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MICROENCAPSULATION OF GLUCOSYL-HESPERIDIN IN ALGINATE/CHITOSAN HYDROGEL BEADS

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Summary

Glucosyl-hesperidin is a water soluble derivate of hesperidin. Both these derivates have many health-promoting properties such as antioxidant, anti-inflammatory and antimicrobial activities. However, the low water solubility of hesperidin disables its wide utilization in the food and pharmaceutical industries so glucosyl-hesperidin has an advantage concerning new product development. The aim of the study was to produce hydrogel beads filled with glucosyl-hesperidin by applying microencapsulation technique using vibration technology. Beads were fabricated under the same operating conditions of the encapsulator and obtained by dropping a mixture of glucosyl-hesperidin and alginate into different hardening solutions (calcium chloride or calcium chloride-chitosan) with different times of complexation (30 min or 90 min). The highest retention ability of glucosyl-hesperidin had chitosan-alginate beads, which were complexed for 30 min (590.93 mg/kg), while the lowest retention ability was observed for alginate beads with a complexation time of 30 min (409.94 mg/kg). Beads were stored for 7 days at ambient temperature and in the presence of light. The highest amount of glucosyl-hesperidin was detected in chitosan-alginate beads as after preparation. Results of this study give insight into glucosyl-hesperidin encapsulation into hydrogel beads and its behavior during storage.

Keywords: microencapsulation, glucosyl-hesperidin, beads, alginate, chitosan

Introduction

Hesperidin is a flavanone found in citrus fruits and is composed of aglycone (hesperetin) and a disaccharide unit (rutinose). Different health-promoting properties of this polyphenol were demonstrated such as anti-inflammatory (Corciovă et al., 2021), antioxidant and antibacterial activity (Balakrishnan et al., 2021), analgesic, antitoxic and metal-chelating properties, neuroprotective and cardioprotective effects, prevention of glucose homeostasis, bone resorption (Sa'Ayinzat et al., 2021) and kidney diseases (Li and Schluesener, 2015). It also controls blood pressure (Chen et al., 2018) and has a lipid-lowering effect (Xiong et al., 2019). However, its low solubility limits its wide utilization in the food or pharmaceutical industries. Hayashibara Co., Ltd. proposed a solution to this problem by improving the solubility of hesperidin using a commercial enzymatic process that involved attaching glucose to its structure. The molecule obtained was called glucosyl-hesperidin and had 100 000 times better solubility than hesperidin (197 g/100 mL). Safety studies were conducted and it was concluded that glucosyl-hesperidin is safe to use as a food ingredient (Matsumoto, 2019). Since polyphenols are very unstable, sensitive to heat, light and oxygen, it is necessary to protect them using various encapsulation techniques (Popović et al., 2019). Also, bioactive compounds are often microencapsulated and then added to various foods as functional ingredients which subsequently enrich the final product (de Moura, 2018). Microencapsulation is a process, in which the final products are particles ranging from 1 to 1000 μm. Produced microcapsules can have different structural forms and various natural and synthetic materials are used as wall material(s) (Whelehan and Marison, 2011). This process can improve the physical properties of bioactive compounds, ensure their targeted release and also prolong their shelf-life by preventing degradation reactions (Macías-Cortés et al., 2020). The microencapsulation techniques are categorized as chemical (polymerization reactions), mechanical (spray-drying, extrusion methods) and physicochemical processes (complex coacervation). Vibrating-jet (nozzle) method is a mechanical procedure based on generating droplets from a polymer that is extruded through a nozzle and broken after it passes through it at a certain flow rate. Prepared alginate particles are gelified into beads upon landing in a bath of calcium chloride. They can be used immediately, stored, or further processed (e.g. adding a new membrane). Size and other characteristics of the beads depend on the nozzle diameter, the flow rate of the laminar jet, the frequency at defined amplitude and the viscosity of the extruded liquid. This procedure allows the production of beads which will protect the encapsulant from external factors, enable its

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controlled release or improve its organoleptic properties (Whelehan and Marison, 2011). Different biopolymers such as proteins and polysaccharides are used as wall material(s) in microencapsulation processes (Capablanca et al., 2017). There is a growing interest in natural polymers such as alginate and chitosan since they are known for their low toxicity, safe usage, biodegradability and biocompatibility. Lower animals and humans can consume edible polymers without any harmful effects and thus the Food and Drug Administration has regarded edible polymers as safe (GRAS) (Ćorković et al., 2021). Encapsulation of different polyphenols in hydrogel beads has been studied in other studies as well (Stoica et al., 2013; Kim et al., 2016; Bušić et al., 2018; Maleki et al., 2020), however as far as we know glucosyl-hespeirdin was not studied. Considering all these aspects, the microencapsulation technique was applied for the production of alginate beads filled with glucosyl-hesperidin. In addition to alginate, the effect of chitosan and different times of complexation on the encapsulation of glucosyl-hesperidin in beads were investigated after preparation, as well as after 7 days of storage.

Materials and methods

Materials

The alginic acid sodium salt, with very low viscosity, was procured from Alfa Aesar (Kandel, Germany). Chitosan was obtained from Sigma-Aldrich (St. Louis, MO, USA) and calcium chloride and ascorbic acid from Gram-mol (Zagreb, Croatia). Glucosyl-hesperidin was the product of Hayashibara Co., Ltd. (Okayama, Japan). Methanol (HPLC grade) was purchased from J.T. Baker (Deventer, Netherlands) and orthophosphoric acid (HPLC grade, > 85%) from Fisher Scientific (Loughborough, UK). Hydrochloric acid (37%) and methanol were purchased from Carlo Erba Reagents (Sabadell, Spain).

Preparation of hydrogel beads

Beads were prepared using Encapsulator B-390 (BÜCHI Labortechnik AG, Flawil, Switzerland) with a 1000 µm vibrating nozzle. The device was operated under fixed conditions: pressure 500 mbar, frequency 200 Hz, electrode 1000 V and ambient temperature. As an encapsulation mixture glucosyl-hesperidin solution (1500 mg/L) with 3.75% alginate was used. Two types of hardening solutions were used, 10% $CaCl_2$ and 10% $CaCl_2$ with the addition of 1.25% chitosan and 2.5% ascorbic acid. The influence of different complexation times (30 and 90 min) was also investigated. One set of the beads was analyzed immediately after preparation and the other was stored for 7 days at ambient temperature and in the presence of light and then analyzed.

Extraction of glucosyl-hesperidin from hydrogel beads

Extraction of the beads was performed according to Kopjar et al. (2021). For the extraction of glucosyl-hesperidin, 1 g of hydrogel beads was weighed and 10 mL of acidified methanol (methanol:hydrochloric acid ratio was 99:1) was added. The mixture was left for 24 h and then filtered. Extracts were further analyzed.

Determination of glucosyl-hesperidin concentration in extracts using high-performance liquid chromatography (HPLC)

The concentration of glucosyl-hesperidin in extracts was determined using Agilent HPLC 1260 Infinity II system (Agilent Technology, Santa Clara, CA, USA) equipped with Poroshell 120 EC C-18 column (4.6 x 100 mm, 2.7 µm), quaternary pump, DAD detector and a vial sampler. As solvent A, 0.1% phosphoric acid solution was used and methanol as solvent B. Elution conditions were: 0 - 38 min from 3% to 65% B and 38 - 45 min 65% B. Injection volume was 5 µL. Prior to injection into the system, extracts were filtered using PTFE filters with a pore size of 0.2 µm. All chromatograms were recorded with DAD in the 190-600 nm range and visualization and peak integration were done at 280 nm. The calibration curve of the glucosyl-hesperidin standard was constructed in concentrations ranging from 100 to 800 mg/L and the linearity was confirmed by $r^2 = 0.9992$. Each extract was injected twice and concentrations were expressed as mg of glucosyl-hesperidin per kg of beads (mg/kg).

Statistical analysis

All results were expressed as the mean values ± standard deviation. Statistical analysis was performed using software STATISTICA 13.1 (StatSoft Inc., Tulsa, OK, USA). Analysis of the variance (ANOVA) and Fisher's least significant difference (LSD) with the significance defined at $p < 0.05$ were used for the data analysis.

Results and discussion

The aim of this study was preparation of hydrogel beads that could be efficient delivery systems of glucosyl-hesperidin. Two sets of hydrogel beads were prepared. The first one based on alginate ALG-30 and

ALG-90 (30 and 90 minutes of complexation, respectively). The second one on alginate and chitosan ALG/CHIT-30 and ALG/CHIT-90 (30 and 90 minutes of complexation, respectively). In Table 1, the concentration of glucosyl-hesperidin in hydrogel beads after preparation as well as after 7 days of storage are presented. In the beads analyzed after preparation, the concentration of glucosyl-hesperidin ranged from 409.94 mg/kg to 590.93 mg/kg with a statistical difference between all samples. Encapsulation of different polyphenols within alginate beads has been the subject of research in numerous studies. The addition of fillers (cocoa and carob powder) improved the encapsulation of dandelion polyphenols since a very porous alginate structure limits its performance as an encapsulation system for small molecules (Bušić et al., 2018). On the other side, molecules such as proanthocyanidins were encapsulated with higher encapsulation efficiencies because their diffusion is limited by their high molecular weights (Kim et al., 2016). Interactions between polyphenols and alginate occur due to hydrogen bonds, which are formed in a presence of hydroxyl groups of phenols and the carboxyl and hydroxyl groups of alginate (Plazinski and Plazinska, 2011).

Table 1. Concentrations of glucosyl-hesperidin in produced hydrogel beads determined using HPLC method

Sample	Concentration after preparation (mg/kg)	Concentration after 7 days of storage (mg/kg)
ALG-30	409.94 ± 2.37^{a}	318.59 ± 6.32^{a}
ALG-90	512.20 ± 5.03^{c}	376.10 ± 0.73^{c}
ALG/CHIT-30	590.93 ± 2.86^{d}	456.23 ± 0.47^{d}
ALG/CHIT-90	425.61 ± 2.73^{b}	357.45 ± 0.13^{b}

ALG and ALG/CHIT: alginate or alginate/chitosan as wall material(s). 30 and 90: different times (minutes) of complexation. Within the column, means followed by superscript different letters are significantly different at $p \leq 0.05$ (ANOVA, Fisher's LSD).

For the beads with only alginate as wall material, it was observed that prolonged complexation had a positive effect on glucosyl-hesperidin concentration since it increased from 409.94 mg/kg to 512.20 mg/kg when the time of complexation was extended from 30 to 90 minutes. The opposite effect was observed for beads that had wall material composed of both alginate and chitosan, where longer complexation time caused a decrease of glucosyl-hesperidin concentration (from 590.93 mg/kg to 425.61 mg/kg). In this study, exposure time affected the concentration of encapsulated glucosyl-hesperidin and the same was observed in the Deladino et al. (2008) study. They concluded that for chitosan-coated beads, a shorter time of complexation was more favorable as opposed to alginate beads.

The addition of chitosan as a wall material caused a higher effect on glucosyl-hesperidin concentration than the extension of complexation time from 30 to 90 minutes (590.93 mg/kg for ALG/CHIT-30 and 512.20 mg/kg for ALG-90). The addition of other biopolymers such as chitosan, pectin, cellulose derivates improved the encapsulation of bioactives in different investigations as well (Bušić et al., 2018). Results in the present study were in accordance with Kim et al. (2016) who concluded that the addition of chitosan enhances encapsulation of polyphenols. The same was observed by Stoica et al. (2013). They observed that with the addition of chitosan, a denser membrane was formed. The reasons for such a phenomenon were hydrogen bonding and van der Waals forces between functional groups of chitosan and polyphenols (Spagna et al., 1996).

Encapsulation efficiencies (%) calculated from the concentration of the glucosyl-hesperidin in the encapsulation mixture and its concentration in beads are depicted in Fig. 1. It was observed that prolonged complexation (90 min) has a better effect on encapsulation efficiency of alginate beads than on alginate/chitosan beads (34.15% for ALG-90 and 28.37% for ALG/CHIT-90). The addition of chitosan caused an increase in encapsulation efficiency only when complexation time was 30 min (from 27.33% to 39.40% for ALG-30 and ALG/CHIT-30, respectively). Lavelli and Harsha (2018) concluded that encapsulation efficiency was affected by the loss of polyphenols from the droplet which occurs prior to gelling in the hardening solution. The diffusion rate of polyphenols through liquid-liquid diffusion is higher than solid-liquid diffusion which occurs after gelling. To obtain higher values of encapsulation efficiency, nozzles with smaller orifices than used in the present study could be a possible solution. It was reported that the encapsulation efficiency of bioactives was improved when smaller particles are produced (de Moura et al., 2018). Also, higher concentrations of alginate solution create a network with a ticker membrane that inhibits the loss of encapsulated material to the environment (Najafi-Soulari et al., 2016).

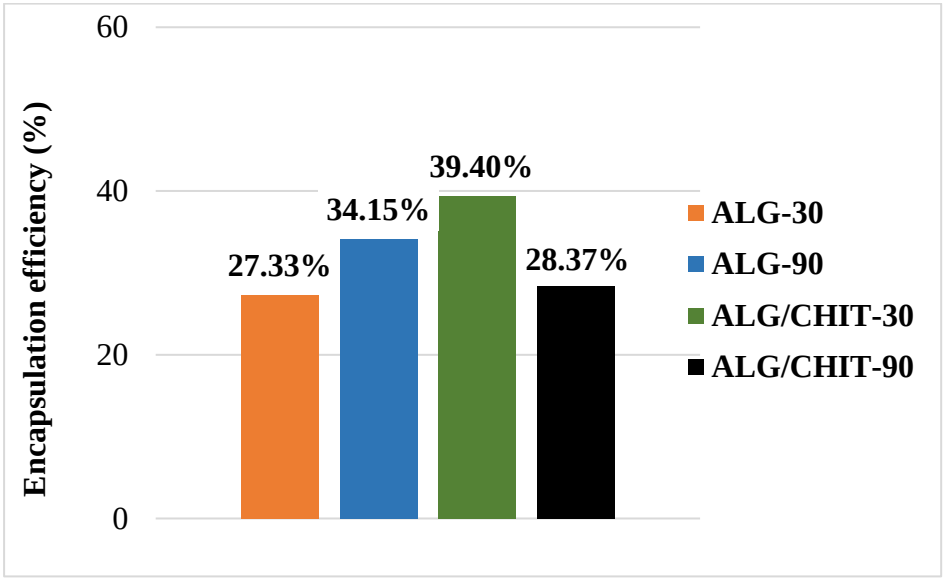

Fig. 1. Encapsulation efficiency of glucosyl-hesperidin in prepared alginate/chitosan beads (ALG and ALG/CHIT: alginate or alginate/chitosan as wall material(s). 30 and 90: different times (minutes) of complexation)

After storage, loss of glucosyl-hesperidin occurred. All of the stored samples had lower concentrations of glucosyl-hesperidin compared to fresh ones, ranging from 318.59 mg/kg to 456.23 mg/kg. The lowest concentration of glucosyl-hesperidin after storage was detected in the alginate beads complexed for 30 minutes, while the highest was determined in beads with chitosan complexed for 30 minutes. Calculating stability of glucosyl-hesperidin (Fig. 2) it was observed that the most stable beads were alginate/chitosan beads complexed for 90 minutes where the retention was 84%. The lowest retention 73.43% was calculated for alginate beads also complexed for 90 minutes, while the other two samples had retention around 77%. A storage study by Maleki et al. (2020) revealed that antioxidant properties, total phenolic compounds, hardness and thickness of alginate beads containing barberry extract decreased during the storage period, but higher alginate concentration showed the best results during the storage period.

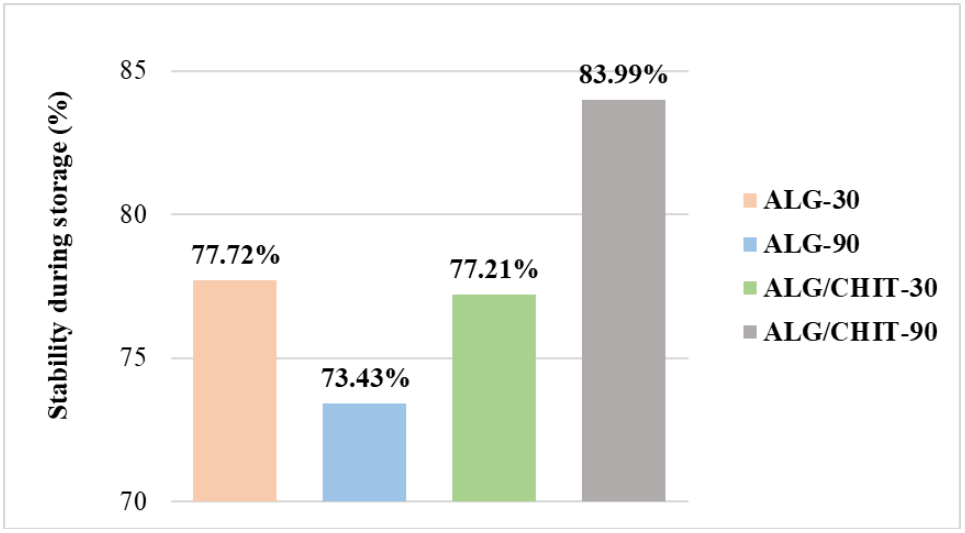

Fig. 2. Stability of glucosyl-hesperidin in alginate/chitosan beads during storage (ALG and ALG/CHIT: alginate or alginate/chitosan as wall material(s). 30 and 90: different times (minutes) of complexation)

Conclusions

The best retention ability of glucosyl-hesperidin had chitosan-alginate beads which were complexed for 30 min (590.93 mg/kg), while the lowest retention ability was observed for alginate beads with a complexation time of 30 min (409.94 mg/kg). From the presented results, it can be concluded that prolonged complexation had a positive effect on the concentration of glucosyl-hesperidin in hydrogel beads just in case when beads were prepared only from alginate. The addition of chitosan caused an increase in glucosyl-hesperidin concentration, but an extension of complexation from 30 to 90 minutes caused its decrease. Different interactions between used wall materials and glucosyl-hesperidin may occur such as hydrogen bonds and affect its encapsulation efficiency. For further investigations, the main challenge will be the application of vibration technology for the production of beads at an industrial scale since until now it was used mainly at a lab scale. Prepared beads could be used for encapsulation of different polyphenols and as antioxidant delivery systems that can be further added as ingredients of functional foods.

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DETERMINATION OF POLYPHENOLS BIOACCESSIBILITY BY IN VITRO GASTROINTESTINAL DIGESTION OF APPLE PEEL

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Summary

Bioaccessible polyphenols represent polyphenols that are released from the food matrix during digestion and become available for absorption. This work aimed to determine the bioaccessible polyphenols from the peel of commercial apple variety 'Idared' throughout oral, gastric, and intestinal simulated digestion. Polyphenols were extracted by the means of chemical and enzymatic extraction. *In vitro* gastrointestinal digestion of the peel of apples was conducted. Polyphenols present in the extracts and oral, gastric, and intestinal digest were analyzed with the use of high-performance liquid chromatography. The amount of polyphenols released during the simulated digestion was lower than the one present in the extracts. Polyphenols bioaccessibility, expressed as a percentage of initial polyphenol concentrations, was 26%, 37%, and 22% for oral, gastric, and intestinal phases, respectively. Flavonols showed to be the most stable group with the intestinal recovery of 34%, followed by phenolic acids (11%) and dihydrochalcones (8%). Flavan-3-ols and anthocyanins were not found in the intestinal phase. These results suggest that polyphenols are released from the peel of apples during digestion and that the amount decreases in the intestines.

Keywords: apples, simulated digestion, polyphenols, bioaccessibility

Introduction

Polyphenols are a group of natural compounds that contain phenolic structural features, i.e. a phenyl group ($-C_6H_5$) to which a hydroxyl group (-OH) is attached. They are widespread in the plants and can generally be divided into phenolic acids, flavonoids, stilbene and lignans (Belščak-Cvitanović et al., 2018; Mancha et al., 2004). Their role in plants has not been fully elucidated, but they are thought to protect plants from pathogens and herbivores. Furthermore, they contribute to plant color and flavor thereby indirectly increasing the chances of seed dispersal (Juadjur and Winterhalter, 2012). A number of scientific studies have been published suggesting a possible negative correlation between a polyphenol-rich diet and the risk of diseases such as cardiovascular disease, specific cancers, and diabetes (Anhê et al., 2103; Mendonça et al., 2018; Giacco et al., 2019 ; Yi et al., 2019; Sajadimajd et al., 2020). In order to show a positive effect on the human body, polyphenols must firstly be released from the food matrix and then absorbed in a certain amount (Jakobek, 2015).

Here we come to the problem of bioaccessibility, which can be defined as the amount of ingested compound that is available for absorption in the digestive tract (Palafox-Carlos et al., 2011). Several authors determined polyphenol bioaccessibility from different sources such as red chicory, white grape, jaboticaba fruit, plum and cabbage varieties (Bergantin et al., 2017; Kaulmann et al., 2016; Lingua et al., 2019; Quatrin et al., 2020). The common conclusion between these papers is that polyphenols are available for absorption throughout the human gastrointestinal system but in an amount that is lower than in the original source. However, Lingua et al. (2019) reported that the bioactivity of digested polyphenols, compared to the nondigested ones, did not change (Lingua et al., 2019). The extent to which polyphenols could be released from certain food depends on several factors among which are the characteristics of the food matrix, processing, and preservation, as well as the pH of the intestine environment (Stübler et al., 2020; Thakur et al., 2020). Out of total released polyphenols, only a small amount is actually absorbed, while the majority of polyphenols reaches the large intestine. Here, polyphenols can interact with gut microbiota and exert beneficial effect, either in terms of promoting bacterial health (prebiotic effect) or by direct antimicrobial effect. Inversely, beneficial gut microbes could utilize and transform certain polyphenols into more easily absorbable phenolic catabolites, thus affecting polyphenol bioaccessibility (Rodríguez-Daza et al., 2021).

Apples, considering that they are one of the most popular fruits worldwide and relatively rich in polyphenols, represent a good day-to-day source of polyphenols in the human diet (Fernández-Jalao et al., 2020). Although traditional apple varieties gained a lot of attention in recent years, due to higher polyphenol content (Jakobek and Barron, 2016), they are not as readily available as commercial varieties. Recovery of both commercial and traditional apple polyphenols

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has been investigated before (Bouayed et al., 2012; Jakobek et al., 2021). However, in the study that assessed commercial apple polyphenol recovery, authors used whole fruit, which could hinder the contribution of peel polyphenols due to significantly lower mass (Bouayed et al., 2012). Furthermore, despite the fact that peel only represent around 10 % of the total apple mass, it contains high amounts of quercetin derivatives, which are not commonly found in the apple flesh (Jakobek and Barron, 2016). Unfortunately, apple peel is often discarded, both by consumers and food industries, which could affect the total intake and the recovery of polyphenols, especially quercetin derivatives.

Hence, we decided to investigate the recovery of polyphenols from the peel of the commercial apple variety 'Idared' by studying in vitro simulated digestion processes in the mouth, the stomach, and the small intestine.

Materials and methods

Chemicals

Calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$ x $6H_2O$), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4) and sodium hydrogen carbonate ($NaHCO_3$) were obtainted from Gram mol (Zagreb, Croatia). Ammonium carbonate ($(NH_4)_2CO_3$) and sodium chloride (NaCl) were purchased from Kemika (Zagreb, Croatia) and Carlo Erba Reagents (Val de Reuil, France), respectively. Orto-phosphoric acid (85% HPLC-grade) was from Fluka (Buchs, Switzerland) while methanol (HPLC grade) was obtained from J.T. Baker (Gliwice, Poland). Polyphenol standards were obtained from Extrasynthese (Genay, France) and Sigma-Aldrich (St. Louis, MO, USA). α-amylase (A3176, 13 U/mg), pepsin (P7000, 632 U/mg), pancreatin (P7545, 8 USP), bile salt (B 8756, microbiology grade) and barley β-D-glucan were from Sigma-Aldrich (St. Louis, MO, USA).

Reagents preparation

Concentrated stock solutions of electrolytes (KCl (0.5 M), KH_2PO_4 (0.5 M), $NaHCO_3$ (1 M), $MgCl_2$ (0.15 M), $(NH4)_2CO_3$ (0.5 M), NaCl (2 M) and $CaCl_2$ (0.3 M)) were used to prepare simulated salivary, gastric and intestinal fluids. According to Menikus et al. (2014), the simulated fluids were prepared with 1.25 times concentration described in their study in order to reach final proper concentrations when used in digestion experiment (Table 1).

Enzyme solution were prepared daily in following concentrations: α-amylase 1,000 mg/L in simulated salivary fluid (SSF), pepsin 31,660.61 mg/L in simulated gastric fluid (SGF), pancreatin 8,000 mg/L in simulated intestinal fluid (SIF). Bile salt was prepared in simulated intestinal fluid in 25,000 mg/L, while β-glucan was prepared in millipore water at 550 mg/L.

Table 1. Preparations of 1.25 times concentrated simulated digestion fluids

	Simulated salivary fluid			**Simulated gastric fluid**			**Simulated intestinal fluid**	
	pH 7			pH 3			pH 7	
Constituent	**Volume (mL)**	**Concentration in SSF (mmol /L)**		**Volume (mL)**	**Concentration in SGF (mmol/L)**		**Volume (mL)**	**Concentration in SIF (mmol/L)**
KCL (0.5 M)	3.775	18.875		4.3125	8.625		8.5	8.5
KH_2PO_4 (0.5 M)	0.925	4.625		0.5625	1.125		1	1
$NaHCO_3$ (1 M)	1.7	17		7.8125	31.25		53.125	106.25
NaCl (2 M)	-	-		7.375	59		12	48
$MgCl_2$ (0.15 M)	0.125	0.05625		0.25	0.15		1.375	0.4125
$(NH_4)_2CO_3$ (0.5 M)	0.015	0.06		0.3125	0.625		-	-
H_20 (Millipore)	93.46			229.375			424	
Total volume	100			250			500	
For pH adjustment								
	mL	**mmol/L**		**mL**	**mmol/L**		**mL**	**mmol/L**
HCL (6 M)	-	-		1	24		1.6	1.92

Apple samples

About 1 kg of commercial apple variety 'Idared' was purchased for local supermarket in Croatia. After peeling, apple peel was pooled and homogenized with a coffee grinder. The samples were stored in plastic bags in a refrigerator at – 18 °C and used for chemical and enzyme assisted extraction of polyphenols and for simulated digestion within one week of storage.

