotech, Aidenbach, Germany) supplemented with 10% FCS and 1% antibiotic–antimycotic solution-complete DMEM/F12 medium. All cell lines were grown in 25 cm^2 tissue culture flasks at 37 °C, 5% CO_2, in a humidified incubator. After reaching 70% confluence, the cells were harvested from flasks with the use of 0.25% trypsin-EDTA solution (Institute for Virology, Vaccines, and Serum "Torlak", Belgrade, Serbia) and seeded in 96-well plates (2×10^4 cells/well for HTR-8/SVNeoand $1{,}5 \times 10^4$ cells/well for JAR, JEG-3 and HeLa cells) in 100 μl of the respective complete medium. The cells were allowed to adhere to the wells for 24 h at 37 °C, 5% CO_2 before the treatment. After incubation, the medium was removed, and treatments with the extracts or fresh medium alone (control) in a total culture volume of 100 μl/well, were added, and left for 24 h at 37 °C. On the next day, treatments from the wells were removed and exchanged with 100 μL of fresh complete medium and 10 μL of MTT reagent (thiazolyl blue tetrazolium bromide, 1 mg/mL, Sigma Aldrich, St. Louis, MO, USA) was added per each well. The cells were allowed to react with MTT for 2 h in the dark at 37 °C, and the formed purple formazan crystals were solubilized by adding sodium dodecyl sulfate (10% SDS in 0.01 M HCl, Sigma Aldrich, St. Louis, MO, USA) at 100 μL/well. The plates were shaken to allow complete solubilization, and absorbance was read using a microplate reader (BioTek ELx800, VT, USA) at 570 nm. Each experiment was performed three times in triplicates, $n = 3$.

Antimicrobial activity

Antimicrobial activity of MAX113, MAX 118–1, MAX 117, MAX 1 was tested against nine laboratory control strains of microorganisms: four Gram-positive bacteria - *Staphylococcus aureus* subsp. *aureus* Rosenbach ATCC 6538, *Staphylococcus epidermidis* ATCC 12,228, *Enterococcus faecalis* ATCC 29,212 and *Bacillus subtilis* ATCC 6633; four Gram-negative bacteria - *Escherichia coli* ATCC 25,922, *Klebsiella pneumoniae* subsp. *pneumoniae* NCIMB 8267, *Salmonella enterica* subsp. *enterica* serovar Abony NCTC 6017 and *Pseudomonas aeruginosa* ATCC 27,853, and one yeast *Candida albicans* ATCC 24,433 (all KWIK-STIK™, Microbiologics, USA). Minimal inhibitory concentrations of MAX 113, MAX 118–1, MAX 117, MAX 1 were determined by broth microdilution test in 96-well microtiter plates according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST, 2022) guidelines. One colony of each strain in exponential phase of growth (i.e. overnight culture) was suspended in a saline solution to a density of 0.5 per McFarland standard (Bio-Merieux, France), which corresponds to a 1.5 x 10^8 CFU/mL of microorganisms. MAX 113, MAX 118–1, MAX 117, MAX 1 were dissolved in DMSO, and further prepared in concentrations ranging from 1 to 1000 μg/mL in fresh Mueller-Hinton broth (MHB, Lab M Limited, UK) for bacteria, or Sabouraud-dextrose broth (MHB, Lab M Limited, UK) for *C. albicans*. The stock solution of samples MAX 113, MAX 118- 1, MAX 117, MAX 1 was prepared in DMSO, and further diluted to concentrations ranging from 1 to 1000 μg/mL in fresh Mueller-Hinton broth (MHB, Lab M Limited, UK) for bacteria, or Sabouraud-dextrose broth (MHB, Lab M Limited, UK) for *C. albicans*. The final concentration of DMSO in serial dilutions of extract was less than 0.0005% in first dilution, and less than 0.5% in the last dilution of extracts. Each concentration was set in duplicate and inoculated with $5{\times}10^5$ CFU/mL of microorganisms. For detection of cell growth and metabolism, MHB was supplemented with redox indicator - 0.05% triphenyltetrazolium chloride (TTC, Sigma-Aldrich, USA). TTC is a colorless dye that is converted to a red metabolite 1,3,5-triphenyformazan with the activity of cellular dehydrogenase in viable cells. After incubation for 20 h at 35°C in aerobic conditions minimal inhibitory concentrations (MIC) were determined as the lowest concentration of extract that inhibits bacterial growth (i.e., shows no visible change of medium color). Positive controls (microorganisms in plane medium and microorganisms in medium with DMSO corresponding to concentrations from 0.0005%−0.5%) and negative controls (only medium with MAX 113, MAX 118- 1, MAX 117, MAX 1) were included in experiments. Antibacterial activity of two standard antibiotics Amikacin and Levofloxacin, and antimycotic agent Fluconazole was also determined according to EUCAST guidelines (https://www.eucast.org/clinical_breakpoints). Each test was repeated three times.