Polyphenol extraction

For the chemical extraction of polyphenols, apple peel (around 3 g) and 22.5 mL of 80% methanol in water were added into a plastic tube (Jakobek & Barron, 2016; Jakobek et al., 2020). After 15 min extraction in the ultrasonic bath (Bandelin Sonorex RK 100, Berlin, Germany), the solution was centrifuged (10 min at 9,500 rpm; SL 8R, Thermo Fisher Scientific, Waltham, MA, USA). The extract was separated from the residue. The process of extraction of the residue was repeated one more time with 10 mL of 80% methanol. After combining two extracts, a total volume of approximately 32.5 mL was obtained. Finally, 1 mL of that extract was taken from a plastic tube, filtered (0.45 syringe filter) and analyzed using HPLC system.
Enzyme assisted extraction followed after chemical extraction (Bergantin et al., 2017). Into the tube with the residue after the chemical extraction, 0.3 mL of pepsin, 0.6 mL of pancreatin, 1.2 mL of bile salts and 21 mL of millipore water were added. The solution was incubated (2 h at 37 °C, water bath with shaking, SW 22, Julabo, Seelbach, Germany), and centrifuged (5 °C, 9,500 rpm, 5 min). 1 mL of the obtained extract was filtered (0.22 μm PTFE syringe filter), placed in an ice bath and analyzed using HPLC system. The residue was extracted one more time. Both extracts were analyzed with HPLC.

Simulated digestion

Around 3 grams of the homogenized peel was weighed into a plastic tube. For the simulation of oral digestion (Bergantin et al., 2017; Minekus et al., 2014), 3.5 mL of SSF, 0.975 mL of H_2O, 25 μL of $CaCl_2$ (0.3 M), 0.5 mL of α-amylase were added into a plastic tube containing the sample of peel. After vortexing (30 seconds), 0.5 mL was taken from the solution, filtered, placed in an ice bath, and analyzed using HPLC system. Then, in order to simulate gastric digestion, 7.5 mL of SGF, 0.295 mL of H_2O, 5 μL of $CaCl_2$ (0.3 M), 0.2 mL of HCl (1 M) and 2 mL of pepsin were added into the solution after simulated oral digestion. The solution was incubated (2 hours, 37 °C, water bath with a shaking device), centrifuged (5 minutes, 5 °C, 9500 rpm), and 0.5 mL was taken from the solution, filtered, put in an ice bathand analyzed using HPLC system. Finally, for the intestinal digestion, 11 mL of SIF, 3.61 mL of H_2O, 40 μL of $CaCl_2$ (0.3 M), 0.15 mL of NaOH (1 M), 5 mL of pancreatin and 0.2 mL of bile salt were added to the solution after simulated oral and gastric digestion. The solution was incubated (2 hours, 37 °C, water bath with a shaking device), centrifuged (5 minutes, 5 °C, 9500 rpm), and 1 mL was taken from the solution, filtered, placed in an ice bath and analyzed using HPLC system.

The recovery was calculated as:

$$recovery\ (\%) = \frac{\gamma_{digestion\ phase}\ (mg/kg)}{\gamma_{before\ digestion}\ (mg/kg)} * 100 \quad (1)$$

where
$\gamma_{digestion\ phase}$ is the concentration of polyphenol after a particular digestion phase (mg/kg fresh weight (FW)), $\gamma_{before\ digestion}$ is a polyphenol concentration in fruit before digestion determined with chemical and enzyme assisted extraction (mg/kg FW).

Reversed phase high performance liquid chromatography

HPLC system (1260 Infinity II, a quaternary pump, a PDA detector, a vialsampler) (Agilent technology, Santa Clara, CA, USA) with Poroshell 120 EC C-18 column (4.6 × 100 mm, 2.7 μm) and a Poroshell 120 EC-C18 4.6 mm guard-column was used to analyze all the samples. 10 μL of each sample was injected into the system and polyphenols were separated using 0.1% H_3PO_4 (mobile phase A) and 100% methanol (mobile phase B). The gradient was: 0 min 5% B, 5 min 25% B, 14 min 34% B, 25 min 37% B, 30 min 40% B, 34 min 49% B, 35 min 50% B, 58 min 51% B, 60 min 55% B, 62 min 80% B, 65 min 80% B, 67 min 5% B, 72 min 5% B, with a flow of 0.8 mL min^{-1} (Jakobek et al., 2020). Spiking samples with authentic standards and by comparing UV/Vis spectrum (200 to 600 nm) of standards and samples was used to identify polyphenols.

Statistical analysis

All experiments were repeated three times. The results were reported as mean ± standard deviation. The differences between results were analyzed using post-hoc Tukey test (Minitab LLC., State College, PA, USA).

Results and discussion

Polyphenols in the peel

Peel polyphenols, are well documented in the literature, and are composed of four or five subclasses depending on the color of the apple peel. Red apples, besides flavan-3-ols, dihydrochalcones, phenolic acids and flavonols, contain anthocyanins (Jakobek and Barron, 2016; Kschonsek et al., 2018). Total of

fourteen polyphenols were found in the chemical extracts of the peel 'Idared' (Fig. 1). Table 2 shows the amount of polyphenols after chemical and enzymatic extractions. The most abundant polyphenol subclass was flavonol subclass (720.9 mg/kg), followed by flavan-3-ols (302.7 mg/kg), phenolic acids (93.8 mg/kg), dihydrochalcones (66.8 mg/kg) and anthocyanins (24.9 mg/kg). Similar amounts were reported earlier (Jakobek et al., 2020; Kschonsek et al., 2018; Lo Piccolo et al., 2019). Enzymatic extractions were used to solubilize polyphenols bound to dietary fiber (Bergantin et al., 2017). The amount of polyphenols after enzymatic extraction was significantly lower than the amount after chemical extraction, and it contributed to approximately 12 % of the total amount of extracted polyphenols.

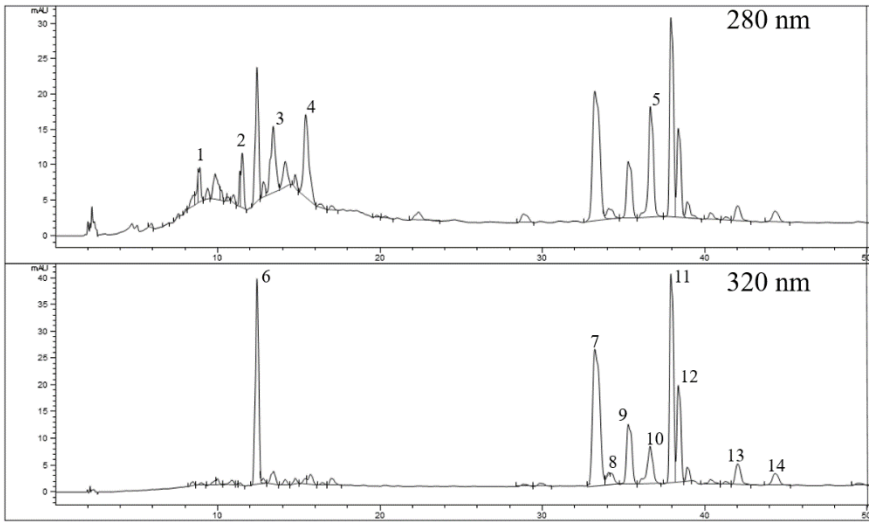

Fig. 1. RP-HPLC chromatographs of the polyphenols extracted from the peel of apple 'Idared. Peaks: 1-Procyanidin B1; 2- (+)-catechin; 3- (-)-epicatechin; 4- Cyanidin-3-galactoside; 5- Phloretin-2-glucoside; 6- Chlorogenic acid; 7- Quercetin-3-galactoside; 8- Quercetin-3-glucoside; 11- Quercein-3-xyloside; 12- Quercetin-3-rhamoside; 9, 10, 13, 14- Querectin derivatives

Table 2. The amounts of polyphenols from the peel of apple 'Idared' obtained after chemical extraction and first and second enzymatic extraction (mg/kg fresh weight (FW))

	Chemical extraction	Enzymatic extraction 1	Enzymatic extraction 2
Anthocyanins			
Cyanidin-3-galactoside	24.9 ± 1.1		
Total	24.9 ± 1.1		
Flavan-3-ols			
Procyanidin B1	45.2 ± 1.0		
(+)-catechin	45.5 ± 7.3	45 ± 14.0	
(-)-epicatechin	149.5 ± 4.4	7.1 ± 2.2	10.4 ± 0.1
Total	240.2 ± 12.7^{a}	52.1 ± 16.2^{b}	10.4 ± 0.1^{c}
Dihydrochalcones			
Phloretin-2-glucoside	60.0 ± 3.4	4.1 ± 1.6	2.7 ± 0.2
Total	60.0 ± 3.4^{a}	4.1 ± 1.6^{b}	2.7 ± 0.2^{b}
Phenolic acids			
Chlorogenic acid	88.9 ± 1.0	4.9 ± 1.9	
Total	88.9 ± 1.0^{a}	4.9 ± 1.9^{b}	
Flavonols			
Quercetin-3-galactoside	175.9 ± 3.5	8.8 ± 0.5	1.6 ± 0.1
Quercetin-3-glucoside	17.2 ± 4.4	1.5 ± 0.3	
Quercetin derivative 1	99.7 ± 4.5	9.9 ± 0.4	4.6 ± 0.1
Quercetin derivative 2	17.4 ± 4.0	4.5 ± 0.1	
Quercetin-3-xyloside	234.3 ± 14.0	20.3 ± 1.3	6.8 ± 0.3
Quercetin-3-rhamnoside	61.0 ± 2.5	9.1 ± 0.1	5.7 ± 0.1
Quercetin derivative 3	19.5 ± 0.7		
Quercetin-derivative 4	23.1 ± 0.9		
Total	648.1 ± 34.5^{a}	54.1 ± 2.7^{b}	18.7 ± 0.6^{c}
Total polyphenols	1062.1 ± 52.7^{a}	115.2 ± 22.4^{b}	31.8 ± 0.9^{c}

The means in a same row that do not share a letter are statistically different according to post-hoc Tukey pairwise comparison test at significance level 0.05. The results are reported as means ± standard deviations.

In vitro gastrointestinal digestion

The peel of apple 'Idared' underwent digestion simulation process and earlier identified polyphenols were tracked throughout the oral, gastric and intestinal phase of digestion. Table 3 shows the amounts of polyphenols before digestion and after each phase of digestion. For majority polyphenols, significantly lower amounts were detected after digestion in comparison to the amount present in undigested apple peel. These findings are similar to the earlier reported studies (Bouayed et al., 2012; Lingua et al., 2019; Quatrin et al., 2020). As can be seen from Fig. 2, the highest amount of total polyphenols was released in the gastric phase of digestion (37 % of the native amount), followed by oral phase (26 %) and intestinal phase (22 %). The same trend (gastric recovery > oral recovery > intestinal recovery) was established in our previous work, which investigated the recovery of traditional apple polyphenols (Jakobek et al., 2021). Similar results were reported in the studies that evaluated the effect of simulated digestion on polyphenols of white and red grapes. 34 %, 37 %, and 13 % of white grape polyphenols were recovered after oral, gastric, and intestinal digestion, respectively (Lingua et al., 2019), while red grapes had a slightly lower recovery (24 %, 29%, and 16 % for oral, gastric and intestinal phase, respectively) (Lingua et al., 2018). Fernández-Jalao et al. (2020) investigated the impact of gastrointestinal digestion on apple phenolic compounds. They reported gastric and intestinal recoveries similar to ours (32 and 28%, respectively) while oral recovery was somewhat higher (43 %).

Table 3. The amounts of polyphenols from peel of apple 'Idared' before digestion and recovered polyphenols after oral, gastric and intestinal digestion (mg/kg fresh weight (FW))

	Before digestion	Oral phase	Gastric phase	Intestinal phase
Anthocyanins				
Cyanidin-3-galactoside	24.9 ± 1.1^{a}	3.6 ± 0.9^{b}	4.4 ± 0.4^{b}	
Total	24.9 ± 1.1^{a}	3.6 ± 0.9^{b}	4.4 ± 0.4^{b}	
Flavan-3-ols				
Procyanidin B1	45.2 ± 1.0^{a}	11.8 ± 0.1^{c}	24.7 ± 0.4^{b}	
(+)-catechin	90.5 ± 21.3^{a}	11.5 ± 1.0^{b}	16.7 ± 1.5^{b}	
(-)-epicatechin	167.0 ± 6.7^{a}	15.2 ± 4.1^{b}	16.1 ± 0.9^{b}	
Total	302.7 ± 39.0^{a}	38.5 ± 5.2^{b}	57.5 ± 2.8^{b}	
Dihydrochalcones				
Phloretin-2-glucoside	66.8 ± 5.0^{a}	15.6 ± 2.6^{b}	17.4 ± 1.0^{b}	5.2 ± 1.2^{c}
Total	66.8 ± 5.0^{a}	15.6 ± 2.6^{b}	17.4 ± 1.0^{b}	5.2 ± 1.2^{c}
Phenolic acids				
Chlorogenic acid	93.8 ± 2.9^{a}	47.2 ± 0.1^{c}	55.2 ± 0.7^{b}	10.7 ± 0.6^{d}
Total	93.8 ± 2.9^{a}	47.2 ± 0.1^{c}	55.2 ± 0.7^{b}	10.7 ± 0.6^{d}
Flavonols				
Quercetin-3-galactoside	186.3 ± 4.1^{a}	47.5 ± 7.9^{c}	67.3 ± 8.2^{b}	48.4 ± 1.6^{c}
Quercetin-3-glucoside	18.7 ± 4.7^{c}	47.0 ± 9.9^{a}	75.2 ± 8.4^{b}	$54.7 \pm 8.2^{a,b}$
Quercetin derivative 1	114.2 ± 5.0^{a}	24.2 ± 4.0^{c}	37.0 ± 3.6^{b}	$29.3 \pm 0.1^{b,c}$
Quercetin derivative 2	21.9 ± 4.1^{a}	12.9 ± 2.0^{b}	24.3 ± 1.9^{a}	20.4 ± 0.2^{a}
Quercetin-3-xyloside	261.5 ± 15.6^{a}	15.3 ± 3.5^{c}	24.8 ± 3.3^{c}	46.7 ± 0.1^{b}
Quercetin-3-rhamnoside	75.8 ± 2.7^{a}	35.0 ± 2.0^{c}	53.9 ± 4.7^{b}	24.9 ± 0.1^{d}
Quercetin derivative 3	$19.5 \pm 0.7^{b,c}$	16.5 ± 2.4^{c}	23.2 ± 4.1^{a}	$21.1 \pm 0.3^{a,b}$
Quercetin-derivative 4	23.1 ± 0.9^{a}	13.5 ± 2.0^{b}	4.4 ± 0.4^{c}	
Total	721.0 ± 37.8^{a}	211.9 ± 33.7^{c}	310.1 ± 34.6^{b}	$245.5 \pm 10.6^{b,c}$
Total polyphenols	1209.2 ± 85.1^{a}	316.8 ± 42.5^{c}	444.6 ± 39.5^{b}	261.4 ± 12.4^{c}

The means in a same row that do not share a letter are statistically different according to post-hoc Tukey pairwise comparison test at significance level 0.05. The results are reported as means ± standard deviations.

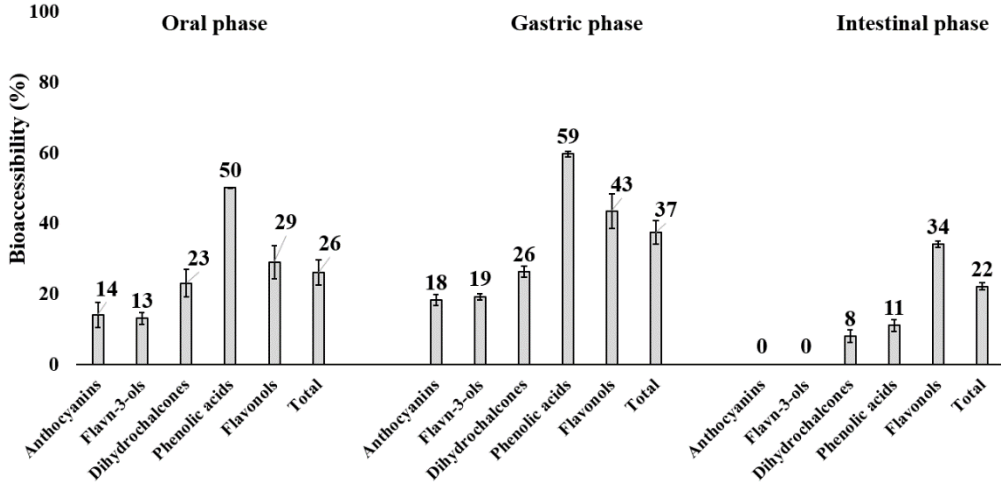

Fig. 2. The percentage recovery of polyphenols from the peel of apple 'Idared' after oral, gastric and intestinal digestion

Anthocyanins, namely cyanidin-3-galactoside, were found in the peel of 'Idared' in small amounts (Table 2). They were released in oral and gastric phases in similar amounts (3.6 and 4.4 mg/kg, respectively) (Table 3) which accounts for 14 and 18 %, respectively (Fig. 2). They were not found in the intestinal phase (Fig. 2). This might be due to their instability at higher pH such as pH 7 of the intestinal phase. At this pH anthocyanins undergo structural transformations from flavylium cation to colorless chalcone which could hinder their detectability (Pérez-Vicente et al., 2002). The disappearance of apple anthocyanins during intestinal digestion was reported in earlier studies (Bouayed et al., 2012; Jakobek et al., 2021). However, anthocyanins were found after intestinal digestion of jaboticaba fruit and strawberries, although in a small amount. This could be due to much higher initial amounts of anthocyanins compared to apples (Fernández-Jalao et al., 2020; Hilary et al., 2020; Stübler et al., 2020; Quatrin et al., 2020).

Flavan-3-ols showed the same trend as anthocyanins, where their amount increased (although not significantly) from oral to gastric phase (from 38.5 to 57.5 mg/kg) which accounts for 13 and 19 % of total flavan-3-ols, respectively (Fig 2). They were not present in the intestinal phase (Table 3). A much longer duration of the gastric phase compared to the oral phase might explain the observed increase of flavan-3-ols. Degradation of flavan-3-ols due to the transfer from the acidic gastric environment to neutral pH of the intestines might explain their disappearance. These findings are in accordance with earlier studies, in which degradation of flavan-3-ols to unknown compounds due to autooxidation, polymerization, transformation or complexation was suggested (Fernández-Jalao et al., 2020; Hilary et al., 2020).

Dihydrochalcones, namely phloretin-2-glucoside, were detected in the peel extracts, as well as in oral, gastric and intestinal phase of digestion. Similar amounts were recovered after oral and gastric phase (15.6 and 17.4 mg/kg, respectively), while the amount recovered after intestinal phase was significantly lower (5.2 mg/kg) (Table 3). Their oral, gastric, and intestinal recovery was 23, 26 and 8 %, respectively (Fig 2). Suggested gastric stability of phloretin-2-glucoside might be the reason for similar amounts in oral and gastric phase (Fernández-Jalao et al., 2020). However, other authors reported an increase in the amount of dihydrochalcones after intestinal phase ((Bouayed et al., 2012; Fernández-Jalao et al., 2020; Jakobek et al., 2021), which was not the case in this study.

Chlorogenic acid was the only phenolic acid identified in the peel of apple 'Idared'. As can be seen from Fig. 2, out of all polyphenol subclasses phenolic acids had the highest recovery throughout oral and gastric phases (50 and 59 %, respectively) (Fig. 2). However, their intestinal recovery decreased to 11 %. These results are in accordance with our previous work (Jakobek et al., 2021). A significant increase of phenolic acids from oral to gastric phase was detected (from 47.2 to 55.2 mg/kg), followed by a significant decrease after intestinal phase (from 55.2 to 10.7 mg/kg) (Table 3). The decrease of phenolic acids in the intestinal phase was reported in other studies of apple polyphenols (Bouayed et al., 2012) or other sources (Hilary et al., 2020; Lingua et al., 2018).

Flavonols were the most abundant polyphenols both in extracts and after gastrointestinal digestion (Table 3). Unlike other polyphenol subclasses, flavonols had a higher recovery in the intestinal phase than in the oral phase. The same trend was observed in other studies as well (Fernánndez-Jalao et al., 2020; Jakobek et al.,

2021). Furthermore, flavonols were the only polyphenol group that did not show a significant decrease from gastric to intestinal phase (from 310.1 to 245.5 mg/kg) (Table 3). It was suggested that quercetin derivatives were resistant to the mild alkaline environment of the intestine since quercetin was not detected, meaning that quercetin derivatives hydrolyzed to a lesser extend than other compounds (Jakobek et al., 2021). Their recovery was 29, 43 and 34 % for oral, gastric and intestinal phase, respectively (Fig. 2). These results are similar to those reported in our previous study (Jakobek et al., 2021).

Conclusion

This study investigated polyphenols from the peel of 'Idared' after gastrointestinal digestion. Five subclasses of polyphenols were found in the peel – anthocyanins, flavan-3-ols, dihydrochalcones, phenolic acids and flavonols. The amount of all polyphenols significantly decreased after the simulated digestion. Gastric recovery of total polyphenols was the highest (37 %), followed by oral (26 %) and intestinal (22 %). All polyphenol subclasses followed this trend as well, except for flavonols. Flavonols showed the best stability in the intestinal environment, while flavan-3-ols and anthocyanins were not found.

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PREVENTION OF GENOTOXIC EFFECT OF OCHRATOXIN A AND AFLATOXIN B1 BY ECHINACEA PURPUREA EXTRACTS

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original scientific paper

Summary

The aim of this work was to examine the polyphenol content in *Echinacea purpurea* (L.) Moench extracts and to determine their antioxidant and antigenotoxic effect. The antioxidant activity of extracts and main components is determined by the application of electronic spin resonance and spectophotometric methods of capturing DPPH, superoxide and hydroxyl radical. The antigenotoxic effect was evaluated with genotoxic mycotoxins – aflatoxin B_1 (AFB_1) and ochratoxin A (OTA). Herbe ehinacea extract contained 13.31% of total polyphenols. Ehinacea extract contained a sufficient amount of phenolic acids (primarily chicoric and caftaric acid). Ehinacea extract showed the ability to neutralize DPPH radicals (EC_{50} = 15.67 μg/mL). Similar results were also identified by testing the capture of hydroxyl and superoxide radicals. The strong antioxidant activity of the dominant components that indicate phenolic acids as the ingredients that contribute the most to the antioxidant and antigenotoxic effect of echinacea extract was also found. Genotoxic suppression of AFB_1 and OTA was established by a comet test, establishing a significant reduction in tail, tail intensity and tail torque in leukocytes co-treated with mycotoxins and different concentrations of extracts.

Keywords: antioxidant activity, comet assay, *Echinacea purpurea*, genotoxicity, polyphenols

Introduction

Numerous epidemiological studies have shown that there is a strong association between fruit and vegetable intake and reduced mortality from heart disease, tumors and other degenerative diseases, and that intake of these foods reduces or delays the development of mentioned diseases and aging (Block et al., 1992; Scalbert et al., 2005). This effect is today attributed to the presence of various antioxidants, especially polyphenols. Among phytochemicals, polyphenols form one of the most numerous and widespread groups of compounds in the plant world, with more than 8.000 currently known structures. Polyphenols, which are normally present in the daily diet, have recently gained great importance due to their redox properties and possible positive impact on health, as well as in the prevention and treatment of diseases associated with oxidative stress, such as cardiovascular diseases and cancer (Zhou et al., 2006; Vinson et al., 1998; Teow et al., 2007).

Many substances of plant origin have been shown to "capture" free radicals, which are known to cause oxidative stress. Purple coneflower - *Echinacea purpurea* (L.) Moench is a good example of such plant species that contains a number of bioactive substances with potential antioxidant properties.

Species of the *Echinacea* L. genus are known immunomodulators, whose phytochemical composition indicates the presence of substances with antioxidant and anti-inflammatory effects (Barnes et al., 2005). Polyphenols from the group of phenol-carboxylic acid derivatives are associated with the prevention of diseases caused by oxidative stress, such as malignant, cardiovascular and neurodegenerative diseases (Mikulášová et al., 2005). Previous research has shown the anti-genotoxic effects of aqueous and alcoholic extracts of aboveground parts of *E. purpurea* (Kopjar et al., 2007), as well as the antimutagenic effect of the most representative phenolic carboxylic acids in *E. purpurea* extracts using the Ames test (Mikulášová et al., 2005). It is assumed that polyphenols with their antioxidant action reduce the formation of free radicals that significantly contribute to the cytotoxic and genotoxic effect of certain mycotoxins (Costa et al., 2007; Kopjar et al., 2007).

The aim of this study was to investigate whether extracts of *Echinacea purpurea* (L.) Moench extract are antigenotoxic *in vitro*. The results determined the concrete effect of the extract, as well as the most

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dominant components of *E. purpurea* after treatment of cells with mycotoxins ochratoxin A and aflatoxin B_1.

Materials and methods

Plant material and chemicals

Plant material

The leaves of the plant (belonging to the aboveground parts of the leaf, flower, stem) of the species Echinacea purpurea (L.) Moench, taken from the company Jan-Spider (Pitomača, Croatia), were used as material. Air-dried and pulverized plant material (20.00 g) was extracted with 200 mL of 70% ethanol using an ultrasonic bath for 30 minutes. The extract was then filtered and the residue was then re-extracted with 200 mL of the same solvent as described above. Obtained extracts were combined and then concentrated to dryness under vacuum at 50 °C using a rotary evaporator.