Statistical analysis

The results from cytotoxicity evaluations were analyzed by one-way Analysis of Variance (ANOVA) with Tukey's multiple comparison post-hoc test. Statistical analysis was performed by using GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). All values were expressed as the mean ± SEM, and $p < 0.05$ was considered statistically significant.

Results and discussion

Phenol profile of four selected pumpkin extracts

The phenolic compounds have been determined using LC/MS-MS technique according to the previous reported method with minor modifications (Orcic et al., 2014). Only six of 45 standard compounds have been quantified. The rest of the compounds were under the limit of quantification and detection.

The obtained results presented in the Table 1. indicated that phenolic acids are dominant quanified phenolics in all pumpkin samples, where the presence of quinic acid stands out and its highest concentration is noticed in the sample **MAX 113** (71 ng/mg). There are some studies

Table 1
Quanitification of the phenolic compounds .

	Concentration (ng mg^{-1} lyophilized sample)			
Phenolic compounds	MAX 113	MAX 118–1	MAX 117	MAX 1
p-Hydroxybenzoic acid	<loq	<loq	<loq	23.12
p-Coumaric acid	1.52	1.78	1.81	<loq
Quinic acid	71.67	12.16	15.66	36.48
Chrysoeriol	<loq	<loq	<loq	3.07
Amentoflavone	<loq	<loq	<loq	100.18

Quantification (ng mg^{-1} sample); **<loq** = less than limit of quantification.

whose results are in accordance with ours (Kulczynski & Gramza--Michałowska, 2019a, 2019b; Peiretti et al., 2017; Zdunić et al., 2016), where some phenolic acids (gallic and vanillic acid) have been detected in the pumpkin seeds using HPLC-DAD.

Kulczynski and Gramza-Michałowska (2019) analyzed the content of phenolic components in different pumpkin varieties and observed that there are significant variations between them. The samples analyzed in that study stood out for the content of phenolic acids, the most abundant of which were gallic, protocatechinic, p-hydroxybenzoic and p-coumaric acids, which is in accordance with results obtained in our study. Also, in the study of Kostecka-Gugała et al. (2020), there can be noticed that phenolic acids are dominant quantified phenolic compounds in different varieties of Curcubita species.

Furthermore, only two phenolic compounds, amentoflavone and chrysoeriol have been detected and quantified in the sample **MAX 1,** wherby it can be noticed that amentoflavone is the most abundant phenol generally (100 ng/mg). Even thougt a few phenolic compounds are determined and quantified, their presence in the pumpkin samples could have an impact on the biological and pharmacological activity of the samples, especially amentoflavone and quinic acid that possess a planty of bioactivities such as anticancer, antimicrobial, antiinflamatory, etc. (Xiong et al., 2021).

Cytotoxic effects of four selected pumpkin extracts

Four different cell lines, one non-malignant, HTR-8/SVneo, and three cancer cell lines JEG-3, JAR and HeLa were used to evaluate the cytotoxicity of the four selected pumpkin extracts at three different concentrations (10, 100 and 1000 μg/mL) and the obtained results are presented in Fig. 1.

All four extracts induced significant increase of cell viability in HTR-8/SVneotrophoblast cells after incubation with the two lower concentrations of 10 and 100 μg/mL while the highest concentration did not exhibit any effect (Fig. 1A). Among the extracts, MAX 113 showed the most pronounced stimulation of cell proliferation. In two choriocarcinoma cell lines, JEG-3 and JAR, similar effect was observed where two lower concentrations produced significant rise in cell viability after 24 h incubation with extracts, and the effect was observed for all four extracts (Fig 1B and C). Again, MAX 113 produced the most effects. In human cervical carcinoma cells, HeLa, cytotoxic effects were not observed after the treatment with the extract MAX 117 and MAX 1 in any of the concentrations, while the highest concentrations of MAX 113 and MAX 118–1 produced modest but significant reduction of cell viability at concentration of 1000 μg/mL (Fig 1D). Some published studies showed that these phenolic acids have submicromolar potency against cancer cells and are selective for these cells over non-cancerous cells. They therefore constitute interesting scaffolding for anticancer agents that specifically cause cancer cells to undergo apoptosis (Menezes et al., 2017; Stefani et al., 2021; Voutilainen et al., 2006).