Chemicals

Acetic acid, aluminium chloride, formic acid, disodium hydrogen phosphate, ethanol, ethylenediaminetetraacetic acid (EDTA), hexamethylenetetramine, methanol, pyrogallol, sodium carbonate, sodium citrate, sodium dihydrogen phosphate, sodium hydroxide, sodium nitrite, sodium phosphate, sulphuric acid, tannic acid (95%), thiourea were purchased from Kemika (Zagreb, Croatia). Acetonitrile (HPLC grade) was purchased from J.T.Baker (Deventer, Netherlands). Ammonium molybdate, chlorogenic acid, caffeic acid, cynarin, echinacoside, chicoric acid, caftaric acid, casein, 2-deoxy-D-ribose, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine), 2,2-diphenyl-1-picryl-hydrazyl (DPPH$^{\bullet}$), hydrogen peroxide, potassium ferricyanide, rosmarinic acid (96%), sodium acetate, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), dimethylsulfoxide (DMSO) and sodium molybdate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Butylated hydroxytoluene (BHT, ≥ 99%), iron (II) chloride and quercetin-3-rutinoside (rutin, ≥ 95%) were obtained from Fluka (Buchs, Switzerland). Ascorbic acid (99%) and trichloroacetic acid (TCA) were purchased from Acros Organics (Geel, Belgium), Folin–Ciocalteu's phenol reagent, 3-*tert*-butyl-4-hydroxyanisole (BHA) and 2-thiobarbituric acid (TBA) were obtained from Merck (Darmstadt, Germany). Iron(III) chloride and hydrochloric acid were obtained from Riedel-de Haën (Seelze, Germany) and POCh (Gliwice, Poland), respectively.

Qualitative and quantitative analysis of E. purpurea extract

Determination of total polyphenols, tannins and flavonoids, phenolic acid

Determination of total tannin as well as total polyphenol contents was performed following the method described in European Pharmacopoeia (EDQM, 2004). Briefly, the extract (0.5 g) was boiled for 30 min in a water bath with water (150 mL), then the filtrate was made up to 250 mL with water and the obtained solution served as stock solution. An aliquot of stock solution was mixed with Folin–Ciocalteu's phenol reagent and sodium carbonate solution. After 30 min, the absorbance was read at 720 nm (A_1), and the quantification of total phenols was done with respect to the standard calibration curve of pyrogallol (6.25-50.00 μg). For the determination of tannins content, stock solution was vigorously shaken with hide powder for 60 min. Since the hide powder adsorbed tannins, phenols unadsorbed on hide powder were measured in filtrate, after addition of Folin–Ciocalteu's phenol reagent in a sodium carbonate medium (A_2). The percentage content of tannins, expressed as pyrogallol, was calculated from the following equation:

$$(\%) = 3.125 \times (A_1 - A_2)/(A_3 \times m) \quad (1)$$

where A_3 is the absorbance of the test solution containing 0.05 g of pyrogallol, and m the mass of the extract (g).

The total flavonoid contents of tested plant extract were determined using the spectrophotometric method of Christ and Müller (1960). Each powdered plant sample (0.2 g) was mixed with 20 mL of acetone, 2 mL of 25% hydrochloric acid and 1 mL of 0.5% hexamethylenetetramine solution and heated under reflux in a water bath for 30 min. The extract was filtered and re-extracted twice with 20 mL of acetone for 10 min. Filtrates were combined and made up to 100 mL with acetone. An aliquot of 20 mL of the acetone extract was mixed with 20 mL of water and then extracted with three quantities, each of 15 mL, of ethyl acetate. Combined ethyl acetate layers were washed twice with water then filtered and diluted to 50 mL. To 10 mL of this solution 0.5 mL of 0.5% solution of sodium citrate and 2 mL of 2% aluminium chloride solution (in 5% methanolic solution of acetic acid) was added and then diluted to 25 mL with 5% methanolic solution of acetic acid. The mixture was allowed to stand for 45 min and the absorbance was measured at 425 nm. A sample solution prepared in the

same manner but without addition of aluminium chloride solution served as a blank. All determinations were performed in triplicate. The percentage content of flavonoids, expressed as quercetin, was calculated from the equation:

$$(\%) = A \times 0.772/b \quad (2)$$

where *A* is the absorbance of the test solution at 425 nm and *b* is the mass of the sample, in grams.

Determination of total phenolic acids

Determination of hydroxycinnamic acid derivatives was performed according to procedure described in European Pharmacopoeia (EDQM, 2004). Briefly, 0.2 g of the powdered plant material was extracted with 80 mL of 50% ethanol under a reflux condenser in a boiling water bath for 30 min. The cooled extract was filtered, the filter rinsed with ethanol, and then combined filtrate and rinsing was diluted to 100 mL with 50% ethanol. An aliquot of 1 mL of the extract was mixed with 2 mL of 0.5 M hydrochloric acid, 2 mL of Arnow reagent (10% aqueous solution of sodium nitrite and sodium molybdate), and 2 mL of 8.5% sodium hydroxide and diluted to 10 mL with water. The absorbance of the test solution was measured immediately at 505 nm against sample blank. The percent of total hydroxycinnamic acid content was calculated and expressed as rosmarinic acid, according to the following expression:

$$(\%) = A \times 5.3/m \quad (3)$$

where *A* is the absorbance of the test solution at 525 nm and *m* is the mass of the sample, in grams. Analysis of each sample was performed in triplicate.

HPLC analysis of E. purpurea extract

Herbal extracts were analyzed according to the method described by Belščak-Cvitanović et al. (2011). The samples were filtered through a 0.45 μm filter (Nylon Membranes, Supelco, Bellefonte, USA) before HPLC analysis. 20 μL of each sample was injected for HPLC analysis using a Varian Pro Star Solvent Delivery System 230 (Varian, Walnut Creek, USA) and a Photodiode Array detector Varian Pro Star 330 (Varian, Walnut Creek, USA) by using a reversed-phase column Gemini-NX C-18 column (Phenomenex, USA) (150×4.6 mm, 2.6 μm i.d.). The solvents consisted of 3% formic acid in acetonitrile (solvent A) and 3% formic acid in water (solvent B) at a flow rate of 0.9 mL/min. The elution was performed with a gradient starting at 10% A, rising to 40% A after 25 min, then to 70% A after 30 min and becoming isocratic for 5 min. Chromatograms were recorded at 278 nm. Detection was performed with a Photodiode Array Detector by scanning between 200 and 400 nm, with a resolution of 1.2 nm. Phenolic compounds were identified by comparing the retention times and spectral data with those of standards. The data acquisition and treatment were conducted using Star Chromatography Workstation Version 5 software. All analyses were repeated three times.

Antioxidant activity of E. purpurea extract

2, 2-Diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) radical scavenging assay

The free radical scavenging activities of the samples were measured using the stable DPPH$^{\bullet}$ radical, according to the method of Blois (1958). Briefly, 0.1 mM solution of DPPH$^{\bullet}$ in ethanol was prepared and 1 mL of this solution was added to 3 mL of sample solution in ethanol at different concentrations (0.39-200 μg/mL). The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm. The capability to scavenge the DPPH$^{\bullet}$ radical was calculated using the following equation:

$$(\%) = [(A_0 - A_1)/A_0] \times 100 \quad (4)$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of sample, corrected for the absorbance of sample itself. Butylated hydroxytoluene (BHT) was used for comparison. All determinations were done in triplicate.

Hydroxyl radical (OH$^{\bullet}$) scavenging assay

Hydroxyl radicals were generated by a Fenton reaction (Fe^{3+}-ascorbate-EDTA-H_2O_2 system), and the scavenging capacity towards the hydroxyl radicals was measured by using a deoxyribose method as described by Halliwell et al. (1987) with a slight modification. The reaction mixture contained, in a final volume of 1 mL, 2-deoxy-2-ribose (2.8 mM), phosphate buffer (0.1 mM, pH 7.4), iron (III) chloride (20 μM), EDTA (100 μM), hydrogen peroxide (500 μM), ascorbic acid (100 μM) and various concentrations (12.5-1600 μg/mL) of the test sample or reference compound. After incubation for 1 h at 37 °C, an aliquot of the reaction mixture (0.5 mL) was

added to 1 mL of 2.8% TCA solution, followed by 1 mL of TBA solution (1% in 50 mM sodium hydroxide) and then the mixture was heated 20 min at 90 °C to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All experiments were performed in triplicate. Hydroxyl radical scavenging activity was evaluated with the inhibition percentage of 2-deoxyribose oxidation by hydroxyl radicals, according to the following equation:

$$(\%) = [A_0 - (A_1 - A_2)]/A_0 \times 100 \quad (5)$$

where: A_0 is the absorbance of the control without a sample, A_1 is the absorbance in the presence of the sample and deoxyribose and A_2 is the absorbance of the sample without deoxyribose. Thiourea was used as a positive control.

Reducing power assay

The reducing power of samples was determined by the method of Oyaizu (1986). An aliquot of 1 mL of the sample at various concentrations (0.20-50 μg/mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After adding 2.5 mL of 10% trichloroacetic acid, the mixture was centrifuged at 650 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% iron (III) chloride (0.5 mL), and the absorbance was measured at 700 nm using appropriate blank. Assays were carried out in triplicate. BHT was used as a reference.

Metal ion chelating assay

The ability of samples to chelate iron (II) ions was estimated using the method reported by Gülçin (2006) and compared with that of reference chelator agent EDTA. Different concentrations of the sample (final concentration 0.78-800 μg/mL) were added to a solution of 2 mM iron (II) chloride (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and the mixture was finally quantified to 4 mL with ethanol, shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. All assays were done in triplicate. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated using the formula given below:

$$(\%) = [A_0 - (A_1 - A_2)]/A_0 \times 100 \quad (6)$$

where A_0 is the absorbance of the control, containing iron (II) chloride and ferrozine only, A_1 is the absorbance in the presence of the tested sample and A_2 is the absorbance of the sample under identical conditions as A_1 with water instead of iron (II) chloride solution.

Total antioxidant capacity assay

The total antioxidant capacity of tested plant extract and their active constituents were evaluated by the phosphomolybdenum method, according to the procedure of Prieto et al. (1999). An aliquot of 0.4 mL of the sample solution in ethanol was combined in a vial with 4 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The effective concentrations of the sample in the reaction mixtures were in the range of 12.5-100 μg/mL. The vials were capped and incubated in a water bath at 95 °C for 90 min. After cooling the mixture to room temperature, the absorbance was measured at 695 nm against a blank. The antioxidant capacity of the sample was expressed as equivalents of ascorbic acid (AAE), utilizing a calibration curve of ascorbic acid in the concentration range from 1.56 to 100 μg/mL. All assays were run in triplicate.

ESR measurements

Hydroxyl radical scavenging activity

As hydroxyl free radicals (•OH) are highly reactive, with relatively short half-lives, the concentrations found in natural systems are usually inadequate for direct detection by ESR spectroscopy. Spin-trapping is a chemical reaction that provides an approach to help overcome this problem. Hydroxyl radicals are identified because of their ability to form nitroxide adducts (stable free radicals form) from the commonly used DMPO as the spin trap (Buettner, 1985). The Fenton reaction was conducted by mixing 200 μL of DMPO (112 mM), 200 μL of DMF, 200 μL of H_2O_2 (2 mM) and 200 μL of $FeCl_2$ (0.3 mM) (control). The influence of *E. purpurea* extract on the formation and stabilization of hydroxyl radicals was investigated by adding investigated extracts in the Fenton reaction system at the range of concentrations 25-1500 μg/mL. ESR spectra were recorded after 5 minutes, with the following spectrometer settings: field modulation 100 kHz, modulation amplitude 0.226 G, receiver gain 5 $x10^5$, time constant 80.72 ms, conversion time 327.68 ms, center field 3440.00 G, sweep width 100.00 G, x-

band frequency 9.64 GHz, power 20 mW, temperature 23 °C.
The SA_{OH} value of the extract was defined as:

$$SA^{\bullet}_{OH} = 100 \times (h_0 - h_x) / h_0 \, [\%] \qquad (7)$$

where h_0 and h_x are the hight of the second peak in the ESR spectrum of DMPO-OH spin adduct of the control and the probe, respectively.

Superoxide anion radical scavenging activity

Superoxide anion radicals ($O_2^{\bullet-}$) were generated in the reaction system obtained by mixing 500 μL of dry dimethylsulfoxide (DMSO), 5 μL of KO_2/crown ether (10 mM / 20 mM) prepared in dry DMSO and 5 μL of 2 M DMSO solution of DMPO as spin trap. The influence of extracts on the formation and transformation of superoxide anion radicals was obtained by adding the DMF solution of *E. purpurea* extract to the superoxide anion reaction system at the range of concentrations 5-100 μg/mL. After that the mixture was stirred for 2 min and transferred to a quartz flat cell ER-160FT. The ESR spectra were recorded on an EMX spectrometer from Bruker (Rheinstetten, Germany) under the following conditions: field modulation 100 kHz, modulation amplitude 4.00 G, receiver gain 1×10^4, time constant 327.68 ms, conversion time 40.96 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23 °C.

The $SAo_2^{\bullet-}$ value of the extract was defined as:

$$SAo_2^{\bullet-} = 100 \times (h_0 - h_x) / h_0 \, [\%] \qquad (8)$$

where h_0 and h_x are the height of the second peak in the ESR spectrum of DMPO-OOH spin adduct of the control and the probe, respectively.

Comet assay

This test was performed using whole human blood in the volume of 5 mL that was taken by venipuncture from a forty-year-old male volunteer (non-smoker). Blood was mixed with cell culture media RPMI 1640 (1:1) and transferred into 96-well plates. Prior to cell treatment water extract of *E. purpurea* was sterilised by filtration trough millipore filter (0.2 nm). Aflatoxin B_1 (AFB_1) was dissolved in dimethyl sulfoxide (DMSO), while absolute ethanol was used as solvent for OTA. Leukocytes were exposed for 2 hours to plant extracts at concentrations 1 mg/mL (E1), 10 mg/mL (E2) and 20 mg/mL (E3), single AFB_1 (3 μM) and OTA (10 μM), as well as combination of each mycotoxin with each concentration of plant extract. Water (10%), DMSO (0.03%) and ethanol (0.3%) were used as controls.
The comet assay was carried out according to Singh et al. (1988). After cell treatment Aliquots of 20 μL of cell suspension were mixed with 80 μL 0.5% low melting point agarose (LMP), and 100 μL of agarose-cell suspension was spread onto a fully frosted slide (Surgipath, Richmond, Il, USA) pre-coated with 1.5% normal melting point agarose, NMP (in Ca- and Mg-free PBS buffer). The slides were allowed to solidify on ice for 10 minutes. After overnight lysis at 4 °C in a mixture of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris (pH 10) supplemented with 1% Triton-X, the slides were placed in denaturation and electrophoresis buffer (10 mM NaOH, 200 mM Na_2EDTA, pH 13), incubated for 20 min, and electrophoresed for 20 min at 25 V and 300 mA. DNA was neutralised with a neutralisation solution (0.4 M Tris/HCl, pH 7.5) three times 5 min each. The slides were kept in a humid atmosphere in a dark box at 4 °C until further analysis. For image analysis, DNA was stained with 100-250 μL ethidium bromide solution per slide for 10 min. Slides were scored using an image analysis system (Comet assay II, Perceptive instruments Ltd., U.K.) connected to a fluorescence microscope (Zeiss, Germany). Images of 100 randomly selected cells were measured. Only comets with a defined head were scored. Comet parameters considered in this study were the tail length, the proportion of DNA in the comet tail (tail DNA or tail intensity), and tail moment, which was calculated as the product of the fraction of DNA in the comet tail and the tail length.

Results and discussion

The total amount of polyphenols in Echinacea purpurea

Echinacea species were investigated regarding their polyphenolic content and separation. The presence of flavonoids, phenolic acids and tannins in ethanolic plant extracts was detected by spectrophotometrical method. The results of spectrophotometric determination of the total amount of polyphenols (prepared as shown in chapter 2.2) are presented in the Table 1.
The determination of the amount of polyphenols in the species *E. purpurea* was performed with the spectrophotometrical method.
Table 1 shows the values of the content of the specimens of the ethanol extract in *E. purpurea* and the estimated content of the total polyphenols, tannin,

phenolic acid, flavonoids. It was determined that the overhead parts of the examined species contain ranging between 12.98% and 13.80% of polyphenols, tannin between 0.85% and 0.92; 3.23% to 3.72% hydroxycinnamic derivatives and portions of flavonoid between 0.123% to 0.131%.

Table 1. Contents of phenolic acids, flavonoids, tannins and total polyphenols in *E. purpurea* selected *Echinacea* species

Plant extracts	Contens (%)			
	Total polyphenols	Flavonoids	Phenolics acids	Tanins
Echinacea purpurea	13.31±0.43	0.126±0.004	3.47±0.25	0.863±0.003

Each value is the mean ± SD of three indenpendent measurements.

Phytochemical analysis of polyphenolic compounds

A large number of HPLC methods for determining phenolic compounds in plant extracts have been published. Essentially, they are adapted to determine the content of the most dominant polyphenols in one plant species or a certain number of compounds of that class in a variety of extracts (Harnly et al., 2006). Given the large number of compounds belonging to the flavonoid group, as well as the fact that most are related in the form of glycosides, it is difficult to find the ideal method for determining their total content. Flavons and flavonoids (quercetin, luteoline, apigenin, routine) in such biological substrates most often appear in glycosylated form, so a specific glycoside is also needed to identify them There are a large number of works relating to the examination of the composition of polyphenol *E. purpurea* (Cech et al., 2006; Lin et al., 2011).

In the extract of echinacea (Fig. 1) the presence of chicoric, caftaric, chlorogenic, and caffeic acids and echinacoside were identified the quantified. The chromatogram (Fig. 1) of the ethanol extract of the echinacea herb clearly shows pronounced peaks of chicoric and caftaric acid.

Chicoric acid derivative is a caffeic acid and is the most represented phenolic component in *E. purpurea*. Similar results were obtained from research and other authors (Kuštrak, 2005; HMPC, 2008; Stanisavljević et al., 2009; Lin et al., 2011). Cynarine was not detected, similar to the work of Lin and associates (2011), who did not detect it in any part of the plant (overhead parts, stems and roots).

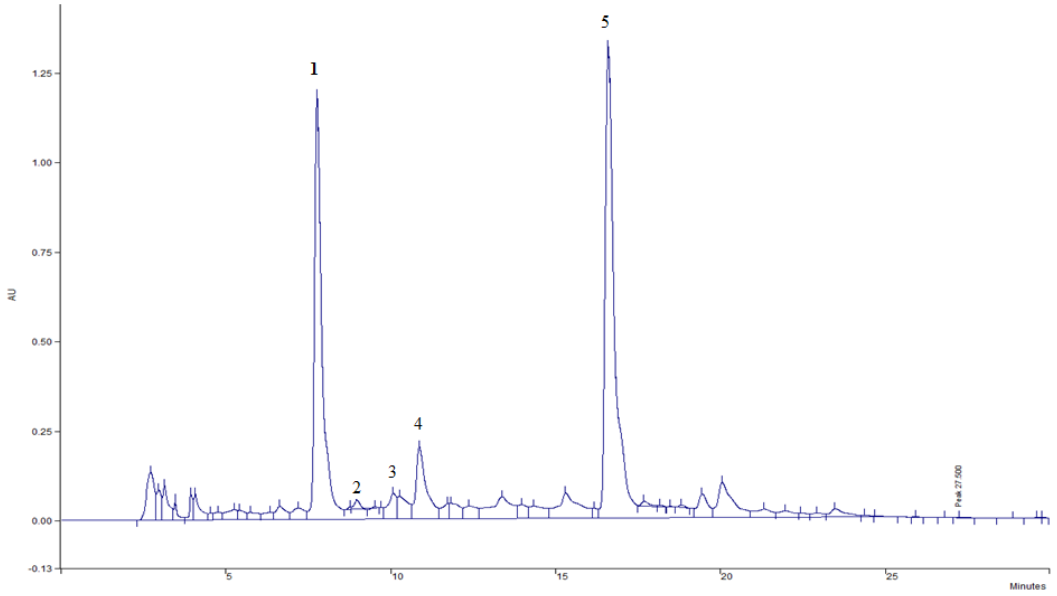

Fig. 1. Chromatogram of extract *Ehinacea purpurea* : 1- caftaric acid, 2- chlorogenic acid, 3- caffeic acid, 4- echinacoside, 5- chicoric acid

Table 2 shows the data on the content of the extract of the plant *E. purpurea*, where the largest share of chicoric and caftaric acid. This agrees with Pellati et al. (2004) that the main phenolic components of the species *E. purpurea* are chicoric and caftaric acid. According to literature sources, the main components

of the overhead part of the plant are alkylamides, polyacetylenes and caffeic acid derivatives, polysaccharides and glycoproteins (Stanisavljević et al., 2009). Established quantities of chicoric acid (Table 2) are in accordance with literature data, where it is stated that it is the dominant component and that *E. purpurea* extracts contain this acid in a very wide range (Lee and Scagel, 2010).

Table 2. Polyphenol components in purple ehinacea extract

Substance	Extract	
	mg/L	*mg/g plants*
Caftaric acid	2447.56	12.23
Chlorogenic acid	34.69	0.17
Caffeic acid	36.19	0.18
Echinacoside	400.04	2.00
Cynarine	nd	nd
Chicoric acid	3144.52	15.72

*nd - not detected

For example, the established share chicoric acid of the extract of overhead parts of ehinacea was 13 mg/g (Becker and Hsieh, 1985) or ranged from 1.4-38.3 mg/g (Bauer and Remiger, 1989; Stuart and Wills, 2000; Wills and Stuart, 1999). Differences in the content of chicoric acid are somewhat expected, as a large number of factors affect the content of bioactive components. Differences in concentrations of chicoric acid and other caffeic acid derivatives in plant material and preparations occurring by a particular analogue method may be affected by genetic variations and many environmental factors, light, temperature, extraction process, formulations and storage conditions (Pellati et al., 2004). Chicoric acid shows immunomodulating properties (Lee and Scagel, 2010; Stanisavljević et al., 2009), stimulates phagocytosis *in vivo* and *in vitro*, shows antihyaluronidase activity and protects collagen from degradation with free radicals. Chicoric acid showed antiviral action and was found to inhibit HIV-1 hiv integration and replication (Lee and Scagel, 2010; Stanisavljević et al., 2009; Lou et al., 2003). The content of chicoric acid, also correlated well when assessing antioxidant activity using different methods: the ability to capture DPPH radicals and the effect of peroxide lipid emulsion on oxygen consumption (Thygesen et al., 2007). Therefore, a significant influx of this component on the antioxidant effect of the extract of overhead parts of ehinacea can be expected.

Caftaric acid may inhibit the mutagenicity of heterocyclic aromatic amines in the Ames and micronucleus assays (Zhang et al., 2011).

It has also been shown to be effective in inhibiting phase I enzymes (cytochrome P450 1A1 and 1A2) and enhancing phase II enzyme activity (UDP-glucuronosyl transferase and GST-glutathione S-transferase) (Zhang et al., 2011). Phenolic acids can also inhibit the formation of mutagenic and carcinogenic N-nitroso components because they inhibit the reactions of their bioactivation *in vitro* (Kono et al., 1995). According to Kosalec (2006), laboratory studies have identified the immunostimulation effect of caftaric acid, similar to other derivatives of caffeic acid from purple echinacea (chicoric, chlorogenic, ferrulic, *p*-coumaric, *p*-hydroxybenzoic and vanillic acid). Caftaric acid is found predominantly in overhead parts of the plant (Perry et al., 2001).

Antioxidant activities of Echinacea ethanolic extracts

The antioxidant activities of polyphenols were attributed to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers, as well as their metal chelating abilities. Polyphenolic compounds such as flavonoids, phenolic acids and tannins are considered to be the major contributors to the antioxidant activity of medicinal plants, fruits and vegetables (Pereira et al., 2009; Rice-Evans et al., 1996). The ability to remove free radicals mostly depends on the structural properties of phenol compounds such as the energy of dissociation of O-H bound, the delocalisation of phenol radicals (PheO*) and steric disturbances caused by the substitutes on the aromatic ring (Sanchez-Moreno et al., 1998). Therefore, in the present study five different assays were employed in order to determine and compare the antioxidant properties of selected *Echinacea* species, as well as to elucidate their mode of action.

Unlike free radicals generated in a lab, such as superoxide and hydroxyl radicals, the use of free stable radicals is an advantage, since it is not influenced by the secondary reactions, such as chelate with metals and enzyme inhibition, caused by additives (Yamaguchi et al., 1998).

After measuring absorptions at 517, the percentage of the inhibition capacity of DPPH$^{\bullet}$ radicals were calculated. The plant extract in lower amounts has quite a weaker effect than the synthetic andtioxidant. Although it lags continually after the effect of BHA, the difference is significantly lowered in the amounts above 50 μg/mL. It was also revealed that the chlorogenic acid, rutin and tannic acid are better catchers of DPPH$^{\bullet}$ than the referent antioxidant. The effect of BHA is equalised with the effect of rutin only at the amount of 12.5 μg/mL when it accomplished the inhibition above 85%.

Abilities of the tested samples to scavenge DPPH• were assessed on the basis of their IC_{50} values which were inversely related to their antioxidant capacities, as they express the amount of the antioxidant needed to decrease the radical concentration by 50%. The IC_{50} values obtained in this study are listed in Table 3. The strongest antiradical activity was determined for the tannic acid which already in the amount of 0.78 μg/mL accomplishes a 50% exhibition of DPPH•. The chlorogenic acid shows the same effect in the amount of 1.56 μg/mL and is equalised with the tannic acid in the concentration of 6.25 μg/mL. The extract of the species also shows a significant scavenging capacity of DPPH•. In the concentration higher than 50 μg/mL the effect of the extract approaches the effect of clear substances and BHA. The results show that flavonoids, phenolic acid and tannins, present in the examined species, equally contribute to the antiradical effect of the extract.

The DPPH assay has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances. Nitrogen centered radicals such as DPPH• react with phenols via two different mechanisms: direct abstraction of phenol H-atoms and electron transfer processes. The contribution of one or the other pathway depends on the nature of solvent and/or the redox potentials of the species involved. DPPH• is a stable free radical compound with a characteristic absorption at a wavelength of 517 nm. Antioxidants upon interaction with DPPH• either transfer an electron or hydrogen atom to DPPH•, thus neutralizing its free radical character. The colour of the reaction mixture changes from purple to yellow with a decrease of the 517 nm absorbance. The degree of discolouration indicates the scavenging potential of the antioxidants (Villaño et al., 2007; Foti et al., 2004). The research of Yokozawa et al. (1998) has shown that tannins and some flavonoids show an activity in relation to DPPH• radicals and that the activity is closely related to their chemical structure. With the increase in galiol groups, the molecular mass and ortho-hydroxy groups in the structure, the activity of tanine increases, and the number and position of hydroxyl groups represent an important characteristic of flavonoids for "quenchers" free radicals.