It should be mentioned that previous research on cytotoxic effects of phytochemicals, such as those found in pumpkins, showed diverse outcomes in different cell types. While in normal cells they are mostly known to exhibit antioxidant and anti-inflammatory response, in malignant cells they showed anti-proliferative effects and altered gene expression and the activation of pro-apoptotic signaling (Koklesova et al., 2020). Thus, elucidating the proper balance in the anti-/-pro-oxidant roles of phytonutrients and the effects that they exhibit in certain types of cells should contribute to the understanding of their safety and health promoting effects.

Pregnancy is an especially vulnerable period of increased metabolic demands that affects the health and well-being of both mother and baby. Proper nutrition prior to and throughout pregnancy is of vital importance for the long-term health of the offspring, and there is a positive contribution of certain antioxidant micro and macronutrients to successful pregnancy (Mistry & Williams, 2011). The potential of bioactive compounds present in Cucurbitaceae family against human reproductive system cells and reproductive cancer cell lines has not been investigated so far. HTR-8/SVneocell line was derived from first trimester of pregnancy placental explant cultures and are widely used as a model which faithfully recapitulate functions of primary trophoblast cells

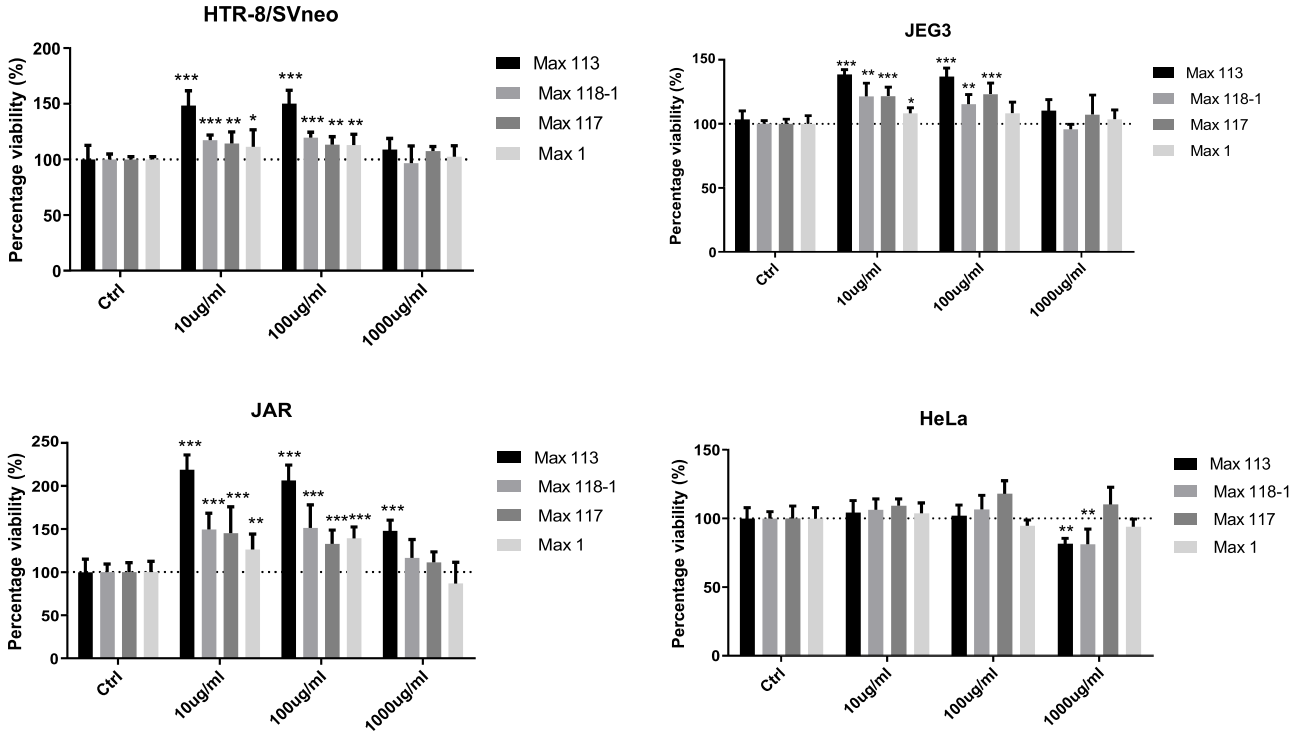

Fig. 1. Cytotoxicity effect of four pumpkin extracts (MAX 113, MAX 118–1, MAX 117, MAX 1) in (A) HTR-8/SVneocells, (B) JEG-3 cells, (C) JAR cells, and (D) HeLa cells. The data are expressed as mean + SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. respective control (by one-way Analysis of Variance (ANOVA) with Tukey's multiple comparison post-hoc test).

(Abou-Kheir et al., 2017). The choriocarcinoma cell lines JEG-3 and JAR possess characteristics of syncytiotrophoblasts and present a convenient experimental models testing of agents that possess anti-cancer properties, where JEG-3 cell line has significantly increased invasive/methastatic capacity compared to the JAR cell line (Bačenková et al., 2022; Jingting et al., 2007). HeLa is a human cervical carcinoma cell line often used as a reference model of malignant cells to test anti-tumorigenic effects modulated by natural compounds (Afroze et al., 2022).

The cytotoxic effect of Cucurbitaceae extracts in HeLa cells was repored previously in literature. *Cucurbita maxima* methanol seed extract showed potent inhibition of HeLa cell growth at 100 μg/mL . In a recent paper by Morales-Vela, extracts from fruit of *C. okeechobeensis martinezii* showed remarkable cytotoxic activity against HeLa cell lines, where ethyl acetate extract (inhibitory effect at 2.5 μg/mL) was more potent than methanol extracts (inhibitory effect at 11 μg/mL) (Morales-Vela et al., 2019). They suggested these effects could be originating from triterpenes e.g. cucurbitacines content. Cucurbitacines isolated from different species of the Cucurbitaceae family are found in roots and fruits of plants, and they have been associated to an extensive range of biological actions such as anti-inflammatory, anti-cancer, hepatoprotective, etc. (Shah et al., 2014). Moreover, the terpenoid cucurbitacin E is a specific marker of the species *C. maxima*, which was detected previously in these four extracts and described in our previos research (Miljić et al., 2021). Namely, in that study we also detected several terpenoid classes, such as carotenoids, cucurbitacins, diterpenoids, monoterpenoids, sesquaterpenoids, sesquiterpenoids, sesterterpenoids, and triterpenoids in the same extracts that we investigated in this research (Miljić et al., 2021). In terms of anti-cancer effects, cucurbitacins previously exhibited differential toxicity in the diverse cell lines (e.g. lung, kidney, skin, breast) and they are known to affect various targets of cancer signaling pathways, which play crutial role in the apoptosis and survival of cancer cells (Duangmano et al., 2012; Kim et al., 2014; *NII-Electronic Library Service*, n.d.; Wang et al., 2017). Another, recent study, which examined methanolic extracts of *Ibervillea sonorae* (Cucurbitaceae) in HeLa cells, showed that a synergistic effect of phytocemical components can be associated to the observed cytotoxic activity of the towards HeLa cells and not only cucurbitacins (Torres Moreno et al., 2020). Furthermore, one of the main phenolic compounds detected in all four extracts in the present study is quinic acid (QA). QA is a cyclic polyol which showed anticancer properties on several types of cancer cells including HeLa (Murugesan et al., 2020). Thus, cytotoxic activity against HeLa cells observed in our study could be a result from synergistic activities of both cucurbitacins and phenolics. Concerning the *Cucurbia maxima* cytotoxic potential, so far there were reports of anticancer effects from extracts of seed and aerial parts, but not from fruits (Abou-Elella & Mourad, 2015; Saha et al., 2011). Therefore, our research is the first report of cytotoxic potential of *Cucurbita maxima* methanol fruit extract against HeLa cell lines.