Fenglin et al. (2004) released the results of the study of the 'scavangers' activity on DPPH radicals of water-methanol extracts of more then 300 medicinal herbs. For 56 of the examined specimens they got EC_{50} values under 0.500 mg of the specimen/mL of the extragent. The same authors attribute the activity of DPPH• radicals of plants to the present flavonoids and tannins in the extract. Chen et al. (2004) discovered that the chlorogenic acid most actively removes DPPH• radicals in plants, and that their activity in the same test is the same and larger than the activity of tocopherol.

Orhan et al. (2009) got similar results when they studied antioxidant activities of the species *E. purpurea* and *E. pallida* by determining the catching capacity of DPPH• of free radicals and chelate ions of iron. A chrloroform extract in air of dry plant material *E. purpurea* showed the greatest capacity of chelate iron ions (Orhan et al., 2009).

Table 3. Comparative overview of IC50 values as well as total antioxidant capacities of *Echinacea* ethanolic extracts, polyphenolic compounds and reference antioxidants

	IC_{50}* (μg/mL)				**Total antioxidant capacity (mg AAE/g)****
	DPPH scavenging activity	**OH scavenging activity**	**Reducing power**	**Iron chelating activity**	
E. purpurea extract	15.7	1071.9	47.69± 2.96	125.86±11.33	2.44±0.23
Rosmarinic acid	-	-	-	-	-
Rutin	1.7	62.0	7.89±0.66	nd	3.19±0.16
Tannic acid	0.78	11.9	2.58±2.96	nd	8.72±0.50
BHT	-	-	4.95±0.36	nd	7.79±0.13
Thiourea	-	81.3	-	-	-
Chlorogenic acid	1.56	260.8	3.48±0.33	nd	6.13±0.05
BHA	2.8	37.6	3.40±0.08	nd	8.70±0.39
EDTA	-	-	-	0.94±0.04	-

*IC_{50} value: concentration at which the DPPH and OH radicals were scavenged by 50%, absorbance was 0.5 for reducing power, iron(II) ions were chelated by 50%, respectively;

**Results are calculated for sample concentrations of 12.5 μg/mL. Each value is expressed as mean ± SD. (n = 3); nd - not determined at tested concentrations; − not tested.

The method based on the determination of the catching ability of OH• free radical was used, according to the principle of oxidising deoxyribose when exposed to the hydroxyl radicals which appear with the Fenton's

reaction. In the reaction the hydroxyl radicals appear with the decomposure of H_2O_2, whereby the high potential of EDTA-Fe^{2+} causes the decomposure of deoxyribose. The oxydative decomposure can be perceived by heating up products with 2-thiobarbituric acid (TBA) in acidic conditions, whereby the pink chromogene (TBARS, thiobarbituric acid reactive species), which has the absorption maximum at 532 nm. The added antioxidants compete with the deoxyribose for the hydroxyl radicals and reduce the amount of chromogenes (Cheng et al., 2003).

The study results showed that the plant extract has a lower scavenging capacity of the free radical $OH^{\bullet}$ in comparison to the pure polyphenol components and standard antioxidants. The highest tested concentration of the extract 1600 μg/mL achieved a 57% inhibition of $OH^{\bullet}$. BHA constantly shows a stronger antiradical activity than thiourea, so that it achieves a 57% inhibition in the concentration of 50 μg/mL, and thiourea in the concentration of 100 μg/mL. In comparison to the tested standard antioxidants, polyphenol compounds achieved an effect according to the following order: tannic acid > BHA > rutin > thiourea > chlorogenic acid. In this case too, tannic acid showed the strongest antiradical activity and performed a 52% inhibition already in the concentration of 12.5 μg/mL.

The extract constantly lags in its scavenging capacity of $OH^{\bullet}$, whereas the chlorogenic acid approaches in the highest tested concentration 1600 μg/mL. The results point out that flavonoids, phenolic acid and tannins, present in the examined plant species, are responsible for the antiradical effect of the extract and that favonoids contributes more to the effect.

Reduction ability

The reduction ability of the extract was studied, along with chlorogenic acid, rutin and tannic acid, in comparison to the BHA and BHT referent antioxidants. The yellow colour of the examined solutions is changed into varios nuances of green and blue depending on the reduction ability of the studied antioxidant specimens. The reduction component can serve as an important indicator of its potential antioxidative ability.

Fe^{3+} from $FeCl_3$ forms Fe^{3+}-ferricyanide in a reaction with the solution $K_3F_3(CN)_6$. The presence of reducents such as a specimen of antioxidants causes the reduction of Fe^{3+}-ferricyanide into the Fe^{2+} form. Fe^{2+} with ferricyanide produces a blue solution, whose colouring intensity can be observed by measuring the absorption at 700 nm (Fe^{2+} +$[Fe(CN)6]^{3-}$ Fe^{3+} + $[Fe(CN)_6]^{4-}$) (Akand and Gülçin, 2008; Prasad et al., 2009). The reduction ability increases with an increase in the concentration of the specimen. Solutions with greater concentration are prominently blue due to the presence of a greater amount of Fe^{2+} and show greater absorption, i.e. a greater reduction ability, thus acting as stronger antioxidants. With a decrease in concentration, the studied specimens become weaker reducents. That is the reason why a greater amount of Fe^{3+}-ferricyanide is present in solutions, making the specimens greener. Without antioxidants the solution of the Fe^{3+}-ferricyanide complex is yellow.

The herbal extract has a visibly lower reduction ability than the synthetic referent antioxidants BHA and BHT and the usual substances such as phenolic acid, flavonoids and tannins. Chlorogenic acid also proved to have a better reduction ability than BHT, showing a better ability even than BHA in concentrations above 25 μg/mL. Rutin has a lower reduction ability than BHA, but shows a greater ability than BHT in concentrations above 25 μg/mL. Tannic acid shows the best reduction ability. In comparison to the tested standard antioxidants, polyphenol compounds achieved the following performance in concentrations above 25 μg/mL: tannic acid > chlorogenic acid > BHA > rutin > BHT.

The 0.5 absorption value can be clearly noticed, i.e. a 50% reduction ability, which is achieved by tannic acid already in the concentration 2.58 ± 0.21 μg/mL and by the *E. purpurea* extract only in the concentration 47.69 ± 2.96 μg/mL. Chlorogenic acid reached a 50% reduction ability in the concentration 3.48±0.36 μg/mL, rutin in 7.89 ± 0.66 μg/mL, BHA in 3.40 ± 0.08 μg/mL and BHT in 4.95 ± 0.36 μg/mL. In higher concentrations chlorogenic acid is a better antioxidant than BHA. In concentrations under 25 μg/mL, the reduction ability of rutin lags a little bit behind BHT; in higher concentrations rutin is a better antioxidant than BHT. By far the best reduction ability is that of tannic acid, that is, it acts as the best antioxitant. On the basis of the examined pohyphenol compounds and referent antioxidants, the extract of *E. purpurea* constantly lags in reduction ability.

The results indicate that flavonoids, phenolic acid and tannins, present in the studied species, contribute to the antioxidant effect of the extract together, tannins contributing the most, followed by phenolic acid and, lastly, flavonoids.

Iron ions chelating capacity (II) was studied for the extract of the *E. purpurea* species, chlorogenic acid, rutin and tannic acid, in comparison to the synthetic referent antioxidants with BHA and BHT. EDTA was used as the standard chelator.

The appearance of reactive oxygen species such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical was catalysed with free iron in the Haber-Weiss reaction (O_2^- + H_2O_2 →O_2+ OH^- +

$OH^{\bullet}$). In the transition metals family, iron is known as the most important pro-oxidant of lipid oxidation. The Fe^{2+} iron state accelerates lipid oxidation, transforming hydrogen and lipid peroxides into free reactive radicals in the Fenton's reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$). Fe^{3+} also creates radicals from peroxide but ten times weaker than Fe^{2+}. Fe^{2+} is the strongest oxidising agent among metal ions. Ferrozine forms quantitatively pink complexes with Fe^{2+}. The presence of chelating agents in a reactional compound inhibits the formation of ferrozine and Fe^{2+} complexes, resulting in the reduction of the pink colour of the solution. Colourimetric measuring of a colour intensity enables the estimation of metal ion chelating ability for the studied element. A lower absorbing value indicates a greater chelating ability. The structures which have one or more functional groups (-OH, -SH, -COOH, $-PO_3H_2$, C=O, $-NR_2$, -S-, -O-) are known to show a good metal ion chelating ability in a convenient configuration (Gülçin et. al., 2010).

As the standard chelator, EDTA was shown to have a great iron ion chelating ability even in low concentrations, that is, a 100% chelating ability in a concentration range of 6.25 – 800 μg/mL. It is only in a concentration at 3.13 that its chelating ability begins to decrease. In lower concentrations, the extract of *E. purpurea* has a noticably lower effect than the standard metal chelator EDTA. Although it continually lags behind the effect of EDTA, the difference is considerably lowered in concentrations above 200 μg/mL. The study also showed that the extract is a better iron ion chelator than the BHA and BHT referent synthetic antioxidants, which did not show metal ion chelating ability at all in the tested concentrations. Unlike chlorogenic acid and rutin, tannic acid showed a certain iron ion chelating ability, although it was weak if compared to the extract of *E. purpurea*.

Unlike the extract of *E. purpurea* and tannic acid, EDTA approaches the maximum iron ion chelating ability in concentrations above 6.25 μg/mL. The value of IC_{50} is noticeable also, i.e. the concentration of the examined specimen which causes a 50% iron ions chelation. With interpolation from a linear regression analysis the concentration of 125.86 ± 11.33 μg/mL is obtained, i.e. the EDTA concentration 0.94 ± 0.04 μg/mL which causes a 50% iron ions chelation. The results showed that out of the studied bioactive substances of *E. purpurea* only tannic acid possessed iron ion chelating ability, and was only slightly responsible for the chelating ability of the studied extract. It was shown that iron ion chelating ability was dependent on the extract concentration and tannic acid. With an increase in concentration, the Fe^{2+}-ferrozine complex absorption is decreased in a linear manner, i. e. iron ion chelating ability is increased. Metal ion chelating ability is a significant trait of antioxidants, since it lowers the concentration of a catalysing transition metal in lipid peroxidation. Chelating agents are effective as secondary antioxidants as they lower the redox potential, thus stabilising the oxidised form of the metal ion.

The total antioxidant capacity of the extract of *E. purpurea*, chlorogenic acid, rutin and tannic acid, in comparison to the synthetic referent antioxidants BHA and BHT was tested. The formation of the complex also depends on the antioxidant concentration. While a greater amount of complexes is formed in higher concentrations and the examined solution shows a more prominent green colour, the solution becomes less green when the concentration is lowered. When there is a lack of antioxidants, the solution is colourless as there is no complex formation.

Antioxidants act as reducents whose ability is based on breaking the chain reaction of creating free radicals by donating a hydrogen atom, i.e. by donating electons they react with free radicals and transform them into more stable products. They also react with peroxide precursors, thus stopping peroxide formation. The antioxidant capacity depends on the number of free –OH groups, so polyphenols exhibit a greater antioxidant ability than monophenol. The greatest antioxidant capacity among phenolic acid is exhibited by gallic acid with three –OH groups on the aromatic ring, i.e. epigallocathechin with three –OH groups in the B ring among flavonoids. Glycoside flavonoids have a weaker antioxidative capacity than aglycones alone (Kim et al., 2004).

The total antioxidative capacity was calculated at 695 nm as a ascorbic acid equivalent from the estimated absorptions. The results showed that the extract of *E. purpurea* had a lower total antioxidative capacity expressed as a ascorbic acid equivalent in relation to pure polyphenol components and standard antioxidants. In comparison to the standard tested antioxidants, polyphenol compounds have a lower total antioxidative capacity. Rutin has the lowest capacity, whereas chlorogenic acid and tannic acid have an equal total antioxidative capacity. BHT in higher concentrations has a clearly weaker effect than BHA. Although it continually lags behind the effect of BHA, the difference decreases considerably in greater dilutions.

One can see that the total antioxidative capacity of standard polyphenol substances visibly lags behind the total antioxidative capacity of standard polyphenol antioxidants. However, the difference is decreased so that in 12.5 μg/mL tannic acid and BHA almost have an equal total antioxidative capacity. It is aslo visible that the total antioxidative capacity of the extract lags behind the standard polyphenol substances. But this

difference also decreases in greater dilutions, so that in 12.5 μg/mL, the extract of *E. purpurea* and rutin almost have an identical total antioxidative capacity. The results indicate that flavonoids, phenolic acid and tannins, present in *E. purpurea*, contribute to the total antioxidative capacity of the extract together, with phenolic acid and tannins contributing the most, followed by flavonoids.

Results ESR

One part of our investigation on antioxidant activity of *E. purpurea* extract was the scavenging activities on hydroxyl and superoxid anion radicals measured by ESR method. Using a spin trap, such as DMPO, it is possible to convert reactive hydroxyl radicals to stable nitroxide radicals (DMPO-OH adducts) with spectral hyperfine splitting that reflects the nature and structure of these radicals. The reaction of Fe_2^+ with H_2O_2 in the presence of the spin trapping agent DMPO generated a 1:2:2:1 quartet of lines with hyperfine coupling parameters (*a*N=*a*H= 14.9 G) (Čanadanović-Brunet, et al., 2005). The intensity of the ESR signal, corresponding to the concentration of free radicals formed, was decreased in the presence of different amounts of *E. purpurea* extract. The total elimination of hydroxyl radical (SA_{OH} = 100%) was obtained in the presence of 1250 μg/mL of extract, which indicates that this applied concentration inhibits the creation of hydroxyl radicals completely. This was confirmed by the calculated EC_{50} values of 210 μg/mL.

Comparing the intensity of the ESR signal of the DMPO-OOH spin adduct blank test and the sample, it was determined that aqueous solutions of Echinacea extract lyophilisates in the range of tested concentrations have an inhibitory effect on $O_2^{\bullet-}$ formation or participate in their transformation. The addition of the extract at a mass concentration of 10 μg/mL causes an inhibitory effect on the formation of $O_2^{\bullet-}$ radicals, and at the same concentration it reaches an antioxidant activity of 53.75%. Lee et al. (2009) using a spectrophotometric method at a concentration of 200 μg/mL of *E. purpurea* extract (whole plants) found 41.7%, and at 1600 μg/mL 91.1% inhibition of $O_2^{\bullet-}$. The investigated extract showed dose-dependent radical scavenging activities. The EC_{50} value, defined as the concentration of extract required for 50% scavenging of superoxid anion radicals under experimental condition employed, is a parameter widely used to measure the free radical scavenging activity (Cuvelier et al., 1992); a smaller EC_{50} value corresponds to a higher antioxidant activity. The EC_{50} value of *E. purpurea* extract (76.7 μg/mL) shows that extract is rich in antioxidant compounds and efficiently scavenge superoxide anion radicals.

Comet assay

Table 4 shows the results of alkaline comet assay following 2 hours exposure to various concentrations of *E. purpurea* extracts (E1, E2, E3), single AFB_1 and OTA as well as combination of extracts and mycotoxins. Plant extracts did not exert genotoxic activity taking into account values of tail intensity and tail moment. However, significant increase in tail length was observed upon exposure to highest concentration of plant extract (P<0.05), while lower concentrations did not provoked increase of this parameter.

Table 4. Evaluation of primary DNA damage measured in human leukocytes following 2-h exposure to *E. purpurea* extracts and single AFB_1 and OTA or combinations of each mycotoxin with plant extracts

Cell treatment	Tail length (μm)				Tail intensity (% DNA)				Tail moment			
	Mean±SD	M	25%-P	75%-P	Mean±SD	M	25%-P	75%-P	Mean±SD	M	25%-P	75%-P
C1 (10% water)	14.19±1.75	14.10	12.82	14.74	0.58±1.23	0.0	0.0	0.52	0.076±0.17	0.0	0.0	0.055
C2 (0.03% DMSO)	13.53±2.15	13.46	12.18	14.74	0.48±0.97	0.0	0.0	0.55	0.060±0.11	0.0	0.0	0.062
C3 (0.3% ethanol)	14.61±2.07	14.74	13.46	13.38	0.53±1.57	0.0	0.0	0.21	0.076±0.23	0.0	0.0	0.023
E1 (1 mg/mL)	13.49±1.35	13.46	12.82	14.10	0.50±1.05	0.13	0.0	0.63	0.063±0.13	0.018	0.0	0.077
E2 (10 mg/mL)	14.09±2.09	14.10	12.82	15.38	0.58±1.25	0.02	0.0	0.62	0.075±0.15	0.002	0.0	0.081
E3 (20 mg/mL)	15.34±2.11	15.38*	13.46	16.67	0.39±0.85	0.0	0.0	0.47	0.054±0.10	0.0	0.0	0.070
AFB1 (3 μM)	16.58±4.63	15.38*	13.46	19.71	1.73±2.49	0.54*	0.0	2.55	0.240±0.33	0.078*	0.0	0.364
E1+AFB_1	14.38±1.81	14.10**	12.98	15.38	0.52±1.46	0.0**	0.0	0.41	0.070±0.18	0.0**	0.0	0.059
E2+AFB_1	15.60±1.84	15.38	14.10	16.67	0.40±0.87	0.0**	0.0	0.44	0.060±0.12	0.0**	0.0	0.065
E3+AFB_1	14.82±2.49	14.74	13.46	15.87	0.96±2.13	0.06	0.0	0.83	0.130±0.27	0.004	0.0	0.125
OTA (10 μM)	17.30±4.85	16.03*	13.62	19.87	1.16±2.10	0.21*	0.0	1.25	0.170±0.30	0.030*	0.0	0.277
E1+OTA	14.48±1.86	14.10**	13.46	15.38	0.56±1.04	0.02	0.0	0.85	0.073±0.13	0.001	0.0	0.106
E2+OTA	14.26±2.35	14.10**	12.82	15.22	0.34±0.67	0.0**	0.0	0.30	0.045±0.08	0.0**	0.0	0.040
E3+OTA	13.68±2.08	13.46**	12.18	14.74	0.41±1.04	0.0**	0.0	0.47	0.052±0.12	0.0**	0.0	0.058

C1, C2, C3 – control solvent; SD- standard deviation; 25%- P- 25% percentile; M – median; 75% -P- 75% percentile; *- compared to control (P<0.05); **- compared to AFB_1 or OTA given alone (P<0.05)

As it was expected, exposure to AFB_1 at 3 μM and OTA at 10 μM significantly increased all three comet parameters comparing to control solvents ($P<0.05$). Leukocytes simultaneously exposed to AFB_1 and E1 or E2 had significantly lower tail intensity and tail moment, as compared to cells exposed to mycotoxin alone ($P<0.05$), showing antagonizing effect of plant extracts. Highest extract concentration (E3) also decreased this comet parameters but without significant difference comparing to AFB_1 given alone. At the same time, tail length was significantly lower only when AFB_1 was simultaneously applied with E1 ($P<0.05$). Higher concentrations of plant extracts also decreased tail length but without significant difference comparing to effect of single toxin.

E. purpurea extracts also antagonized genotoxicity of OTA. Tail intensity and tail moment were significantly lower in cells exposed to combination of OTA and E2 or E3 than in cells treated with OTA alone ($P<0.05$). All of three extract concentrations showed significant protective effect considering measurements of tail length.

Since both AFB_1 and OTA are food contaminants with genotoxic activity the purpose of this study was to see whether their genotoxic action could be antagonised if leukocytes are simultaneously exposed to this mycotoxins and water extracts of *E. purpurea.* Results of comet assay showed that plant extract did not induced DNA damage taking into account tail intensity and tail moment but significant increase of tail length was observed when highest concentration of extract was used. These results could be explained by the theory of comet tail formation. Tail length is the length of relaxed DNA loops, which migrated from the core during electrophoresis, while tail intensity is the number of DNA breaks in the loop (Colins et al., 2008) mining that high concentration of extract increases relaxation of DNA strands rather than causing DNA fragmentation. Genotoxicity of AFB_1 is well documented. It is known that AFB_1 is metabolized to AFB -8,9B epoxide by cytochrome *P*450. Epoxide could covalently bind to DNA and form 8,9- dihydro-8-(N(7)-guanyl)-9-hydroxy-AFB_1 (Wogan, 1992; Nakai et al., 2008). Results of our study support the findings of AFB_1 genotoxicity observed by comet assay in hepatocytes, whole blood (Miele et al., 1999; Anderson et al., 1999; Williams et al., 2004). Besides DNA adductation, AFB_1 can induce oxidative DNA damage contributing to AFB_1 genotoxicity (Halliwell et al., 1999). Extracts of *E. purpurea* given at 1 mg/mL and 10 mg/mL were able to prevent DNA strand breaks rather than increase of tail length, which could be attributed to antioxidant capacity of extract. Previously we demonstrated that OTA given at 5 μM for 1 h increases tail intensity and tail moment in human leukocytes (Šegvić Klarić et al., 2010) and this study confirms that short time exposure to OTA leads to DNA damage. The mechanism of OTA genotoxicity is still under debate. According to current literature, OTA genotoxicity may be assigned as direct (DNA adduct formation) and indirect (oxidative DNA damage) mechanisms of action. An Fpg-modified comet assay showed that oxidative DNA damage in rat kidney was significantly higher than damage observed by standard alkaline comet assay (Domijan et al., 2006). Taking into account antioxidative activity of *E. purpurea* extract, which showed concentration-dependent antagonizing activity to OTA, we could conclude that oxidative stress plays a key role in OTA genotoxicity.

Polyphenols could act as antioxidants and bind ROSs that is produced by mycotoxins such as OTA. Alternatively, there is evidence that polyphenols can act as prooxidative *in vivo* and promote antioxidant cell protection that removes ROSs (Hail et al., 2008.; Long et al. 2010). Flavonoids also have an inhibitory effect on the activity of many prooxidant enzymes, including lipoxygenase (Laughton et al., 1991; Schewe et al., 2002), cyclooxygenase (Laughton et al., 1991), inducible nitric monoxide synthase (Raso et al., 2001), monooxygenase (Siess et al., 1995), thyroid peroxidase (Doerge and Chang, 2002), xanthine oxidase (Sheu et al., 1998), NADH-oxidase (Hodnick et al., 1994), etc. They also inhibit β-glucuronidase (Kim et al., 1994), phosphodiesterase (Picq et al., 1989), phospholipase A_2 (Gil et al., 1994) and protein kinase (Cushman et al., 1991). Some of the best phenol chemoprevention studies have used green tea and its predominant polyphenol, epigallocatechin gallate. Inhibition of DNA adducts formation and induced chemical carcinogenesis in experimental animals (Xu et al., 1992), was observed, as well as inhibition of DNA methyl transferase and reactivation by methylation-silenced expression of genes important in the process of carcinogenesis (Fang et al., 2003), etc.

Polyphenols can reduce or inhibit the mutagenic potential of mutagens and carcinogens (Miadakova et al., 2008). Controlling cell mutation with natural antimutagens can result in a variety of ways to prevent mutations that are essential, both in the case of cancer and in diseases caused by genotoxic agents (Birt et al., 2001).

After many laboratory and epidemiological studies, it has been found that diet is responsible for approximately 35% of all human cases of cancer (Doll and Peto, 1981). Based on epidemiological research, Block et al (1992) and Steinmetz et al. (1996) have estimated that the incidence of cancer can be reduced by at least 20-30 % with a healthy diet. The central role

of diet in preventing carcinogens has also been confirmed by the World Cancer Research Foundation (World Cancer Research Fund, 2007). The interactions between diet and the biological processes that lead to cancer are very complex. Although a large number of carcinogenic substances have been found in food, the human body has its own defense mechanisms that are sufficient if the exposure is not quantitatively excessive and chronic.

Conclusion

The content of total polyphenols was 13.31% in herba purple ehinacea extract. The analysis of phenolic acids, flavonoids, tannins and proantocyanidine in the samples identified the multiple proportion of phenolic acids in herba ehinacea extract.

Qualitative and quantitative analysis of echinacea extract determined the presence of chicoric, caftaric, chlorogenic, caffeic and echinacoside acid. The most represented were chicoric and caftaric acid.

The echinacea extracts studied showed relatively poor ability to capture DPPH radicals (EC_{50} = 15.67 μg/mL) compared to reference antioxidant and ingredient standards. Efficiency order: tanic acid > chlorogenic acid > routines > BHA > ehinacea extract.

The use of another method of comparative testing of plant extract and reference substances has proven that ehinacea extract has a lower ability to capture $OH^{\bullet}$ compared to DPPH radical. The EC_{50} value for extract (1071.91 μg/mL) was thirteen times less than the value obtained for tioureu. According to the ability to capture radicals $OH^{\bullet}$ the following order has been established: tanic acid > BHA > routines > tiourea > chlorogenic acid > ehinacea extract.

The ESR spectral analysis, by comparing the intensity of the ESR signal of the DMPO-OH spin ducts, found that ehinacea extracts, in the range of studied concentrations, inhibit the formation of hydroxyl radical and/or affect its transformation.

The EC_{50} value established according to this radical was 210 μg/mL. The results obtained found that phenolcarbonic acids, hydroxycymetic acid derivatives present in the plant species tested contribute the most to the action of extract according to this radical.

The ESR analysis of the DMPO-OOH spin adduct identified the antioxidant activity of ehinacea extracts according to superoxide radical. The EC_{50} extract value was 76.7 μg/mL.

Ehinacea extract at applied concentrations mainly showed no genotoxic effect in the comet test according to established tail intensity and tail torque values compared to control. However, a significant increase in the length of the comet's tail was noticed at exposure to the highest concentration of extract.

Leukocytes that were simultaneously exposed to AFB_1 and ehinacea extract had significantly lower tail torque, tail intensity and tail length at lower tested extract concentrations, compared to cells that were exposed only to mycotoxin. This demonstrates the neutralizing effect of herba purple ehinacea extract in the specified range of concentrations.

Ehinacea extracts also neutralized the genotoxicity of OTA. Tail intensity and tail torque were significantly lower in cells exposed to the combination of OTA and the two highest concentrations than in cells treated only with OTA. All three concentrations of extracts showed a significant protective effect given the length of the comet's tail.

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APPLICATION OF COCOA BEAN SHELL EXTRACTS IN THE PRODUCTION OF CORN SNACK PRODUCTS

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Summary

Extruded snacks products are group of products that are consumed by all age groups in significant amounts. Although these products are mainly made from raw materials rich in starch (corn grits, wheat grits, etc.), recently nutritional values is improved by adding various supplements which contain components like polyphenols, food fibers, vitamins, etc. The aim of this study was to examine the possibility of application of extracts from cocoa shell, which is by-product in the processing of cocoa beans, in production of corn snack products. The expansion ratio, bulk density, color, hardness and fracturability, total polyphenol content and antioxidant activity were tested for the obtained extrudates, thereby determining the quality of the obtained products. It has been found that there is a reduction in the expansion ratio and increase in bulk density, as well as increased hardness and reduced fracturability of tested extrudates with te addition of extracts from cocoa shell. Also samples with the addition of extracts were darker. Addition of cocoa shell extracts resulted in increased polyphenol content and antioxidant activity.

Keywords: cocoa bean shell extracts, corn grits, snack products, physical properties, total polyphenol content

Introduction

Extrusion is a process without which today's food industry is unthinkable, and due to its multiple applications, it is indispensable process in food production. It is mehanical and thermal process during which food raw material, which is rich in starch and proteins, and that is mostly corn or wheat grits, goes under different tecnological operations, like heating, mixing and compression. Finally, material passes through special shaped nozzle, forms and dries with expansion to the finished product which is called extrudate. Extrusion products are increasingly enriched, in order to increase their nutrional value, by using different additives that contain valuable components such as polyphenol, dietary fibers, vitamines etc. (Jozinović et al., 2019; Ačkar et al., 2018; Jozinović, 2015). One of the valuable by–product is cocoa bean shell, which remains as a by-product of cocoa bean processing, and since it represents about 10% of the bean, it is produced in a relativly large quantity. It is commonly used as a fuel (energy source), adsorbent or less frequently as the animal feed because it contains theobromine (Barišić et al., 2020; Jozinović et al., 2019). However, due to its chemical composition, cocoa bean shell has a great potential for use in the production of new food products, since it contains a large amount of dietary fibers (50-60 %), proteins (11-18 %) and polyphenols (from 1.8 to 5.8 %) (Okiyama et al., 2017; Nsor-Aindana et al., 2012). In the case of polyphenols, catechins, epicatechins and procyanidins are the most important components. Also, a higher content of polyphenols was recorded in non-fermented and unroasted beans, as opposed to fermented and roasted cocoa beans. Polyphenols migrate from cocoa beans into the shell during the various cocoa beans processing operations, such as fermentation, roasting and alkalization (Panak Balentinć et al., 2018). The extraction of these valuable components from the cocoa bean shell has been a subject of many researches (Jokić et al., 2019), and one of the newer extraction methods is certainly application of high voltage electrical discharge (HVED) (Li et al., 2019; Boussetta and Vorobiev, 2014). The method is based on a electrical discharge during which a series of physical and chemical reactions take place in the water, which ultimately lead to the destruction of the cell structure and the extraction of the target compounds. There are two phases of the process in the aqueous medium: the phase that occurs before the destruction, so-called *streamer*, and discharge phase. By applying a very high voltage between the two electrodes, electrons that have enough energy to stimulate physical and chemical processes in water are accelerated. At the moment when dischare reaches the electrode, shock

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waves, cavitation bubbles (main bubble and numerous small bubbles) and turbulence in the liquid occur. Mass transfer increases turbulence, while shock waves and burst of cavitation bubbles are responsible for fragmentation (Lončarić et al., 2020; Boussetta and Vorobiev, 2014).

The aim of this study was to produce water extracts of cocoa bean shell by using HVED extraction as an innovative technique and to examine the possibility of their application in the production of corn snack products with the aim of obtaining nutritionally more valuable extrudates.

Materials and methods

Materials used in this research were:

- Corn grits, kindly provided by the „Đakovo Mill" of the Žito Ltd. Osijek, Croatia, suitable for production of expanded snack products;
- Cocoa bean shell, kindly provided by Kandit Ltd. Osijek, Croatia.

Water extracts of coca bean shell used in the mixtures for extrusion were prepared by using the high voltage electric discharge (HVED) technique as an innovative technology. Namely, the extracts used in this study are part of our previous study, which examined the possibility of extraction of bioactive components from cocoa bean shell using the HVED procedure (Jokić et al., 2019). In that paper, HVED extraction under different conditions of extraction time (30, 60 and 90 min), frequency (40, 70 and 100 Hz) and cocoa shell:water ratio (1:10, 1:30 and 1:50) was investigated, by application of the Box-Behnken design of the experiment, where 17 extraction experiments were performed. Based on the results obtained for total polyphenols content, antioxidant activity (% DPPH), as well as a share of individual bioactive components for this study were selected extracts obtained in the listed survey during run no. 4 and run no. 10:

1) Extract 4 (water:cocoa bean shell = 1:30; HVED frequency 100 Hz; time 30 min);
2) Extract 10 (water:cocoa bean shell = 1:50; HVED frequency 100 Hz; time 60 min.

These extracts were chosen because they had the highest content of two dominant methylxanthines, theobromine and caffeine, namely Extract 4 (5246.36 mg/kg of theobromine; 752.32 mg/kg of caffeine) and Extract 10 (6031.51 mg/kg of theobromine; 849.88 mg/kg of caffeine). Furthermore, Extract 4 had the highest content of catechin (284.33 mg/kg) and a high content of epicatechin (270.13 mg/kg), while Extract 10 with a total polyphenol content of 92.39 mg GAE/g and an antioxidative activity of 45.45% DPPH proved to be one of the best (Jokić et al., 2019). The obtained extracts were used as a substitute for water in the preparation of corn grits for extrusion, whereby the extracts being added in the amount necessary to adjust the moisture of the mixture to 15%. A control sample was prepared with the addition of distilled water in corn grits. The mixtures were prepared on the base of 1 kg d. m., by using laboratory mixer (Kenwood KMM020, JVCKenwood, Uithoorn, Netherlands), and left overnight in the plastic bags at temperature of 4 °C, in order to distribute moisture evenly. Extrusion was conducted in a laboratory single-screw extruder (Brabender GmbH, Model 19/20DN, Duisburg, Germany) under the following conditions:

- screw with compression ratio: 4:1;
- round die head with 4mm nozzle diameter;
- screw speed: 100 rpm;
- dosing speed: 20 rpm;
- temperature profiles (in dosing/compression/ejection zone): 135/170/170 °C and 155/185/185 °C.

The extrudates were air-dried overnight at room temperature, and after that expansion ratio, bulk density, color, hardness and fracturability, as well as total polyphenol content and antioxidative activity were determined.

Expansion ratio

On expanded dry samples the diameter (in millimetres) by calliper was measured Five parallel measurements were performed for each sample, and the expansion ratio was calculated, which represents the value of the ratio of the diameter of the extrudate and the diameter of the extruder nozzle (4 mm) (Brnčić et al., 2008). The results were expressed as mean with standard deviation of measurements and shown graphically.

Bulk density

The bulk density was determined by Alvarez-Martinez et al. (1988), and the mass, diameter, and length of the extrudate were measured in five parallel measurements. The results were expressed as mean with standard deviation of measurements and shown graphically.

Color

For the color determination of non-extruded corn grits and extruded (milled) products it was used Chroma Meter CR-400 (Konica Minolta, Japan) with granular

materials attached according to Jozinović et al. (2016). Before the color measurement in CIE-Lab system (L* - whiteness/darkness); a* - redness/greenness; b* - yellowness/blueness) the device was calibrated using a white standard calibration plate. For each sample five measurements were performed and the total colour change (ΔE) was calculated by Equation 1:

$$\Delta E\sqrt{(\mathrm{L}-\mathrm{L}_0)^2+\ (\mathrm{b}-\mathrm{b}_0)^2+\ (\mathrm{a}-\mathrm{a}_0)^2} \qquad (1)$$

where the parameters with subscript „0“ indicates the color value for control non-extruded sample of corn grits. Results are shown graphically as a mean value and standard deviation.

Texture analysis

Extrudate texture was determined using texture analyzer TA.XT2 Plus (Stable Micro Systems, Godalming, United Kingdom), by the method for hardness (N) and fracturability measurement (mm) using the Warner–Bratzler shear blade with guillotine probe (Ačkar et al., 2018). The results were expressed as the mean value and the standard deviation of 10 replications and shown graphically.

Total polyphenol content

The total polyphenol content was determined by colorimetric method accordnig to Wang and Ryu (2013). The absorbance of developed blue color was measured at 725 nm, using 80% methanol as a blank, and test was perforemed in two parallel measurements. Obtained results were expressed in mg of gallic acid equivalents (GAE) per 100 g of dry matter.

Antioxidant activity by DPPH method

Determination of antioxidant activity was conducted by Wang and Ryu (2013) method. The absorbance was measured at 517 nm, and the inhibition percentage of DPPH radicals was calculated by the Equation 2:

$$\%\ \text{inhibition} = \frac{\mathrm{Ao}-\mathrm{As}}{\mathrm{Ao}} \times 100 \qquad (2)$$

where it is:
Ao – absorbance of control,
As – absorbance of sample.

Results and discussion

The aim of this study was to examine the possibility of applying aqueous extracts of cocoa bean shell prepared using HVED tehnique as an innovative extraction method in the production of corn snack products. After extrusion, the physical properties, antioxidant activity and total polyphenol content in obtained extrudates were determined.
Texture is a sensory characteristic, that is crucial in determining the quality of the snack products, and depends on a number of factors, among which the most prominent are expansion ratio, bulk density, hardness and fracturability of the extrudate. The expansion ratio is important for the physical properties of extrudates and plays an important role in the acceptance of products by the customers (Obradović et al., 2015). The addition of the Extract 4 of cocoa bean shell, as well as the Extract 10, during extrusion at a temperature profile 135/170/170 °C, resulted in a significant reduction in the expansion ratio of extrudates compared to the control corn extrudate produced with water (without extract) (Fig. 1). A possible reason for the decrease in the expansion ratio when applying extracts instead of water is that the extracts contain various soluble components from cocoa bean shell, including soluble dietary fiber, which negatively affects the expansion of pure corn grits (Jozinović, 2015). Namely, cocoa shell contains high fiber content, about 57%, with cellulose as dominant polyssacharide and lower amount of pectin and hemicellulose (Redgwell et al., 2003). The obtained results are in accordance with our previous study, in which extruded products enriched with different by-products with a high content of fiber were investigated (Ačkar et al., 2018), and where the addition of brewer's spent grain, sugar beet pulp and an apple pomace caused significant reduction in the expansion ratio. The addition of whole grains or wild legumes rich in fiber to the extrusion mixture also shows a decrease in the expansion ratio (Pastor-Cavada et al., 2011). At the temperature profile 155/185/185 °C the expansion ratio was slightly lower in relation to the lower extrusion temperature profile, and it was found that the addition of extracts resulted in a slight increase in the expansion ratio in relation to the control sample.

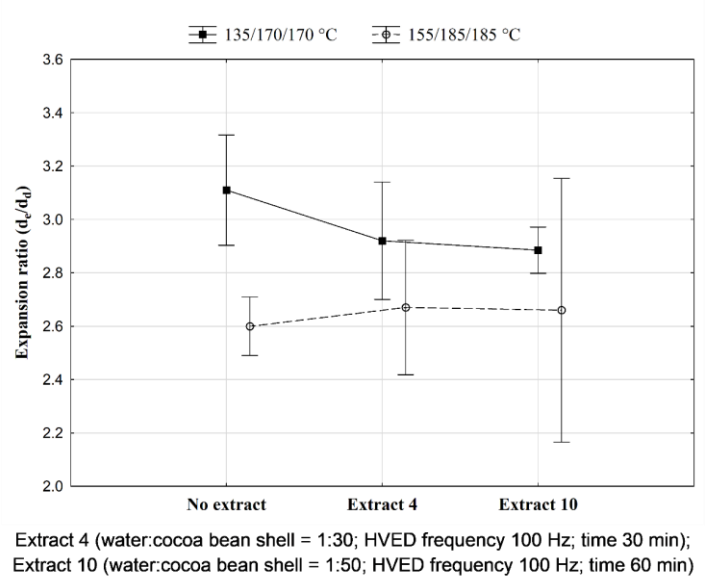

Fig. 1. The effect of the cocoa shell extracts addition on the expansion ratio of the extruded corn grits at different extrusion temperatures

The bulk density is important as a parameter for physical assessment of the quality of produced extrudates. It gives information on how much mass occupies a certain volume, and it is desirable to have as little value. At the temperature profile 135/170/170 °C there was an increase of bulk density with the addition of Extract 4 compared to the bulk density of extrudates without extract addition, while extrudates with the addition of Extract 10 had higher bulk density in relation to corn grits extrudate with water (without extract), but lower than extrudates produced with Extract 4 (Fig. 2). The increase of bulk density in extrudates with extracts is consistent with research by other authors, where the materials with high protein content (Adamafio, 2013) or dietary fibers caused an increase of bulk density (Bishart et al., 2013; Stojceska and Ainsworth, 2008). At the temperature profile 155/185/185 °C there was a slight decrease of products' bulk density with extracts addition in relation to corn grits extrudates without extract.

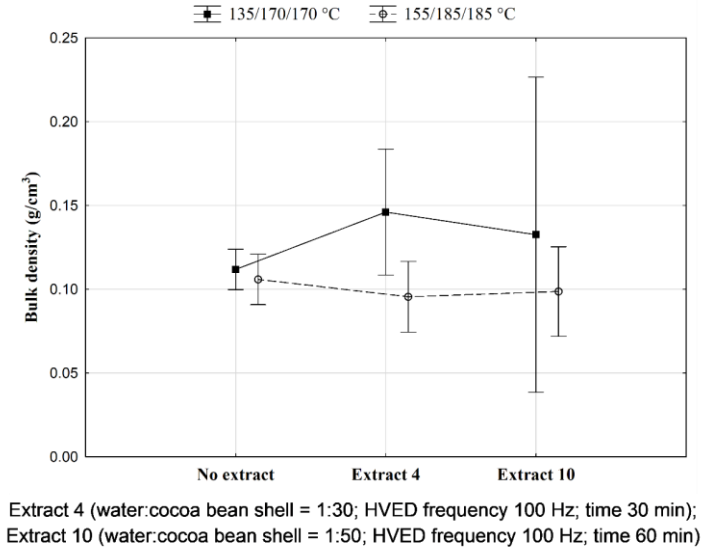

Fig. 2. The effect of the cocoa shell extracts addition and extrusion temperatures on bulk density of extrudates

The addition of cocoa bean shell extracts at the temperature profile 135/170/170 °C reduced the total color change in relation to control sample which was extrudated with water (without extracts) (Fig. 3). At the temperature profile 155/185/185 °C higher values of the total color change were recorded in relation to the lower temperature profile. Furthermore, at a higher extrusion temperature profile with the addition of Extract 4 there was a higher change in color, while the addition of Extract 10 there was no significant change in color compared to the control sample.

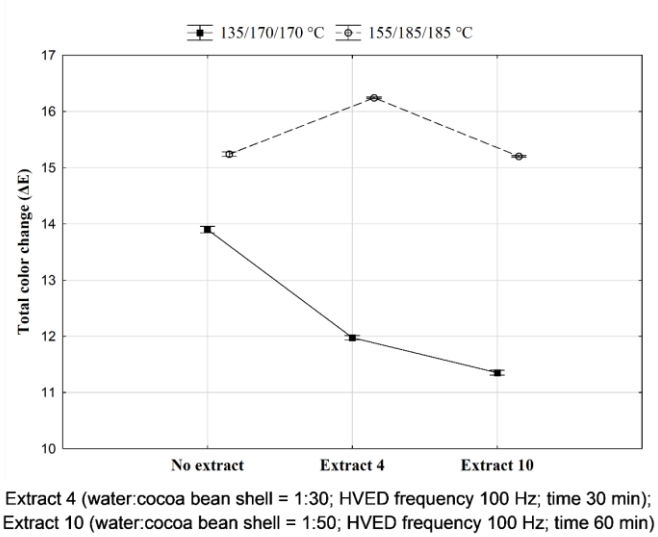

Fig. 3. Total color change of the extrudates with the addition of cocoa shell extracts at different extrusion temperatures

Texture was determined by the parameters for hardness and fracturability, and it is directly related to expansion and bulk density (Anton et al., 2009; Stojceska et al., 2009). At the temperature profile 135/170/170 °C there was an increase of the products' hardness with the addition of both extracts, which is not desirable (Fig. 4). Changes in texture are the result of loss of moisture, formation or decomposition of emulsions and gels, hydrolysis of polymeric carbohydrates and coagulation and/or hydrolysis of proteins (Akdogan et al., 1997). At the temperature profile 155/185/185 °C there was no significant change in products' hardness in relation to control sample.

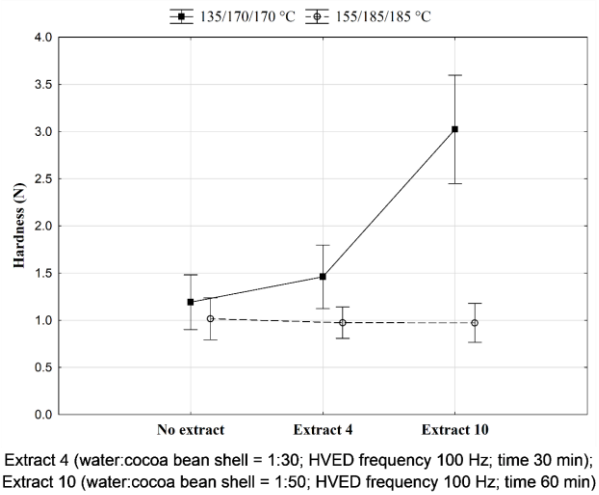

Fig. 4. The effect of the cocoa shell extracts addition and the extrusion temperatures on hardness of extrudates

With the addition of Extract 4 to the extrusion mixture fracturability of extrudates did not significantly changed at a both temperature profiles. On the other hand, with the addition of Extract 10 there was a significant decrease in fracturability at temperature profile 135/170/170 °C, while at the temperature profile 155/185/185 °C there was an increase of fracturability (Fig. 5). Increasing the amount of fibers in the extrusion mixture also increases the hardness of the extrudates. In accordance with the increase in hardness, the fractuarbility of extrudates decreases with the addition of Extract 4, while with Extract 10 at higher temperatures this was not the case.

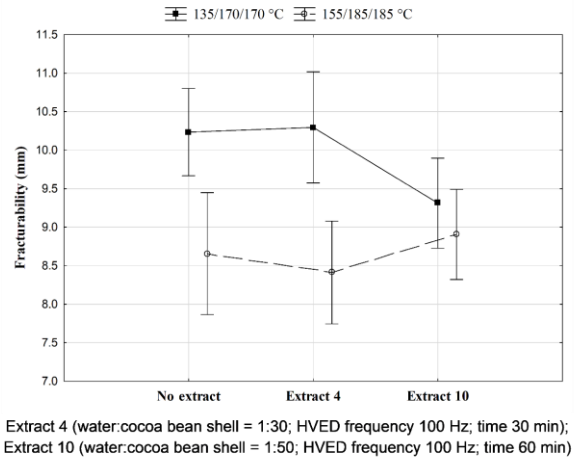

Fig. 5. The effect of cocoa shell extracts addition and extrusion temperatures on fracturability of extrudates

At both temperature profiles of extrusion there was a significant increase of total polyphenol content with the addition of cocoa bean shell extracts (Fig. 6), from which it can be concluded that cocoa shell is rich in polyphenolic compounds, which was confirmed for the used extracts in our previous study (Jokić et al., 2019). Since, the total polyphenol content in Extract 10 (92.39 mg GAE/g extract) was significantly higher than in Extract 4 (72.39 mg GAE/g extract) (Jokić et al., 2019), so the increase of total polyphenol content in extrudates was in line with this (Fig. 6). Other studies have also shown that cocoa shell is rich in polyphenolic compounds, so Vriesmann et al. (2011) in their study recorded high polyphenol content in cocoa shell, but did not investigate in detail which those compounds are. On the other hand, Nsor-Atiandana et al. (2012) stated that about 60% of total polyphenols in cocoa shell are epicatechin and catechin. Amin and Chew (2006) found that the total phenolic content of cocoa shell was 112.9 ± 0.6 mg GAE/g extract, extracted with 70% ethanol, while the extract with 80% ethanol gave total phenolic content of 23.36 ± 1.59 mg GAE/g extract, indicating that the total polyphenol content depends on the extraction technique used.

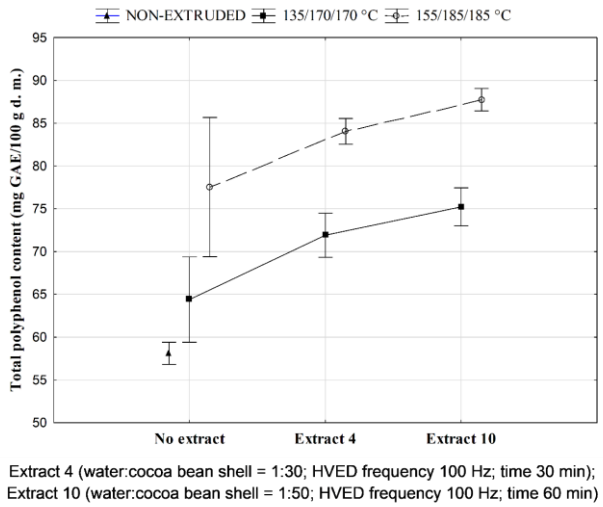

Fig. 6. Total polyphenol content in non-extruded corn grits and extruded products with addition of cocoa shell extracts extruded at different temperatures

Since the addition of cocoa shell extracts had an effect on total polyphenol content in the obtained extrudates, an increase in antioxidant activity was also expected, as the polyphenols are carriers of antioxidant activity. The addition of Extract 4 to the mixture for extrusion at both temperature profiles resulted in a slight increase of antioxidant activity (Fig. 7), while the addition of Extract 10 at temperature profile 155/185/185 °C caused significant increase of antioxidant activity. The higher increase of antioxidant activity with the addition of Extract 10 regardless to the temperature profile is consistent with the higher antioxidant activity of this extract (45.45% DPPH) in relation to Extract 4 (40.37% DPPH) (Jokić et al., 2019). It can be concluded that the antioxidant activity increased with the addition of both cocoa shell extracts, with a significant increase at the temperature profile 155/185/185 °C, which confirms that cocoa shell is rich in antioxidants, and this is consistent with researches of other authors (Jokić et al., 2019; Karim et al., 2014).

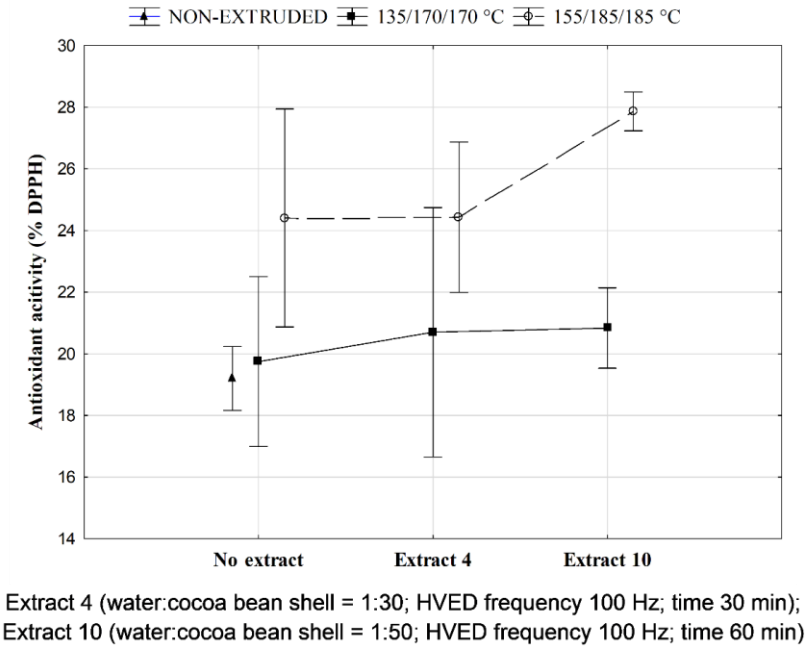

Fig. 7. Antioxidant acitivity of non-extruded corn grits and extruded products with addition of cocoa shell extracts extruded at different temperatures

Conclusions

Based on the obtained results in this study, it can be concluded that the addition of cocoa bean shell extracts to the extrusion mixtures resulted in a decrease of expansion ratio and an increase of bulk density of extrudates at lower extrusion temperature profile, while at higher extrusion temperatures there was an increase in expansion ratio and bulk density. With the addition of cocoa shell extracts at lower extrusion temperatures there was no significant change in the color of the extrudate, while at higher temperatures the change in color was more pronounced. At lower extrusion temperature profile there was an increase in hardness and a decrease in fracturability of extrudates with the addition of cocoa shell extracts, compared to the control sample, while at higher temperatures there was no significant change in hardness, while fracturability increased slightly. The addition of cocoa shell extracts increased total polyphenol content and antioxidant activity of extrudates at both extrusion temperature profiles, which means that cocoa shell is rich in polyphenolic compounds. From all the above, it can be concluded that the addition of cocoa bean shell extracts leads to changes in the physical propetries of extrudates, which may affect the acceptability of those products by consumers, but at the same time the addition of cocoa shell extracts significantly improves their antioxidant activity.