In terms of cell stimulating effects of *Cucurbita maxima* pulp extracts observed in trophoblast cell lines in our research, we believe that extracts could have exhibited mitogen-induced proliferation, mediated by the activation of surface receptors (Proliferation & Death, 2021). The proliferation inducing effect showed dose-dependent fashion, with maximum effect at concentration of 10 μg/mL. Highest concentrations (1000 μg/mL) of four extracts did not induce further proliferation, because of the saturation effect via mitogenic receptors. One of the components that could influence cell surface receptors and is found in all four extracts is zeaxantin. Namely, Cenariu et al. showed that zeaxanthin activity has differential activity in regulating expression of membrane receptors. They futher showed that at mechanistic level, zeaxanthin-rich extracts were able to influence selectively the mitogen-activated protein kinases (MAPK) in normal versus malignant cells (Bunea et al., 2021). The role of the MAPK signal transduction pathway in the proliferation of mammalian cells has been well established before. Thus, it is plausible the effects of extracts from Cucurbita species in our work, could have influenced the MAPK pathway, altering proliferation in trophoblast cells. Similar to our research, Aristatile & Alshammari observed that extract of *Cucurbita ficifolia* enhanced the cell proliferation at lower concentrations (Aristatile & Alshammari, 2017). It is interesting to mention that in trophoblast cells the effect of the pumpkin seed extract, rich in lignans and flavones as phytoestrogens from the species *Curcurbita pepo*, showed stimulative effects on estradiol production in malignant choriocarcinoma cell lines JEG3 and BeWo. In the same study the effects of extracts on MCF-7 breast cancer cell proliferation was examined, and it determined biphasic effects in a concentration range 10.50 and 100 μg/mL, similar to ours. They showed that cells treated with 10 μg/ml or 50 μg/ml significantly increased proliferation while the 100 μg/mL concentration did not seem to affect cell proliferation (Richter et al., 2013). Thus, from our results it can be concluded that *Cucurbita maxima* pulp extracts may be a source of compounds that possibly could modulate the activity of trophoblast cells. It requires future studies to elucidate the exact mechanisms of effect in trophoblast cells as they are crucial for early pregnancy establishment and if disturbed, could lead to unfavorable results regarding the succes of early gestation.

Antimicrobial effects of four selected pumpkin extracts

The antimicrobial activities of MAX 113, MAX 118–1, MAX 117, MAX 1, are presented in Table 2.

The antimicrobial action of the extracts against Gram-negative bacteria was not observed. Three out of four selected extracts did not display antimicrobial activity against tested Gram-positive bacteria (S. aureus, S. epidermidis, and E. faecalis) and yeast C. albicans in the tested range of concentrations, while MAX 117 accession showed antimicrobial effect against *S. aureus* and *S. epidermidis* with MIC concentrations from 1000 μg/mL. These two Gram-positive bacterial species are the leading cause of skin and soft tissue infections (Chessa et al., 2015). It can be observed that there is no corrrelation between the amount of quantified phenolics and antimicrobial activity. Although the sample MAX 117 is not the richest in phenolic compounds, the detected phenolic acids p-coumaric acid (1.81 ng mg^{-1}) and quinic acid (15.66 ng mg^{-1}) possess antimicrobial potential. Thus, p-coumaric acid can destroy bacterial cell membranes and bind to bacterial genomic DNA to inhibit cellular functions, ultimately leading to cell death. Also, the antimicrobial activity can be attributed to other compounds identified in our previous study. Thus, the cucurbitacin and some carotenoids such as β-carotene and zeaxanthine that were the most abundant in the extract MAX 117, could aslo contribute to antimicrobial activity.

Staphylococcus aureus is also food borne pathogen known to cause food poisoning, and methicillin-resistant (MRSA) strain has become an increasing problem in healthcare due to resistance to beta-lactam antibiotics (Chessa et al., 2015). Whereas *Staphylococcus epidermidis* has been regarded as an ubiquitous commensal microorganism on the human skin, it is increasingly being seen as an important opportunistic pathogen that often spreads via medical devices such as peripheral or central intravenous catheters causing life-threatening infections (Otto, 2009). Antibiotic resistance has become a huge public health issue that encourages the search for new antimicrobial molecules. New bioactive compounds from plants hold promise for the control of antibiotic-resistant bacteria, esspecially in underdevolped countries since they are more affordable and would prevent the use of poor quality antibiotics that produce sub-inhibitory concentration in vivo (Ayukekbong et al., 2017; Subramani et al., 2017).

Previous investigation on antibacterial activity of flavonoids extract of different parts of pumpkin leaves, carried out on Gram-positive and Gram-negative pathogenic bacteria, showed variable susceptibilities of microorganism for different concentrations of pumpkin leaves extracts (AL-Ghazal, 2012). Although the mechanisms of antimicrobial activity of pumpkin extracts are still unknown, there are some indications antimicrobial activity of pumpkin extracts is associated with high

Table 2
Antimicrobial ability of four pumpkin extracts (MAX 113, MAX 118–1, MAX 117, MAX 1).

Minimal inhibitory concentration (μg/mL)							
Microorganism	MAX 113	MAX 118–1	MAX 117	MAX 1	Amikacin	Levofloxacin	Fluconazole
S. aureus subsp. *aureus* Rosenbach ATCC 6538	>1000	>1000	1000	>1000	16	0.001	n.t.*
S. epidermidis ATCC 12,228	>1000	>1000	1000	>1000	8	0.001	n.t.
E. faecalis ATCC 29,212	>1000	>1000	>1000	>1000	n.t.	4	n.t.
B. subtilis ATCC 6633	>1000	>1000	>1000	>1000	n.t.	0.001	n.t.
E. coli ATCC 25,922	>1000	>1000	>1000	>1000	4	0.25	n.t.
K. pneumoniae subsp. *pneumoniae* NCIMB 8267	>1000	>1000	>1000	>1000	8	0.25	n.t.
S. enterica subsp. *enterica* serovar Abony NCTC 6017	>1000	>1000	>1000	>1000	8	0.125	n.t.
P. aeruginosa ATCC 27,853	>1000	>1000	>1000	>1000	16	0.5	n.t.
C. albicans ATCC 24,433	>1000	>1000	>1000	>1000	n.t.	n.t.	2

* n.t. -not tested.

concentration of phenolic content and there is a synergistic antimicrobial action between these bioactive substances. (Salehi et al., 2019). There are numerous reports on animicrobial effects of oils from seeds of pumpkin species such as *Cucurbita moschata, Cucurbita pepo, Cucurbita maxima* etc. (EI-Aziz & EI-Kalek, 2011; Ghaffar et al., 2018; Moniruzzaman et al., 2022; Sener et al., 2007). In terms of specific antimicrobial activity against *Staphylococcus aureus*, methanolic extracts of *Cucurbita pepo* showed high to moderate inhibitory activity against ATCC strain but also against clinical isolates from human urinary tract infections (AL-Ghazal, 2012; Chonoko & Rufai, 2011). Latest study by Mokhtar et al. showed that polyphenol extract of the mature fruits of *Cucurbita moschata* displayed inhibitory activity against *Staphylococcus aureus* with MIC of 0.75 mg/L and antimicrobial effects were ascribed to the high levels of phenolic acids and flavonoids (Mokhtar et al., 2021). In the study published by Hussein at al pumpkin flesh extracts from *Cucurbita maxima* exhibited improved antibacterial activities as compared to pumpkin peel and seeds extracts, while pumpkin seed aextracts showed better antifungal activities (Hussain et al., 2021). In vitro antibacterial activity of methanol pumpkin *Cucurbita moschata* pulp extract against *Staphylococcus aureus* was also reported by Glória et al. (2017). These results are in line with ours that showed that MAX 117 extract from fruit of *C. maxima* exhibits antibacterial activities against *Staphylococcus epidermidis* and *Staphylococcus aureus*. This effect is likely due to the synergistic action of several bioactive molecules present in pumpkin extract MAX 117. Due to the fact that the extract showed activity against common skin infection agents, antibacterial effect of extract should be investigated against clinical isolates and also different applications in dermathology could be explored as a next step of investigation. Finnaly, it can be stated that due to the multiple beneficial properties exhibited by *Cucurbita maxima* pulp extracts used in this research, further examination is required for exploring their biologically active metabolites contents with more in-depth biological analyses.

Conclusion

The results obtained in the present study indicated that the extracts of Serbian *Cucurbita maxima* accession MAX 117 showed superior animicrobial activity against *S. aureus* and *S. epidermidis* and accession MAX 113 demonstrated the best antiproliferative activity. In spite of this, they could be selected for more comprehensive investigations on additional bioactivity. On the other hand, since the detected phenols cannot be fully prescribed considered biological activity, more extensive phytochemical research is also needed to get a better understanding of the relationship between phytochemical composition and biological activity

In considering the fact that this is the first study on the phenolic profile, proliferative/antiproliferative, and antimicrobial activity of pumpkin pulp extracts from a Serbian *C. maxima* breeding collection and the species *C. maxima* in general, the results of the current study warrant further investigation for use in medicine and cosmetics, with particular caution regarding their effects on choriocarcinoma cells.

Declaration of Competing Interest

The authors report no competing interests.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Acknowledgments

This research was funded by the Ministry of Education, Science and Technological Development of Serbia through Grant Agreement with University of Novi Sad-Faculty of Sciences, University of Belgrade-Institute for the Application of Nuclear Energy-INEP and University of Belgrade-Faculty of Pharmacy [Grant No. 451–03–68/2022–14/200125; 451–03–68/2022–14/200019; 451–03–68/2022–14/200161]

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.focha.2023.100395.

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