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ULTRASOUND-ASSISTED EXTRACTION OF ACTIVE COMPOUNDS FROM COCOA BEAN SHELL

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Summary

In today's overcrowded world with declining food supplies and the constant struggle against waste accumulation, scientists are increasingly trying to discover new ways to solve these problems. Like many industries, the food industry generates, disposes and accumulates waste thus creating an environmental and economic problem. In this study the one of the green extraction technologies, ultrasound-assisted extraction (UAE), for isolation of bioactive compounds from cocoa bean shell (CBS), a by-product in the chocolate production, was applied. Different temperature (40, 60, 80 °C), extraction time (30, 60, 90 minutes), liquid/solid ratio (10, 30, 50 mL/g) and ultrasound power (30, 50, 70 %) were used to obtain the cocoa bean shell extracts. Six active compounds were detected in the extracts by high performance liquid chromatography with a diode array detector as follows: theobromine (2.077-5.916 mg/g), gallic acid (0.110-1.407 mg/g), caffeine (0.276-0.785 mg/g), catechin (0.033-0.457 mg/g), while the highest obtained concentrations for epicatechin and caffeic acid were 0.100 and 0.527 mg/g of CBS. The highest total phenolic content (TPC) and % scavenging activity measured were 132.897 mgGAE/$g_{extr.}$ and 86.377%. From all investigated parameters, liquid/solid ratio had the greatest influence on the concentrations of obtained compounds. Study proved how UAE is an efficient method for the extraction of bioactive compounds from food by-product - CBS. It should also be emphasized that such application could find the purpose at the industrial level for the discarded waste that still contains valuable compounds, while the enriched extracts could be further used as raw material in other processes.

Keywords: cocoa bean shell, by-products, bioactive compounds, ultrasound-assisted extraction

Introduction

By-products of the food industry that are discarded in the production process as inedible parts are considered as waste material. Such waste accumulates andbecoming an increasing economic and environmental problem today (Jokić et al., 2018; Pavlović et al., 2019; Viganó et al., 2015). Consequently, people are starting to think in the direction of utilizing this type of waste and its possible application. Various authors state that by-products still contain significant amounts of useful bioactive compounds which have been proven to possess the antioxidant, anti-inflammatory, as well as antiviral activities (Viganó et al., 2015). One such by-product is CBS in the production of chocolate and chocolate products (Hamzat and Adeola, 2011). Okiyama et al. (2017) and Panak Balentić et al. (2018) gave a detailed insight regarding new uses of CBS in the food industry, feedstuff for livestock, usage as biofuel as well as adsorbent or composite while Rojo-Poveda et al. (2020) focused on the latest advances of CBS applications for human health mostly from a nutritional and biofunctional point of view. Interesting chemical and nutritional composition of CBS opens up some new possibilities not only as food, feed or industrial usage but also had potential in medical applications (Rojo-Poveda, 2020). It has been scientifically proven that significant amounts of certain phenolic compounds, which are found in the cotyledons of cocoa beans, are lost during the process of fermentation, due to migration into the CBS (Hernández-Hernández et al., 2019, Utami, Armunanto & Rahardjo, 2016), as well as some methylxantines (Beckett, 2009). Green extraction techniques, as highly efficient processes, can provide higher yields from renewable sources without hazardous chemicals (Mustafa and Turner, 2012). By using alternative solvents (mainly water-based), with lower energy consumption and a reduced number of experiments in the extraction process, it is possible to obtain stable extracts without contaminants (Chemat et al., 2012). With longer extraction time, and usage of organic solvents, conventional extraction techniques are uneconomical (Ramos et al., 2002) and demanding at the same time. The reason is mostly the frequent need to purify such extracts, less solvent selectivity and small concentrations of desired thermolabile compounds (Mustafa and Turner, 2011). Ultrasound-assisted extraction (UAE) significantly reduces the time required to extract specific compounds, with higher yields achieved and better quality of the extract. It is considered to be a good option for the extraction of different organic compounds from various matrices because it provides more efficient contact between the solid matrix and the solvent due to increased pressure (better penetration and transport) and

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increased temperature (improves solubility and diffusion) (Grumezescu and Holban, 2017; Chemat et al., 2011). Mechanical effect of cavitation leads to cell wall damage, which facilitates the penetration of solvent to cell content and mass transfer (Drmić and Režek Jambrak, 2010; Patist and Bates, 2008). In the field of Sustainable Chemistry, the advantages of UAE are the use of non-toxic solvents, reduced energy consumption, shorter time and lower temperature of the process what is suitable for the extraction of thermolabile compounds (Medina-Torres et al., 2017).

The main objective of this research was to investigate the impact of the UAE process on the extraction of selected bioactive compounds from CBS as well as to determine a % scavenging activity and TPC in obtained CBS extracts. Those highly enriched CBS extracts could possibly be further used as functional food or as raw material in different industries.

Material and methods

Chemicals

All standards and chemicals were purchased from commercial suppliers and were of analytical grade. Solvents were purchased from J.T. Baker (Phillipsburg, USA). All standards for HPLC analysis, including theobromine standard (purity ≥98%), theophylline (purity≥99%), gallic acid (purity≥99%), epicatechin (purity ≥98%), catechin (purity ≥99%), and caffeic acid (purity ≥99%), were purchased from Sigma Aldrich (Germany), while the caffeine standard (≥98%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MI, USA).

Material

CBS was obtained from Kandit d.o.o. Chocolate Factory, Osijek, Croatia, in the summer of 2017. The material was primarly separated from the cotyledon and roasted at 135 °C for 55 min. The geographical origin of CBS was West Africa blend.

UAE extraction of bioactive compounds from CBS

Prior to the extraction process, the samples of CBS were crushed in an IKA A11 Basic laboratory mill to increase the matrix area. After grinding and weighing 1 g of CBS, different concentrations of samples were prepared for extraction by addition of water as solvent. UAE of bioactive compounds from CBS was performed on an ELMA ultrasonic bath, Elmasonic P 120 H (Elma Schmidbauer GmbH, Germany). The extraction was performed at a frequency of 37 kHz in sweep mode for homogeneous distribution of ultrasonic waves within the water bath. Twenty-nine experiments, including five replicates, were performed according to the Response Surface Methodology (RSM) and the Box-Behnken design (BBD). The effect of temperature, extraction time, liquid/solid ratio and ultrasound power on the yield of bioactive compounds in the obtained CBS extracts were examined. The temperature was set from 40 °C to 80 °C, liquid/solid ratio at 10 to 50 mL/g, ultrasound power from 30 to 70 % and the extraction time from 30 to 90 minutes.

Determination of bioactive compounds of CBS by HPLC Analysis

Immediately after the extraction, the obtained extracts were diluted with water (1:10) and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) syringe filter. Identification and quantification of the obtained bioactive compounds in extracts was performed on the High-Performance Liquid Chromatograph (HPLC) (Infinity 1260 Agilent Technologies, Santa Clara, USA), which contained an autosampler G7129A, quaternary pump G7111B 1260 and diode array detector (DAD) G7117C 1260 DAD HS. The column used for the measurement was Zorbax C18 150 mm x 4.6 mm x 5μm, which was thermostated on 30 °C. The wavelenght was set to 276 nm and the injection volume was 20 μL. Mobile phase was gradient, starting by 1% formic acid and acetonitrile (95:5) at the beginning, changing to (80:29) till 9 min, and returning to (95:5) till 13 min. The flow was set to 1 mL/min and the analysis was done in triplicate.

Determination of % scavenging activity of obtained extracts

The antiradical activity of the obtained extracts was determined using the prior described DPPH procedure (Jokić et al., 2016) on a UV/Vis spectrophotometer (Thermo Spectronic, Cambridge, Great Britain). Measurement solutions were prepared by adding 1.2 mL of CBS (10 mg/mL) and 0.5 mL 0.2 mM freshly prepared DPPH solution in methanol. The samples were left to incubate for 30 minutes in the dark space at ambient temperature, after which the apsorbance was measured at 517 nm. All measurements were done in triplicate.

Determination of total phenol content of obtained extracts

The total phenol content (TPC) of the extracts was determined according to modified spectrophotometric method using Folin-Ciocalteu reagent, with gallic acid as a calibration standard (Jakobek et al., 2007) on a Selecta

UV/Vis spectrophotometer (UV-2005, Spain). Measurement solutions were prepared by adding 20 µl of sample, 1580 µl distilled water, 100 µl Folin-Ciocalteu reagent and 300 µl Na_2CO_3 solution (200 g/L). Prepared samples were placed in a thermostat at 40 °C for 30 minutes after which the measurement was performed. The results were calculated according to the calibration curves derived from the three analyzes and expressed as mg gallic acid (GAE) equivalent per g of extract. The measurement was performed in triplicate at the wavelength of 765 nm.

Statistical Analysis

A BBD with four numerical factors at three levels was used for statistical analysis of the obtained parameters. The design consisted of 29 experiments with five replications at the center point. For the statistical analysis of obtained experimental data the commercial Design-Expert® software, v.9 (Stat Ease Inc. Minneapolis, MN, USA) was used as well as the analysis of variance (ANOVA) to estimate the quality of the obtained models. The test of the statistically significant difference was based on the total error criteria with the level of confidence of 95.0%. The same software was used to generate the response plots in order to better understand the correlation of independent and response variables.

Results and discussion

The results obtained by UAE extraction method for isolation of selected bioactive compounds from CBS are given in Table 1. In our previous study (Pavlović et al., 2019) we investigated the extraction of bioactive compounds from CBS, specifically methylxantines, (theobromine and caffeine) by selected green extraction techniques (supercritical CO_2 extraction, UAE, cold atmospheric plasma extraction, and deep eutectic solvent extraction) in comparison to conventional Soxhlet extraction. It was proved the significantly better efficiency of innovative green extraction techniques compared to conventional Soxhlet extraction in the isolation of targeted compounds as well as the better antioxidant activity of obtained extracts. Opposite to the new technologies, the conventional method did not show practicable due to long duration of the process and the application of high temperatures. In addition to this preliminary research as well as to the available literature, eleven (11) compounds were analyzed in this study that were assumed to be pottentially present in CBS (theobromine, caffeine, theophylline, gallic acid, epicatechin, epigalocatechin, catechin, chlorogenic acid, caffeic acid, vanillin, and 5-Hydroxymethylfurfural (5-HMF).

Table 1. Bioactive compounds, TPC and % scavenging activity of CBS in obtained UAE extracts

RUN	T (°C)	t (min)	Liquid/solid ratio (mL/g)	Power (%)	Gallic acid (mg/g)	Theobromine (mg/g)	Caffeine (mg/g)	Caffeic acid (mg/g)	Catehin (mg/g)	Epicatechin (mg/g)	TPC (mgGAE/g extract)	% scavenging activity
1.	40	60	30	30	0.504	2.077	0.276	0.000	0.165	traces	54.692	36.420
2.	60	90	30	70	0.517	2.696	0.410	0.255	0.266	0.077	87.000	65.108
3.	40	60	10	50	0.904	3.767	0.486	0.000	0.065	0.024	21.103	14.624
4.	40	90	30	50	0.622	2.493	0.360	0.000	0.317	0.055	82.385	56.907
5.	60	60	30	50	0.368	4.122	0.295	0.274	0.409	0.082	92.385	67.703
6.	40	60	30	70	0.642	3.120	0.444	0.398	0.342	0.020	96.487	64.783
7.	60	90	10	50	0.968	3.872	0.497	0.000	0.080	0.005	23.667	16.923
8.	80	60	30	70	0.738	4.098	0.580	0.447	0.457	0.029	132.897	74.943
9.	60	60	30	50	0.807	4.545	0.618	0.527	0.333	0.060	108.282	85.106
10.	60	30	30	70	0.749	3.424	0.486	0.443	0.321	0.078	109.821	73.244
11.	40	60	50	50	1.197	5.916	0.570	0.000	0.134	0.042	91.872	74.812
12.	80	90	30	50	0.727	3.725	0.537	0.431	0.354	0.100	123.410	86.377
13.	60	60	30	50	0.570	3.610	0.508	0.434	0.309	0.085	114.436	70.598
14.	60	60	50	30	1.077	5.195	0.726	0.000	0.208	0.054	118.538	82.326
15.	60	60	30	50	0.769	4.134	0.589	0.000	0.336	0.067	91.615	73.795
16.	60	60	50	70	1.407	5.431	0.785	0.000	0.341	traces	108.282	77.594
17.	60	30	10	50	0.866	3.642	0.433	0.000	0.033	traces	21.872	13.683
18.	40	30	30	50	0.605	2.255	0.315	0.000	0.185	0.023	78.538	59.304
19.	60	30	30	30	0.584	3.325	0.486	0.392	0.245	0.000	105.718	68.167
20.	60	60	10	70	0.110	2.916	0.698	0.000	0.111	0.012	34.436	27.471
21.	60	90	30	30	0.469	3.419	0.472	0.381	0.446	traces	93.410	81.569
22.	80	30	30	50	0.520	2.402	0.358	0.000	0.289	traces	91.872	82.941
23.	60	90	50	50	1.249	5.600	0.587	0.000	0.175	0.023	124.179	14.034
24.	60	60	30	50	0.785	3.456	0.537	0.000	0.340	traces	101.103	81.815
25.	80	60	50	50	1.080	4.128	0.678	0.000	0.247	0.070	123.923	85.485
26.	80	60	30	30	0.586	2.568	0.400	0.000	0.243	0.036	105.974	74.944
27.	60	60	10	30	0.854	3.193	0.439	0.000	0.044	0.006	20.590	14.130
28.	60	30	50	50	1.118	5.297	0.646	0.000	0.170	0.023	106.487	76.097
29.	80	60	10	50	0.687	3.234	0.421	0.000	0.051	0.006	22.128	17.334

T: temperature; t: time; % scavenging activity.

Processes like roasting could eventually lead to thermal degradation and consequently to formation of 5-HMF which could have adverse effect on human health (Kowalski et al., 2013). According to the obtained results shown in Table 1, the most abundant compound in CBS extracts obtained by UAE was theobromine, followed by gallic acid, caffeine, catechin, epicatechin and finally caffeic acid in traces. Theobromine, as the most abundant methylxanthine, was extracted in concentrations from 2.077 mg/g to 5.916 mg/g, while caffeine was extracted in concentrations from 0.276 mg/g to 0.785 mg/g. The highest concentration of theobromine (5.916 mg/g) was extracted under conditions with 50% ultrasonic power, 50 mL/g liquid/solid ratio, temperature 40 °C in the extraction time of 60 minutes. The highest concentration of caffeine (0.785 mg/g) was extracted under conditions of 70% ultrasonic power, 50 mL/g liquid/solid ratio, at a temperature of 60 °C in the extraction time of 60 minutes. The most abundant phenolic compounds extracted by this technique were gallic acid in concentrations from 0.110 mg/g to 1.407 mg/g and catechin from 0.033 mg/g to 0.457 mg/g. The highest concentrations of caffeic acid and epicatechin were 0.527 mg/g and 0.100 mg/g depending on the given extraction parameters. The highest concentration of gallic acid (1.407 mg/g) was extracted at 70% ultrasonic power, 50 mL/g liquid/solid ratio, at 60 °C in the extraction time of 60 minutes. The highest concentration of catechin (0.457 mg/g) was extracted at 70% ultrasonic power, 30 mL/g liquid/solid ratio, at 80 °C in the extraction time of 60 minutes. The extract with the highest catechin concentration was also the extract with the highest TPC (132.897 mgGAE/$g_{extr.}$) while the extract with the highest epicatechin concentration was also the extract with highest % scavenging acitvity (86.377%). The highest concentration of caffeic acid (0.527 mg/g) was extracted at 50% ultrasonic power, 30 mL/g liquid/solid ratio, at 60 °C in the extraction time of 60 minutes. The highest concentration of epicatechin (0.100 mg/g) was extracted at 50% ultrasonic power, 30 mL/g liquid/solid ratio, at 80 °C in the extraction time of 90 minutes. Other phenolic compounds that were found in low concentrations in the CBS extracts were below the limit of detection and could not be quantified while theophylline and 5-HMF were not even detected.

If we look at other similar studies, Esclapez et al. (2011) mentioned that temperature, as one of the important UAE parameter, affects the yield of each compound individually, due to improved mass transfer. McDonell and Tiwari (2017) noted that increased temperature reduces solvent viscosity due to the increased kinetic energy of molecules in the solution. Although some studies have shown that fewer phenolic compounds can be extracted at higher temperatures due to their possible thermal degradation or polymerization, this is not the case in this study. Such processes generally occur at much higher temperatures than in the above experiment (110 °C) (De la Calle and Costas -Rodriguez, 2017). McDonnel and Tiwari (2017) gave an insight into the effectiveness of UAE in the extraction of polyphenols, antioxidants and other bioactive compounds from different matrices (pomegranate peel, grapefruit seeds, olive leaves, etc.). Yusof et al. (2019) applied different concentrations of ethanol (70-90 v/v%), temperature (45-65 °C) and different extraction time (30-60 min) for the extraction of flavonoids from Malasian CBS by UAE. Except epicatechin, authors detected the certain flavonoid oligomers (procyanidin B2, procyanidin C1, procyanidin B4, procyanidin A2, procyanidin trimer, procyanidin tetramer, procyanidin pentamer) in the CBS by the ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS). The calculated optimal extraction conditions for these compounds were 80% aqueous ethanol solution, temperature of 55 °C and the extraction time of 45 minutes. Quiroz-Reyes et al. (2013) demonstrated better UAE efficiency unlike maceration due to the higher antioxidant activity in UAE extracts as well as a good correlation between antioxidant activity and TPC. They also pointed out the higher content of catechins and epicatechins in extracts of cocoa bean cotyledons compared to CBS extracts. They stated how UAE is an excellent extraction method for isolating antioxidants given the shorter extraction time, higher reproducibility as well as small lost of bioactive compounds. Oliveira et al. (2018) extracted methylxanthines (caffeine and theobromine) from cocoa beans by proton ionic liquid 2HEAA (2-hydroxy ethylammonium acetate) and ultrasound. They emphasized excellent efficiency of proton ionic liquids in the extraction of these compounds and highlighted the solid/liquid ratio as the main influential variable in the extraction, while the power of ultrasound had no significant effect, which is also the case in this study. Jiménez and Cañizares-Macías (2013) proved that the UAE is more efficient in isolating caffeine (by 57.7%) and theobromine (by 43.6%) from cocoa beans in comparison to conventional extraction by mixing. As a reason for more efficient extraction, they pointed out the importance of ultrasonic waves that do not cause modification of the extract. Dent et al. (2015) also

compared some conventional methods for extraction of phenolic compounds from sage (*Salvia officinalis* L.) using two different UAE techniques (ultrasonic device with direct mixing and direct sonication with a probe). Studies have shown that UAE with ultrasonic device with direct mixing achieved the highest yield of total and individual polyphenols while direct sonication was also more efficient than conventional extractions. Bamba et al. (2018) were the first who to investigate the influence of UAE conditions on the yield of phenolic compounds from blueberries (*Vaccinium angustifolium*) as well as the antioxidant activity of the obtained extracts. They proved that the efficiency of this extraction largely depends on the ethanol content in the aqueous extract, the solid/liquid ratio, temperature, time and pH. UAE with a 50% ethanol gave higher yields of flavonoids and anthocyanins as well as higher TPC, while a decrease in the solid/liquid content increased the polyphenol content as well as the antioxidant activity of the extracts. With the use of 50% ethanol and higher temperatures, the total flavonoids and antioxidant activity increased while the TPC decreased. The weakly basic pH had a positive effect on the antioxidant activity and TPC in contrast to the acidic pH, while the anthocyanin content reduced. Thus, prolonged extraction time in water increased the anthocyanin content. The authors also stated the possibility of different phenolic fractions with selected UAE conditions. Papoutsis et al. (2018) optimized the UAE for the isolation of rutin from lemon by-products (*Citrus limon* L.) (residual endocarp, seeds, and exocarp). The results of total TPC, total flavonoids and antioxidant activity were compared with hot water-assisted extraction and conventional extraction with organic solvents. Hot water extraction has been shown to be the most effective in isolating the highest flavonoid content and the highest antioxidant activity while UAE and conventional extraction showed similar results. However, they recommended use of UAE due to the shorter duration of extraction.

Response Surface Analysis and Process Optimization

To explore the influence of process parameters on the extraction yield, TPC and % scavenging acitvity of selected compounds in CBS extracts, a response surface analysis was performed. The results showed that six responses (theobromine, caffeine, gallic acid, catechin, TPC and % scavenging activity) (Table 1) were detected in all 29 experiments and they were included in further statistical analysis. The selected responses were evaluated by analysis of variance (ANOVA) and the results are summarized in Tables 2-7. For methylxanthines (theobromine and caffeine, respectively), a statistically significant effect was shown in linear term of liquid/solid ratio (p = 0.0003 and 0.0068), as well as in its quadratic term (p = 0.0041 and 0.0079). The quadratic term of temperature also showed a significant influence on these two compounds (p = 0.0203 and 0.0436). The model F-value of 3.96 for theobromine and 2.94 for caffeine imply that model is significant. There is only a 0.73% chance for theobromine and 2.63% chance for caffeine that an F-value this large could occur due to noise (Tables 2 and 3). For gallic acid, statistically significant effect was shown in linear term of liquid/solid ratio (p = 0.0008), as well as in its quadratic term (p = 0.0005). The interaction between the liquid/solid ratio and the ultrasound power (X_3X_4), also showed a statistically significant effect (p = 0.0119). The model F-value of 3.70 for gallic acid implies that model is significant. There is only a 1.00% chance that an F-value this large could occur due to noise (Table 4). Three process parameters for catechin showed statistically significant effect in linear term, temperature (p = 0.0486), liquid/solid ratio (p = 0.0005) and ultrasound power (p = 0.0291) while the quadratic term of the liquid/solid ratio also showed statistically significant effect (p <0.0001). The interaction between the extraction time and the ultrasound power (X_2X_4) also showed statistically significant effect (p = 0.0437). The model F-value of 8.03 for catechin implies that model is significant. There is only a 0.02% chance that an F-value this large could occur due to noise (Table 5). TPC (Table 6) as well as in the antioxidant activity (Table 7), except for the linear term (p <0.0001 for both responses), the quadratic term of the liquid/solid ratio showed a statistically significant effect (p <0.0001 and 0.0002). Temperature also showed statistically significant effect for both responses (p = 0.0014 and 0.0404). The interaction between the extraction time and the liquid/solid ratio (X_2X_3) showed statistically significant effect only for the antioxidant activity (p = 0.0436). The model F-value of 14.83 for TPC and 5.44 for % scavenging activity imply that models are significant. There is only a 0.01% chance for TPC and 0.16% chance for % scavenging activity that an F-value this large could occur due to noise.

Table 2. Analysis of variance (ANOVA) for the response surface quadratic model for theobromine

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Model	23.72	14	1.69	3.96	0.0073*
X_1-Temperature	0.0232	1	0.0232	0.0543	0.8191
X_2-Time	0.1774	1	0.1774	0.4150	0.5298
X_3-Liquid/solid ratio	9.98	1	9.98	23.35	0.0003*
X_4-Power	0.3033	1	0.3033	0.7095	0.4138
X_1X_2	0.2946	1	0.2946	0.6892	0.4204
X_1X_3	0.3939	1	0.3939	0.9214	0.3534
X_1X_4	0.0591	1	0.0591	0.1383	0.7155
X_2X_3	0.0013	1	0.0013	0.0031	0.9563
X_2X_4	0.1690	1	0.1690	0.3953	0.5397
X_3X_4	0.0658	1	0.0658	0.1539	0.7008
X_1^2	2.93	1	2.93	6.85	0.0203*
X_2^2	0.9173	1	0.9173	2.15	0.1651
X_3^2	5.01	1	5.01	11.73	0.0041*
X_4^2	1.38	1	1.38	3.24	0.0935
Residual	5.98	14	0.4275		
Lack of Fit	5.21	10	0.5211	2.69	0.1760
Pure Error	0.7737	4	0.1934		
Cor Total	29.71	28			
R^2	0.7985				

Table 3. Analysis of variance (ANOVA) for the response surface quadratic model for caffeine

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Model	0.3512	14	0.0251	2.94	0.0263*
X_1-Temperature	0.0228	1	0.0228	2.67	0.1245
X_2-Time	0.0016	1	0.0016	0.1912	0.6686
X_3-Liquid/solid ratio	0.0859	1	0.0859	10.07	0.0068*
X_4-Power	0.0304	1	0.0304	3.56	0.0801
X_1X_2	0.0044	1	0.0044	0.5209	0.4823
X_1X_3	0.0075	1	0.0075	0.8850	0.3628
X_1X_4	0.0000	1	0.0000	0.0041	0.9496
X_2X_3	0.0038	1	0.0038	0.4422	0.5169
X_2X_4	0.0010	1	0.0010	0.1120	0.7429
X_3X_4	0.0099	1	0.0099	1.17	0.2985
X_1^2	0.0420	1	0.0420	4.92	0.0436*
X_2^2	0.0235	1	0.0235	2.76	0.1189
X_3^2	0.0818	1	0.0818	9.59	0.0079*
X_4^2	0.0018	1	0.0018	0.2136	0.6511
Residual	0.1194	14	0.0085		
Lack of Fit	0.0543	10	0.0054	0.3336	0.9275
Pure Error	0.0651	4	0.0163		
Cor Total	0.4706	28			
R^2	0.7463				

Table 4. Analysis of variance (ANOVA) for the response surface quadratic model for gallic acid

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Model	1.79	14	0.1277	3.70	0.0100*
X_1-Temperature	0.0015	1	0.0015	0.0444	0.8362
X_2-Time	0.0010	1	0.0010	0.0286	0.8681
X_3-Liquid/solid ratio	0.6523	1	0.6523	18.10	0.0008*
X_4-Power	0.0007	1	0.0007	0.0193	0.8915
X_1X_2	0.0091	1	0.0091	0.2625	0.6164
X_1X_3	0.0025	1	0.0025	0.0714	0.7932
X_1X_4	0.0000	1	0.0000	0.0014	0.9703

X_2X_3	0.0002	1	0.0002	0.0060	0.9392
X_2X_4	0.0035	1	0.0035	0.1001	0.7564
X_3X_4	0.2884	1	0.2884	8.35	0.0119*
X_1^2	0.0008	1	0.0008	0.0223	0.8835
X_2^2	0.0009	1	0.0009	0.0262	0.8738
X_3^2	0.6944	1	0.6944	20.10	0.0005*
X_4^2	0.0447	1	0.0447	1.29	0.2744
Residual	0.4837	14	0.0346		
Lack of Fit	0.3411	10	0.0341	0.9561	0.5689
Pure Error	0.1427	4	0.0357		
Cor Total	2.27	28			
R^2	0.7870				

Table 5. Analysis of variance (ANOVA) for the response surface quadratic model for catechin

Source	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	***p*-Value**
Model	0.3733	14	0.0267	8.03	0.0002*
X_1-Temperature	0.0155	1	0.0155	4.66	0.0486*
X_2-Time	0.0130	1	0.0130	3.90	0.0683
X_3-Liquid/solid ratio	0.0662	1	0.0662	19.93	0.0005*
X_4-Power	0.0196	1	0.0196	5.91	0.0291*
X_1X_2	0.0011	1	0.0011	0.3361	0.5713
X_1X_3	0.0040	1	0.0040	1.22	0.2885
X_1X_4	0.0003	1	0.0003	0.1021	0.7541
X_2X_3	0.0004	1	0.0004	0.1302	0.7236
X_2X_4	0.0163	1	0.0163	4.91	0.0437*
X_3X_4	0.0011	1	0.0011	0.3231	0.5788
X_1^2	0.0088	1	0.0088	2.64	0.1267
X_2^2	0.0071	1	0.0071	2.14	0.1656
X_3^2	0.2240	1	0.2240	67.46	< 0.0001*
X_4^2	0.0002	1	0.0002	0.0618	0.8073
Residual	0.0465	14	0.0033		
Lack of Fit	0.0408	10	0.0041	2.85	0.1624
Pure Error	0.0057	4	0.0014		
Cor Total	0.4197	28			
R^2	0.8893				

Table 6. Analysis of variance (ANOVA) for the response surface quadratic model for TPC

Source	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	***p*-Value**
Model	33668.56	14	2404.90	14.83	< 0.0001*
X_1-Temperature	2555.82	1	2555.82	15.76	0.0014*
X_2-Time	32.48	1	32.48	0.2003	0.6613
X_3-Liquid/solid ratio	23363.05	1	23363.05	144.06	< 0.0001*
X_4-Power	408.33	1	408.33	2.52	0.1349
X_1X_2	191.72	1	191.72	1.18	0.2953
X_1X_3	240.65	1	240.65	1.48	0.2433
X_1X_4	55.29	1	55.29	0.3409	0.5686
X_2X_3	63.18	1	63.18	0.3896	0.5425
X_2X_4	27.63	1	27.63	0.1704	0.6860
X_3X_4	145.23	1	145.23	0.8956	0.3600
X_1^2	168.18	1	168.18	1.04	0.3258
X_2^2	31.60	1	31.60	0.1949	0.6656
X_3^2	6280.86	1	6280.86	38.73	< 0.0001*
X_4^2	0.3391	1	0.3391	0.0021	0.9642
Residual	2270.42	14	162.17		
Lack of Fit	1876.16	10	187.62	1.90	0.2801
Pure Error	394.27	4	98.57		
Cor Total	35938.99	28			
R^2	0.9368				

Table 7. Analysis of variance (ANOVA) for the response surface quadratic model for % scavenging activity

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Model	16501.16	14	1178.65	5.44	0.0016*
X_1-Temperature	1105.42	1	1105.42	5.10	0.0404*
X_2-Time	229.85	1	229.85	1.06	0.3205
X_3-Liquid/solid ratio	7812.42	1	7812.42	36.06	< 0.0001*
X_4-Power	54.55	1	54.55	0.2518	0.6236
X_1X_2	8.51	1	8.51	0.0393	0.8458
X_1X_3	15.85	1	15.85	0.0732	0.7907
X_1X_4	201.12	1	201.12	0.9284	0.3516
X_2X_3	1066.11	1	1066.11	4.92	0.0436*
X_2X_4	115.97	1	115.97	0.5353	0.4765
X_3X_4	81.66	1	81.66	0.3769	0.5491
X_1^2	43.62	1	43.62	0.2014	0.6605
X_2^2	309.58	1	309.58	1.43	0.2518
X_3^2	5602.18	1	5602.18	25.86	0.0002*
X_4^2	8.07	1	8.07	0.0372	0.8498
Residual	3032.98	14	216.64		
Lack of Fit	2813.55	10	281.36	5.13	0.0646
Pure Error	219.43	4	54.86		
Cor Total	19534.14	28			
R^2	0.8447				

In general, the liquid/solid ratio showed the largest statistically significant effect for all tested responses (p <0.05), while the extraction time did not show statistically significant effect on any response. Temperature showed statistically significant effect for catechin (p = 0.0486), TPC (p = 0.0014) and % scavenging activity (p = 0.0404), while ultrasound power had statistically significant effect only on catechin concentration (p = 0.0291). Regression models for all examined responses showed a statistically significant effect (p-values from <0.0001 to 0.0263) with satisfactory coefficients of determination (R^2) ranging from 0.75 to 0.94. Given that p-value for all regression models were below 0.05 means that there is a statistically significant influence between the independent variables and the variables of the observed responses. The Lack of Fit in all cases was not statistically significant (p>0.05), which means that the obtained second order polynominal equation is adequate for accurate estimation of experimental values and can be used to make predictions about the response for given levels of each factor. The high levels of the factors were coded as +1 and the low levels as -1, by default. The coded equations are useful for identifying the relative impact of the factors by comparing the factor coefficients (Table 8).

Table 8. Polynominal equations calculated after implementation of BBD (in terms of coded factors)

Regresion Coefficient	2nd Order Polynominal Equation
Gallic acid (Y_1)	$0.6599 - 0.0113X_1 + 0.0091X_2 + 0.2283X_3 - 0.0075X_4 - 0.0109X_1^2 + 0.0118X_2^2 + 0.3272X_3^2 - 0.0830X_4^2 + 0.0476X_1X_2 + 0.0248X_1X_3 + 0.0035X_1X_4 + 0.0072X_2X_3 - 0.0294X_2X_4 + 0.2685X_3X_4$
Theobromine (Y_2)	$3.97 + 0.0440X_1 + 0.1216X_2 + 0.9120X_3 + 0.1590X_4 - 0.6720X_1^2 - 0.3760X_2^2 + 0.8792X_3^2 - 0.4620X_4^2 + 0.2714X_1X_2 - 0.3138X_1X_3 + 0.1216X_1X_4 + 0.0183X_2X_3 - 0.2055X_2X_4 + 0.1282X_3X_4$
Caffeine (Y_3)	$0.5095 + 0.0436X_1 + 0.0117X_2 + 0.0846X_3 + 0.0503X_4 - 0.0804X_1^2 - 0.0602X_2^2 + 0.1123X_3^2 + 0.0168X_4^2 + 0.0333X_1X_2 + 0.0434X_1X_3 + 0.0030X_1X_4 - 0.0307X_2X_3 - 0.0155X_2X_4 - 0.0499X_3X_4$
Catechin (Y_4)	$0.3454 + 0.0359X_1 + 0.0329X_2 + 0.0743X_3 + 0.0404X_4 - 0.0367X_1^2 - 0.0331X_2^2 - 0.1858X_3^2 + 0.0056X_4^2 - 0.0167X_1X_2 + 0.0318X_1X_3 + 0.0092X_1X_4 - 0.0104X_2X_3 - 0.0639X_2X_4 + 0.0164X_3X_4$
TPC (Y_5)	$101.56 + 14.59X_1 + 1.65X_2 + 44.12X_3 + 5.83X_4 - 5.09X_1^2 - 2.21X_2^2 - 31.12X_3^2 + 0.2286X_4^2 + 6.92X_1X_2 + 7.76X_1X_3 - 3.72X_1X_4 + 3.97X_2X_3 - 2.63X_2X_4 - 6.03X_3X_4$
% scavenging activity (Y_6)	$75.80 + 9.60X_1 - 4.38X_2 + 25.52X_3 + 2.13X_4 - 2.59X_1^2 - 6.91X_2^2 - 29.39X_3^2 - 1.12X_4^2 + 1.46X_1X_2 + 1.99X_1X_3 - 7.09X_1X_4 - 16.33X_2X_3 - 5.38X_2X_4 - 4.52X_3X_4$

X_1: temperature; X_2: time; X_3: liquid/solid ratio; X_4: ultrasaund power

Three-dimensional plots for two most abundant methylxanthines in CBS, theobromine and caffeine (Figs. 1 and 2) showed very similar shapes. The plots show that by increasing the liquid/solid ratio,

concentration of those two compounds significantly increase. By increasing the temperature to 60 °C, the ultrasonic power up to 50% as well as the longer extraction time up to 60 minutes, the concentration of theobromine first slightly increases and then decreases (Fig. 1). In caffeine, also by increasing the temperature to 60 °C and extracting to 60 minutes, the concentration first slightly increases and then decreases while the increase in ultrasound power had no effect on the concentration of this compound (Fig. 2). The increase in the liquid/solid ratio affected the increase in gallic acid concentration while the increase in temperature, ultrasound power as well as the length of the extraction time had no significant effect on the concentration of this compound (Fig. 3). By increasing the liquid/solid ratio from 10 to 30 mL/g the catechin concentration also increased, while by further increasing of this parameter, catechin concentration decreased. The graph also showed the slight increase of catechin concentration with increase in temperature, ultrasound power and extraction time (Fig. 4). Three-dimensional plots for TPC (Fig. 5) and % scavenging activity (Fig. 6) showed similar shapes, which was expected due to their already proven correlation. Both observed responds showed the tendency to increase by increasing liquid/solid ratio and the slight increase with temperature increase. The increase in ultrasound power as well as the extraction time showed no significant effect for either response (Figs. 5 and 6).

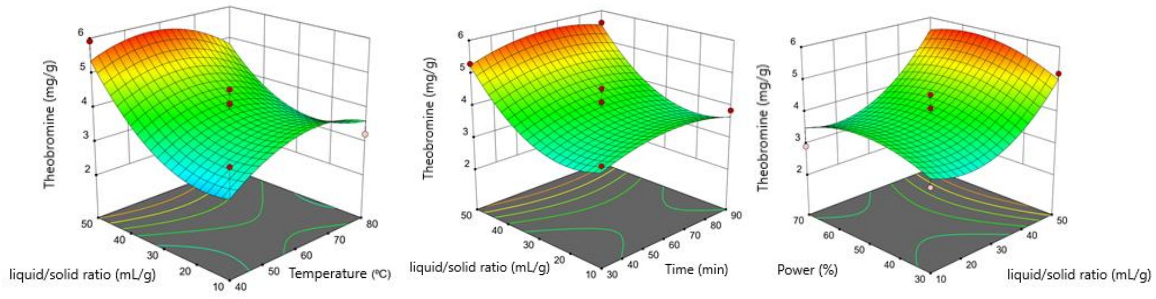

Fig. 1. Three-dimensional plots for theobromine content in CBS extracts obtained by UAE

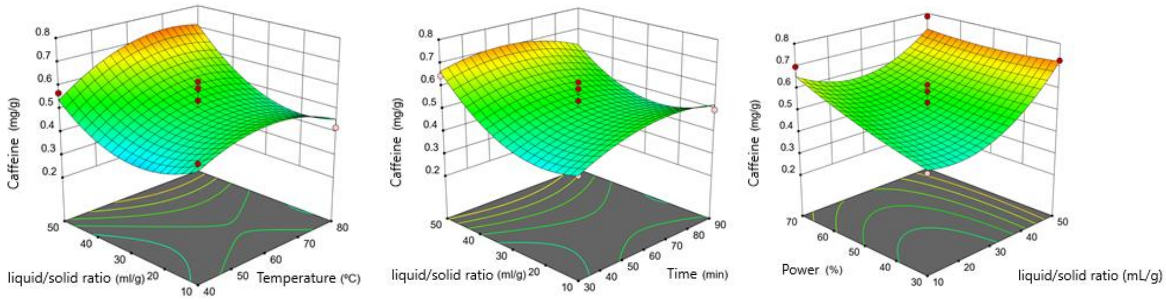

Fig. 2. Three-dimensional plots for caffeine content in CBS extracts obtained by UAE

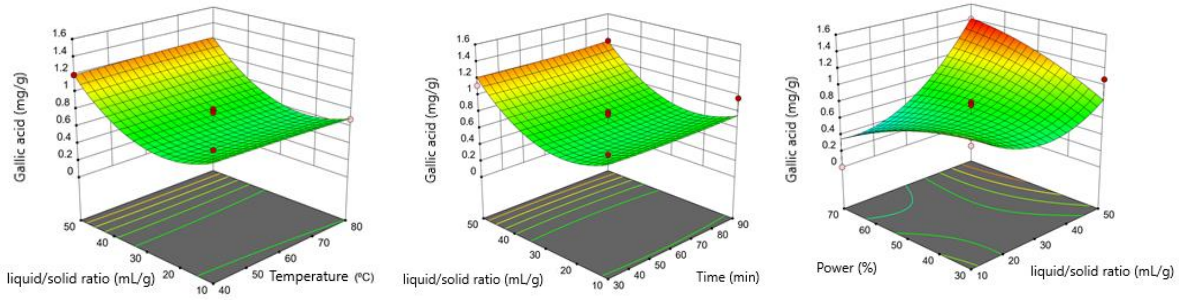

Fig. 3. Three-dimensional plots for gallic acid content in CBS extracts obtained by UAE

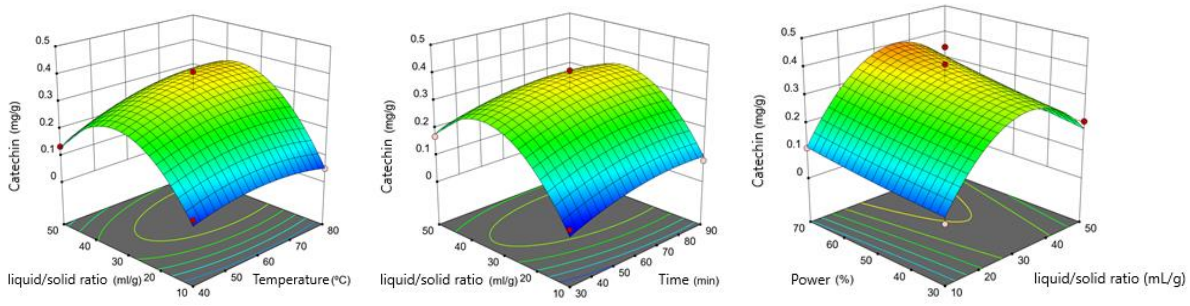

Fig. 4. Three-dimensional plots for catechin content in CBS extracts obtained by UAE

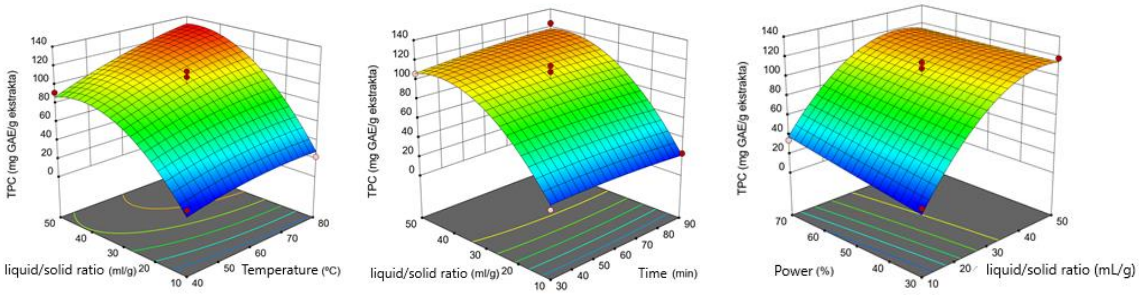

Fig. 5. Three-dimensional plots for TPC in CBS extracts obtained by UAE

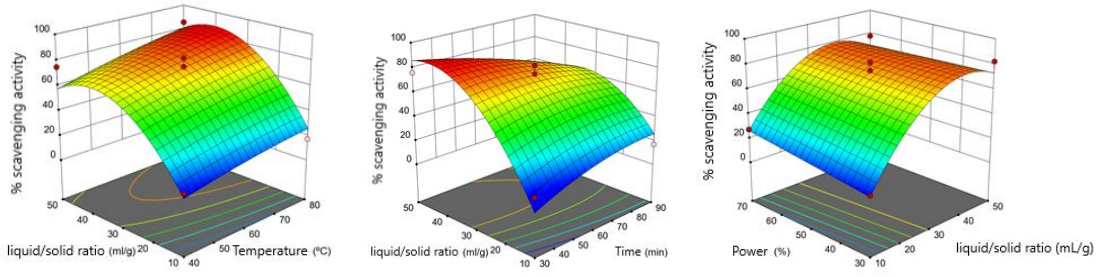

Fig. 6. Three-dimensional plots for % scavenging activity in CBS extracts obtained by UAE

The RSM in this study gave optimal conditions for the UAE extraction of bioactive compounds from CBS, taking into account their maximum: temperature of 69.45 °C, liquid/solid ratio 49.99 mL/g, ultrasonic power of 69.99% and an extraction time of 44.26 minutes. Under these conditions, the predicted concentration of theobromine was calculated to be 5.234 mg/g, caffeine 0.741 mg/g, catechin 0.341 mg/g, gallic acid 1.434 mg/g, TPC 118.380 mg GAE/$g_{extr.}$ and DPPH 81.846% which agrees with the experimentally obtained data.

Conclusions

Given today's popular zero-waste strategy, prevention of waste accumulation and its possible utilization, the study focused on investigating and proving how UAE, as the novel, green extraction method, is very successful for isolation of bioactive compounds from by-product CBS. According to the obtained results, the liquid/solid ratio had the greatest influence on the content of theobromine, caffeine, gallic acid and catechin in the obtained extracts. Although on a lab-scale level, the paper demonstrates how by optimizing

of UAE extraction, the desired composition of CBS extracts enriched in bioactive comounds can be achieved. In addition, the UAE has proven to be an excellent replacement for the conventional extraction techniques. High-valued extracts rich in bioactive compounds, obtained from by-products such as CBS, could eventually find purpose in the other industries such as the pharmaceutical, chemical, cosmetic or food industries.

Author Contributions: Conceptualization - Nika Pavlović, Stela Jokić; Investigation - Nika Pavlović, Martina Jakovljević, Maja Molnar, Stela Jokić; Statistical analysis - Nika Pavlović; Writing—original draft preparation - Nika Pavlović, Stela Jokić; Writing—review and editing - all authors; Funding acquisition - Stela Jokić, Maja Molnar.

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Declaration of interest:
None.

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DIETARY SALT INTAKE IN PREGNANCY AND HYPERTENSIVE PREGNANCY DISORDERS – NARRATIVE REVIEW

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review paper

Summary

Salt is crucial for human health, but it`s excess is associated with the development of many diseases including arterial hypertension which is a major feature of hypertensive pregnancy disorders (*HPDs*). Maternal nutrition during pregnancy can affect cardiometabolic disease development during pregnancy and later in life, but it can also affect fetal growth and disease development in adulthood. Recent studies suggest that excessive salt intake often combined with low potassium intake throughout pregnancy, can suppress renin-angiotensin-aldosterone system (*RAAS*) with adverse effects on fetoplacental development and can increase the risk of *HPDs*. Although salt restriction has been considered potentially harmful in the non-pharmacological treatment of arterial hypertension in pregnancy and current guidelines do not recommend it during pregnancy to prevent *HPDs*, especially gestational hypertension and the development of preeclampsia, its role should be reconsidered in light of the recent evidence. However, one key question remains: How much salt, upper and lower limit of daily intake, in a balanced diet is not harmful in uncomplicated pregnancies as well as HPDs in general?

Keywords: hypertension, pregnancy, hypertensive pregnancy disorders, preeclampsia, salt

Dietary salt intake in general population and in pregnancy

Salt (sodium chloride, *NaCl*), is crucial for human health, however, it`s excess is associated with the development of many diseases, namely: cardiovascular diseases (Aburto et al., 2013; Cogswell et al., 2016; He et al., 2013), gastric cancer (D'Elia et al., 2012; Ge et al., 2012; Poorolajal et al., 2020), nephrolithiasis (Lin, 2020), kidney disease (McMahon et al., 2013) and osteoporosis (Caudarella et al., 2009; Teucher et al., 2008). Sodium is the main chemical component in table salt and an essential nutrient necessary for the maintenance of fluid and acid-base balance, and cellular homeostasis in the human body. The recommended dietary salt intake is less than 5 g (or 2 g of sodium) per day for adults and less than 3 g (1.2 g sodium) for children under 7 years of age, and even less than 2 g (0.8 g soidum) for those under 4 years of age (Campbell et al., 2014; Campbell et al., 2015; WHO, 2016). The amount of sodium needed to maintain homeostasis in adults is very low (<500 mg) (He et al., 2009; Holbrook et al., 1984) compared to current global average intake of 2.4-4.8 g per day (WHO, 2016; WHO, 2020). According to the *US* Centers for Disease Control and Prevention (*CDC*) and European Food Safety Authority (*EFSA*), approximately 70-75% of the sodium intake is in the food we buy (Anderson et al., 2010; Centers for Disease Control and Prevention, 2012; European Food Safety Authority, 2005), while 11% comes from sodium added at the table or cooking at home (Harnack et al., 2017*).* Salt is mainly in food due to its role in sensory appreciation, processing technology and preservation (Elias et al., 2019). High salt intake is often accompanied with high consumption of sugar and fat (Hu, 2013; Gupta et al., 2018). Interestingly, the preference for sweetness and saltiness significantly increases during pregancy peaking highest during the third trimester (Oh et al, 2011). Cioffi et al. (2018), have shown that pregnant women were more likely to consume salt and sugar than nonpregnant women of the same age. There are a growing number of studies showing increased prevalence of cardiometabolic diseases related to high salt and sugar consumption in pregnant and nonpregrant populations (Ponzo et al, 2021; Seo et al, 2020).

Maternal nutrition during pregnancy can affect cardiovascular disease development during pregnancy and later in life, but it can also affect fetal growth and disease development in adulthood (Arvizu et al., 2020; Fogacci et al., 2020; Gluckman et al., 2008). Women of reproductive age have a relatively low prevalence

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of arterial hypertension, 8–9 % (Azeez et al., 2019; Bateman et al., 2012). Arterial hypertension in pregnancy may be chronic (predating pregnancy or diagnosed before 20 gestational weeks) or *de novo* (either preeclampsia or gestational hypertension) (Brown et al., 2018). Hypertensive pregnancy disorders (*HPD*) are among the most common medical complications and affect 5-10 % of all pregnancies worldwide (Hutcheon et al., 2011; Umesawa et al., 2017). In the case of associated comorbidities, the incidence increases up to 15 % (Umesawa et al., 2017). These hypertensive disorders include preexisting (chronic) arterial hypertension, gestational hypertension (*GH*) and pre-eclampsia (*PE*). *PE* itself is now defined as persistent arterial hypertension (systolic pressure ≥140 mmHg or diastolic pressure ≥90 mmHg) that develops after 20 gestational weeks or during the post-partum period, associated with proteinuria (≥ 300 mg in 24 h) and/or other maternal organ dysfunction (Brown et al., 2018). Of note, pregnant woman with chronic arterial hypertension may develop *PE* superimposed on chronic arterial hypertension. *HPDs* are a major cause of maternal, fetal, and neonatal morbidity and mortality (Cantwell et al., 2011; Chappell et al., 2021; Khan et al., 2006; Stevens et al., 2015).

Nutritional interventions during pregnancy for the prevention or treatment of maternal morbidity and preterm delivery often result in reduced energy intake and reduced micro and macronutrient consumption (Villar et al., 2003). Van der Maten (1995) has shown that the restriction of salt consumption (low sodium intake) in pregnant women also causes a significant reduction in the intake of proteins, carbohydrates, fat and minerals.

Current national and international guidelines do not recommend salt restriction during pregnancy to prevent *GH* and the development of *PE* (ACOG, 2013; Hanson et al., 2015; NICE, 2010; WHO, 2011; Magee et al., 2014; Regitz-Zagrosek et al., 2018).

The role of renin-angiotensin-aldosterone system in normal pregnancy and hypertensive pregnancy disorders

Pregnancy is characterized by normal blood pressure (*BP*) and elevated circulating levels of renin, angiotensin II (*Ang II*), and aldosterone (Brown et al., 1997; Langer el al., 1998; Uddin et al., 2015). Hyperactivation of renin-angiotensin-aldosterone system (*RAAS*) in pregnancy is necessary for an increase in the circulating maternal plasma volume (*PV*) which is crucial in maintaining sufficient uteroplacental perfusion (Verdonk et al., 2014). *PV* correlates positively with fetal birth weight, while reduced *PV* correlates with intrauterine growth restriction (Brown et al., 1989). During normal pregnancy, there is a compensatory decline in peripheral vascular resistance to maintain normal maternal *BP*. This happens due to the vasodilating effect of progesterone (Hill et al., 2008) and decreased sensitivity to *Ang II* vasoconstriction (Elsheikh et al., 2001; Irani et al., 2011; Langer et al., 1998). In preeclampsia, *RAAS* is suppressed, circulating maternal *PV* is reduced, and vascular reactivity to *Ang II* is increased compared to normal pregnancy (Brown et al., 1997; Langer el al., 1998; Uddin et al., 2015; Washburn et al., 2015). Furthermore, decreased secretion and utilization of aldosterone in pregnancy can lead to insufficient fetoplacental development, and thus the development of perinatal complications and poorer maternal and fetal outcomes (Bellamy et al., 2007; Birukov et al., 2019; Gennari-Moser et al., 2011; Todkar et al., 2012).

Relation between renin-angiotensin-aldosterone system and salt intake

RAAS activity during pregnancy is influenced by dietary sodium and potassium intake (Birukov et al., 2019; Nielsen et al., 2016), importantly adrenal aldosterone synthesis is stimulated directly by potassium. In a randomized, cross-over, double-blinded, dietary interventional study by Nielsen et al. (2016), high-salt intake decreased renin and *Ang II* concentrations significantly in healthy pregnant and nonpregnant women, however preeclamptic patients failed to demonstrate the same effect. Decreased aldosterone and increased brain natriuretic peptid (*BNP*) were observed in all groups (Nielsen et al., 2016). Importantly, findings from other authors showed that pregnant women who later developed *PE* retained more of a given sodium load than those with uncomplicated pregnancies (Brown et al., 1988; Scaife and Mohaupt, 2017), even after furosemide administration (Brown et al., 1994). This suggests an impaired kidney ability to excrete sodium in *PE* despite suppressed *RAAS* and arterial hypertension, which normally promotes natiuresis (Kjolby et al., 2008). Renal hyperreabsorption of sodium at a site distal to the thick ascending limb of Henle's loop could be explained by abnormal activity of apical sodium transport proteins, i.e. epithelial sodium channels (*ENaC*) (Nielsen et al., 2016). Preeclamptic women display an abnormal presence of plasmin in the urine which activates collecting duct *ENaCs*. This is achieved by cleavage of the apical exodomain of the g-subunit of the *ENaC* (Buhl et al., 2012; Svenningsen et al., 2009). Enhanced renal sodium retention in preeclamptic women could explain subsequent

suppression of *RAAS* and salt-sensitive hypertension. Furthermore, Martillotti et al. (2013), showed that *BP* response to salt was significantly increased in women with a history of *PE* compared with controls.

Relation between hypertensive pregnancy disorders and salt intake

Arterial hypertension is a major feature of hypertensive disorders in pregnancy (Brown et al., 2018) and excessive salt is one of the proven causes of arterial hypertension (Blaustein et al., 2012; Kotchen et al., 2013; Mozaffarian et al., 2014). Moreover, excessive salt plays a role in endothelial dysfunction, induction of inflammation and has differential effects on immune cell activity (Asayama et al., 2018; Edwards, 2016; van der Meer et al., 2013). Inflammation is involved in the pathogenesis of *PE* (Michalczyk et al., 2020; Sanchez-Aranguren et al., 2014; Weissgerber et al., 2016). Gluckman et al. (2008), have demonstrated that impaired endothelial function is present in pregnant women who later develop *PE*.

The studies in pregnant and nonpregnant populations indicated that a combined effect of dietary sodium excess and potassium insufficiency is greater than either alone, as a mediator of high *BP* (Morris et al., 1999; Yılmaz et al., 2017). Furthermore, the decreased efficacy of lowering *BP* with low sodium and potassium intake has also been reported in the Dietary Approaches to Stop Hypertension (*DASH*) trial (Sacks et al., 2001). In a recent meta-analysis conducted by Binia et al. (2015), they found a significant correlation between reduced *BP* with daily urinary sodium to potassium ratio. Yılmaz et al. (2017), reported positive correlations between urinary sodium to potassium ratio and *BP* levels in pregnant women with *PE*. During this study the estimation of daily salt and potassium intake was based on a calculation of 24-hour urinary sodium and potassium excretion. The pregnant women with *PE* (n=150) were divided into tertiles according to urinary sodium to potassium ratio (*U[Na/K]*): low, medium and high Na/K groups. The mean systolic *BP* (*SBP*) and diastolic *BP* (*DBP*) levels were significantly lower in the low Na/K group compared with medium or high Na/K groups. The frequency of severe *PE* was lower in the low Na/K group compared with medium and high Na/K groups. Birth weight and gestational age at birth were higher in the low Na/K group compared with the high Na/K group (newborn weight difference mean was 306 g; 2 weeks difference in gestational age) (Yılmaz et al., 2017). These findings suggested that pregnant women with *PE* with high dietary sodium and low potassium intake had a greater maternal and neonatal morbidity risk compared to their low dietary salt and high potassium intake counterparts. Additional analysis by Birukov et al. (2019) showed that salt intake > 6 g per day in pregnancy was associated with a greater risk of developing *PE* (hazard ratio: 5.68, 95 % CI: 1.51; 21.36). Further, a large analysis from the Danish National Birth Cohort (*DNBC*), carried out on 66,651 singleton pregnancies, demonstrated that women with the highest sodium intake (median 3.70 g/day (range: 3.52–7.52 g/day)) had a 54% (95 % CI: 16% - 104 %) higher risk of *GH* and a 20% (95 % CI: 1 % 42%) higher risk of *PE* than women with the lowest intake of sodium (median 2.60 g/day (range: 0.83–2.79 g/day)) (Arvizu et al., 2020). This indicated that salt intake during pregnancy was positively related to the occurrence of *HPDs* among pregnant Danish women.

Conclusion

Sodium intake and retention in the early stages of pregnancy is essential for physiologic maternal extracellular volume expansion, which regulates maternal blood pressure and uteroplacental circulation (Scaife and Mohaupt, 2017). Excessive salt intake often combined with low potassium intake throughout pregnancy, can suppress *RAAS* with adverse effects on fetoplacental development and can increase the risk of *HPDs* (Arvizu et al., 2020; Asayama et al., 2018; Bellamy et al., 2007; Birukov et al., 2019; Fogacci et al., 2020; Gennari-Moser et al., 2011; Nielsen et al., 2016; Todkar et al., 2012; Yılmaz et al., 2017). Women with a history of *HPDs* have increased risk of cardiovascular and cerebrovacular disease in later life (Bellamy et al., 2007; Honigberg et al., 2019; Umesawa et al., 2017). Cardiovascular risk after *HPD* is largely mediated by development of chronic arterial hypertension (Chappell et al., 2021; Haug et al., 2019). The risk of subsequent cardiovascular events increases with severity, parity and recurrence of *HPDs* (Chappell et al., 2021; Lykke et al., 2009). In addition to the increased risk of morbidity in women, *HPDs* seem to have a clinical impact on the health outcomes of offspring not only in the perinatal period, but also during childhood and adolescence (Jansen et al., 2019; Kanata et al., 2021). Offspring exposed to *HPDs* are more likely to have cardiovascular, renal, endocrine, gastrointestinal, immune and neurocognitive disorders (Jansen et al., 2019; Kanata et al., 2021). *HPDs* may affect fetal programming, namely neurohormonal adaptation (alterations in *RAAS*, hypothalamic-pituitary axis (*HPA*)), immune system, angiogenesis and fetal organ development (Kanata et al., 2021). These alterations may play a role in the future development of the diseases in the exposed offspring. According to current evidence, direct causal relations

between aforementioned disoders and HPDs cannot be established. Thus, closer monitoring of this population and further studies are necessary.

Although salt restriction has been considered potentially harmful in the non-pharmacological treatment of arterial hypertension in pregnancy (Farese et al., 2006; Gennari-Moser et al., 2014) and current guidelines do not recommend it during pregnancy to prevent *GH* and the development of *PE* (ACOG, 2013; Hanson et al., 2015; Magee et al., 2014; NICE, 2010; Regitz-Zagrosek et al., 2018; WHO, 2011), its role should be reconsidered in light of the recent evidence outlined in this article (Table 1). However, one key question remains: How much salt (upper and lower limit of daily intake) in a balanced diet is not harmful in uncomplicated pregnancies as well as *HPDs* in general?

Table 1. Studies on the effect of excessive salt intake on maternal, fetal and offspring morbidity and mortality outcomes during pregnancy and later in life

Study (First author and year)	Type of study	Study population	Outcomes	Note
Yılmaz et al. (2017)	Prospective case – control	200 pregnant women (50 control - healthy pregnancy, 150 with newly diagnosed PE)	Pregnant with PE with high dietary salt and low potassium intake had greater maternal and neonatal morbidity risk than pregnant with PE under low dietary salt and high potassium intake	Women at risk not included
Birukov et al. (2019)	Longitudinal, observational cohort	569 pregnant women from 29 gestational week	Salt intake > 6 g per day in pregnancy was associated with a greater risk of developing PE (HR: 5.68, 95 % CI: 1.51; 21.36).	Women with HPDs or GDM at the time of sampling not included
Arvizu et al. (2020)	Observational cohort	66 651 singleton pregnancies from 62 774 women	Women with the highest Na^+ intake (median 3.70 g/day) had a 54% higher risk of GH and a 20% higher risk of PE than women with the lowest intake of Na^+ (median 2.60 g/day)	Women with history of HPDs not included
Nielsen et al. (2016)	Randomized, cross-over, double-blinded, dietary interventional	22 pregnant women with singleton pregnancies in gestational weeks 28–38: PE patients (n=7), healthy pregnant women (n=15); and healthy nonpregnant women (n=13)	High-salt intake decreased renin and Ang II concentrations significantly in healthy pregnant and nonpregnant women, but not in PE ones.	
Brown et al. (1988)	Combined cross - secional and prospective	158 primigravid women	Na+ excretion after saline solution loading varied according to prestudy Na^+ intake and was reduced between the second and third trimesters, independent of dietary salt intake. Normal pregnant women retain more administered Na^+ in late pregnancy than in midpregnancy despite further increases in PV and no alterations to BP or GFR. Those with established proteinuric PIH (i.e. PE) retain Na^+ avidly without stimulation of PRA or PAC (i.e. RAAS).	
Brown et al. (1994)	Prospective, randomized	25 third-trimester pregnant and 16 non-pregnant women	Normal pregnant women exhibited natriuresis and stimulation of plasma renin after frusemide similar to that of non-pregnant women. PE women had significantly impaired renin stimulation after frusemide (blunted response), but a similar natriuresis to that of normal pregnant women.	All women were on an ad libitum diet. Pregnant women - 10 normal and 9 with PE received frusemide, and 6 control received saline. Non-pregnant women - 10 received frusemide and 6 received saline

Ang II – angiotensin II, BP - blood pressure, GDM- gestational diabetes mellitus, GFR - glomerular filtration rate, GH- gestational hypertension, HPDs – hypertensive pregnancy disorders, HR –hazard ratio; Na+ - sodium, PAC -plasma aldosterone concentration, PE- preeclampsia, PIH- pregnancy induced hypertension, PRA- plasma renin activity, PV - plasma volume, RAAS - renin - angiotensin - aldosterone system

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THE ROLE OF INOSITOL, FOLIC ACID AND POLYUNSATURATED FATTY ACIDS IN PREGNANCY AND FETAL DEVELOPMENT

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review paper

Summary

Historical reasons have led to knowledge that would not have been possible to obtain through research without gross violations of ethical norms. Quantification of macro- and micro-nutrient intake is hampered by a number of barriers. It has been observed that changes in fetal nutrition and its endocrine status can result in developmental adjustments that permanently alter the structure, physiology, and metabolism of children, thus exposing individuals to the risk of metabolic, endocrine, and cardiovascular diseases in adulthood. In research on the process better known as "fetal programming", the influence of the *in utero* environment on the epigenetic mechanisms of the fetus has been observed. Decreased or increased amounts of food intake may interfere with placental function and interfere with fetal growth. Altered placental function can lead to endothelial dysfunction, leading to changes in fetal growth and development. More recently, there has been increasing research on the impact of dietary supplementation on pregnant women and perinatal outcome. Among the more frequently examined variables are micronutrients such as folic acid, antioxidants, iron, magnesium and zinc, but also polyunsaturated fatty acids. The Covid-19 pandemic further highlighted the need to create disease registries and systematically monitor data, especially given the differences in health care availability on one hand and the incredible global differences in nutrient availability on the other.

Keywords: gestational diabetes, fetal development, inositol, folic acid, polyunsaturated fatty acids, supplementation, perinatal outcome

Introduction

There is a growing number of studies on the impact of certain nutrients that could prevent the development of pregnancy-related diseases. These are primarily metabolic diseases that can be treated by changing lifestyle. If healthy habits were to be accepted, with the possibility of using nutrients that in some way preserve the health homeostasis of the organism, it is likely that the perinatal outcome would be improved. Some nutrients have been studied more frequently, such as myoinositol, folic acid, and omega fatty acids and deserve consideration for their impact on the prevention of a very common metabolic disease in pregnancy - gestational diabetes mellitus (GDM).

The role of inositol in fetal development through the prevention of gestational diabetes mellitus

During pregnancy, significant metabolic and hormonal changes occur in pregnant women, which are necessary for normal fetal growth and development (Bozzetti et al., 1988). One of the important physiological hormonal changes is the occurrence of maternal hyperinsulinemia and insulin resistance (Hadden et al., 2009). Insulin resistance increases with gestational age and is the highest in the third trimester (Catalano et al., 1991). This physiological change enables and improves the fetal glucose supply during pregnancy. However, if this condition is not accompanied by adequate insulin secretion it may contribute to an increased risk of developing gestational diabetes (*GDM*) (Phelps et al., 1981). In addition, certain conditions such as overweight and obesity may increase insulin resistance due to increased endocrine activity of increased visceral adipose tissue in such patients and thus increase the risk for *GDM* (Catalano et al., 1991). *GDM* is the most common metabolic complication in pregnancy and it is defined as any degree of glucose intolerance with an onset during pregnancy (ADPSG, 2010). It is a risk factor for the cardiometabolic disorders development in both mother and their child later in life (Bellamy et al., 2009; Schwartz et al., 2015; Teh et al., 2011). In addition, there are other multiple risk factors for *GDM*; they are the following: pre-gestational obesity (*BMI*≥ 30 kg/m^2), weight gain during pregnancy in overweight or obese women (Hedderson et al., 2010; Morisset et al., 2010), physical inactivity (Chasan-Taber et al., 2008), older maternal age (Morisset et al., 2010), diets low in fiber

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and with a high glycemic load (Zhang et al., 2006), previous history of macrosomic baby or *GDM* (Petry, 2010), family history or first-degree relative with diabetes, or polycystic ovarian syndrome (Reece, 2010).

Systematic review of 23 randomized controlled trials (*RCT*s) on the prevention of *GDM* by combined diet and exercise interventions has shown 15% reduced risk for *GDM* in 19 *RCT*s, 5% reduced risk of caesarean section in 14 *RCT*s, and in 16 *RCT*s less gestational weight gain (mean difference (MD) -0.89 kg) in the diet and exercise intervention group compared to the control (no intervention) group (Shepherd et al., 2017). Further, there were no significant differences between aforementioned groups (moderate- to very low- quality evidence) in hypertensive pregnancy disorders, large-for-gestational age, neonatal hypoglycemia, perinatal mortality and childhood adiposity (Shepherd et al., 2017). The authors of this Cochrane review concluded that currently available evidence suggests that combined diet and exercise interventions may be effective for preventing *GDM*, but due to the variability of the diet and exercise components tested in the *RCT*s included in the review, the evidence is limited for clear recommendations in clinical practice (Shepherd et al, 2017). Recently, there is increasing number of interventional studies of some potentially effective and safe supplements in preventing the onset of *GDM* in high-risk pregnant women, or in improving glucose homeostasis in pregnant women with preexisting diabetes. One of these supplements with insulin-sensitizing properties is inositol (Corrado et al., 2011; D'Anna et al., 2013; Larner et al., 2010). Inositol (cyclohexane-1,2,3,4,5,6-hexol) is a sugar alcohol that can be synthesized by humans from glucose (National Center for Biotechnology Information, 2021). There are nine possible stereoisomers of which myo-inositol (*MI*) and D-chiro-inositol (*DCI*) are the most abundant in nature (Thomas et al., 2015; Turneret al., 2002). MI is naturally found in food of plant origin, predominantly in fresh fruits and vegetables, or of animal origin, such as meat, fish and milk (Clements et al., 1980). Inositol is a phospholipid structural component of the cell membrane. Moreover, it mediates osmoregulation (Murthy, 2006). Inositol forms the structural base for a number of signaling molecules and secondary messengers thus participating in a number of biological processes. Its phosphorylated derivatives participate in protein phosphorylation (Saiardi et al., 2004), control of gene transcription (Odom et al., 2000), chromatin remodeling (Shen et al., 2003), and facilitate the export of *mRNA* from the nucleus (York et al., 1999). Further, *MI* and *DCI* are involved in glucose and insulin metabolism (Larner, 2002; Larner et al., 2010; Nacimento et al., 2006). Thus, inositol imbalance can result with the development of different diseases (Chhetri, 2019).

In our review we will present the results of interventional studies on *MI* supplementation alone or in combination with other isomers such as *DCI*, or with folic acid (*FA*) in prevention of *GDM*. In *RCT* on pregnant women (n=200) with *GDM* risk factors Amaeful et al. (2018) have found that *MI* supplementation (4 g daily) from early 2nd trimester until the end of pregnancy resulted in reduction of macrosomia and 60% reduction of *GDM* compared to placebo. In *RCT* on non-obese singleton pregnant women (n=180, pre-gestational *BMI* $\leq$25 kg/m^2) at risk for *GDM* (elevated fasting glucose in the first or early second trimester) on different inositol stereoisomers (*MI*, *DCI*, combined *MI* and *DCI*) supplementation Celentano et al. (2020) have found that those on *MI* alone had a lower incidence of abnormal oral glucose tolerance test (*OGTT*) compared to placebo group. Moreover, all exposed groups required less insulin for glycemic control compared to placebo, and this effect was the most pronounced in the group on *MI*. Further, singleton GDM patients (n=80, BMI not specified) at different dosages of inositol isomers (*MI* group - 4g *MI* plus 400 mg *FA*; *DCI* group - 500 mg *DCI* plus 400 mcg *FA*, *MI plus DCI* group - 1100/27.6 mg *MI/DCI* plus 400 mcg *FA*) showed a significant decrease in *HOMA-IR* index and lower variation in average weight gain (at delivery vs. pre-pregnancy and *OGTT* period) in *MI* group compared to control group (*FA* 400 mcg only) after 8 weeks of treatment. In addition, women on *MI* and *MI plus DCI* treatment required significantly less intensified insulin treatment. Interestingly, women treated with inositol had lower birth weight compared to the control group (Fraticelli et al., 2018). In a small RCT on non-obese singleton pregnant women (n=75, BMI <30 kg/m^2) with *GDM* risk factors Matarrelli et al. (2013) found that women randomized to receive *MI* (n=36) from the first or early second trimester to delivery had significantly lower incidence of *GDM* in mid-pregnancy, required less insulin therapy, delivered at a later gestational age, had significantly smaller babies with fewer episodes of neonatal hypoglycemia compared to placebo. In another *RCT* on pregnant non-obese but overweight women (n=220, pre-pregnancy body mass index (BMI) $\geq$ 25-30 kg/m^2) supplementation with *MI* (4 g daily) together with *FA* (400 mcg daily) from the first trimester to delivery resulted in significantly lower incidence of *GDM* compared to control group on *FA* only. Further, *MI plus FA* treatment was associated with a 67% risk reduction of developing *GDM* (OR

0.33; 95% CI 0.15–0.70), but with no effect on macrosomia, hypertensive pregnancy disorders, shoulder dystocia and pre-term delivery (Santamaria et al., 2016). In another *RCT*, *MI* plus *FA* supplementation (4g *MI* plus 400 mcg *FA* daily) from the first trimester to delivery in pregnant obese women (n= 220, pre-pregnancy *BMI*≥30 kg/m^2) resulted in significantly reduced *GDM* rate compared to control group on *FA* only. Further, women on *MI plus FA* treatment showed a significantly greater reduction in *HOMA-IR* index compared with the control group (D'Anna et al., 2015). In meta-analysis on pregnant women at risk for *GDM* (n=965, patients' heterogeneity: variable *BMI*, singleton and unspecified pregnancies, most Caucasians) inositol supplementation (*MI* 4 g daily or *MI* 1100 mg plus *DCI* 27.6 mg daily) was associated with 51% lower rate of *GDM* (OR 0.49, 95% CI 0.24–1.03, p = 0.01) and 65% lower preterm delivery rate (OR 0.35, 95% CI 0.17–0.74, p = 0.006). After adjustment for the type of intervention (*MI* vs. *MI plus DCI*) significant effect was found only in patients receiving *MI*. In the study there was no adverse effects reported (Vitagliano et al., 2019). In the Cochrane systematic review Crawford et al. (2015) showed a potential benefit of antenatal dietary supplementation with *MI* during pregnancy (first and second trimesters to delivery, n = 567) in reducing the incidence of *GDM*, while it was not possible to conclude on perinatal outcomes (neonatal morbidity and mortality) due to lack of data about the same. Based on the recent evidence presented in this review, preventing *GDM* with antenatal inositol supplementation we improve early maternal and perinatal outcome (except for lower birth weight compared to the control group) and prevent the development of chronic diseases later in life for both mother and their offspring.

Impact of folic acid and omega fatty acids on fetal growth and development

Pregnancy is a period of rapid tissue growth, cell differentiation and organogenesis, processes crucial for normal fetal development. Consequently, it is a period of altered and increased need for dietary supply for both mother and the fetus. If micro and/or macronutrients are given at an inappropriate time of fetal development (different organs develop at different times during pregnancy) and/or inappropriate dose, they may be ineffective and even harmful. Therefore, inappropriate nutrition leads to an increased risk of death *in utero*, but also to alterations in birth weight and functional changes in the neonatal organs (McArdle et al., 1999). Therefore, in interpreting impact of nutrition on fetal growth and development, it should be borne in mind that inadequate nutrition may be due to socio-economic factors, inappropriate eating habits, or failure to fully absorb nutrients.

For several decades, folate has been known to be associated with a reduction in pregnancy complications including neural tube defects, congenital malformations (congenital heart diseases), haemorrhage, pre-eclampsia, spontaneous abortions, and fetal growth restriction (Jonker et al., 2020; Ramakrishnan et al., 1999). Because of its role in nucleic acid synthesis, the need for folate increases during times of rapid dividing of cells, such as in fetal development. Therefore, situations with folate deficiency may lead to alterations in DNA synthesis with variety of problems in fetal growth and development.

Long-chain omega-3 polyunsaturated fatty acids (*PUFA*) are essential fatty acids and nutrients which mostly derive from fish and other seafood. They cannot be synthesized by humans and must be ingested through the diet or from supplements. The most biologically active *PUFA* are eicosapentaenoic acid (*EPA*) and docosahexaenoic acid (*DHA*). During pregnancy, *PUFA* requirements increase to support fetal growth, particularly of the brain and eyes. There is evidence that deprivation of *PUFA* during pregnancy is associated with visual and behavioral deficits that cannot be reversed with *PUFA* postnatal supplementation (Coletta et al., 2010).

In this paper we review the effect and impact of prenatal and/ or antenatal *PUFA*, lipid-based nutrient and *FA* supplementation with or without other micronutrient(s) on fetal growth and development. Most of the studies included in this review are from socially deprived, mostly low-income countries. Thus, there is poor or inadequate *PUFA* dietary intake worldwide, especially in low-income countries. Effect of wealth on response to different nutrient supplementation during pregnancy on perinatal outcomes (birth weight, duration of gestation and perinatal mortality) was investigated in the double-blind *RCT* in rural China (Zeng et al., 2011). The assessment of wealth was based on the inventoried household assets. Pregnant women from the poorest households on multimicronutrient (*MMN*) supplementation had significantly increased birth weight by 68 g, reduced low birth weight by 60% and tended to have reduced early neonatal mortality by 52% compared with *FA* group. Further, women in poorest households on iron plus *FA* supplements had significantly increased duration of gestation by 0.41 weeks, reduced pre-term birth by 45% and reduced early neonatal mortality by 90% compared with those on *FA* alone. In this study, pregnant women from the

poorest households had the most improved perinatal outcomes by *MMN* supplementation, while iron plus *FA* supplementation in these women provided more protection for neonatal survival than the *MMN* one. Interestingly, no significant effects of iron plus *FA* and *MMN* supplementation on perinatal outcomes were observed in women from wealthier households in this study (Zeng et al., 2011). In socially deprived, multi-ethnic population from East London, *MMN* supplementation including iron and *FA*, from the first trimester to delivery resulted in higher mean hemoglobin, higher median concentrations of serum ferritin, erythrocyte folate and 25-hydroxyvitamin D later in gestation compared to controls. Moreover, placebo treated women had more small-for-gestational age (*SGA*) infants than treated ones (Brough et al., 2010). Further, another interventional study on undernourished pregnant women in India showed that additional *MMN* supplementation (29 vitamins and minerals once a day) to FA and iron supplements, from second or early third trimester (24 to 32 weeks of gestation) until delivery resulted in lower incidence of low birth weight (a 70% decrease; relative risk, 0.30; 95% CI, 0.13-0.71; P=.006) and lower incidence of early neonatal morbidity (a 58% decrease; relative risk, 0.42; 95% CI, 0.19-0.94; P=.04) compared to placebo (FA plus iron) (Gupta et al., 2007). Maternal *MMN* supplementation in rural Burkina Faso showed significantly higher both birth weight (52 g; 95% CI: 4, 100; P = 0.035) and birth length (3.6 mm; 95% CI: 0.8, 6.3; P = 0.012) compared to those exposed to iron and FA alone. Further, in *MMN* group the risk of large-for gestational-age infants was higher (OR:1.58; 95% CI: 1.04, 2.38; P = 0.03), and this affected mainly primiparous women (OR: 3.44; 95% CI: 1.1, 10.7; P for interaction = 0.11) (Roberfroid et al., 2008). In another study the same group of authors investigated association between cumulative micronutrient intake (*CMI*) and fetal growth. They found moderate improvement in fetal growth. *MMN* supplementation increased birth weight by 69 g compared to iron plus FA alone. Similar results were observed for thoracic and cephalic circumferences. In the same study, *MMN* supplementation together with iron plus *FA* intake during pregnancy showed cumulative effect on fetal growth (Roberfroid[a] et al., 2012). Further, the same authors in another study demonstrated that prenatal *MMN* supplement for singleton pregnant women when compared with the iron and *FA* supplement (*IFA*) treated women, resulted in a 27% (HR: 0.73; 95% CI:0.60, 0.87; P = 0.002) reduction of stunting over the whole observation period. This difference was lost by age of 30 months. The effect of *MMN* supplementation on postnatal growth continued over the observation period with 14% reduction in reported episodes of fever. No other differences in childrens' morbidity and mortality compared to *IFA* group were observed (Roberfroid[b] et al., 2012). Sebayang et al. (2011) showed that maternal *MMN* supplementation increased mean birthweight by 33 g and significantly reduced low birth weight by 21% compared to infants whose mothers received *IFA* supplements. In contrast to previous studies, Ramakrishnan et al., (2016) demonstrated that preconceptional weekly supplementation with *IFA* or the same amount of aforementioned nutrients plus other *MMN* had no effect on birth outcomes (birth weight, gestational age, preterm delivery and small for gestational age) compared to *FA* alone in Vietnamese women.

Prenatal lipid-based nutrient supplementation *(LNS*, 20g daily) for primiparous women in Ghana resulted in greater mean birth length, weight, *BMI* and head circumference than the groups treated with *IFA* (60 mg iron/400 mg FA) or with *MMN* (18 micronutrients including 20 mg iron) (Adu-Afarwuah et al., 2015). The same group of authors demonstrated that maternal *LNS* supplementation during pregnancy and 6 months postpartum together with their infant *LNS* supplementation from 6 to 18 months of their age resulted in significantly greater baby mean length and weight, but not head or midupper arm circumference, compared to those treated with *IFA* or with *MMN*. Further, the prevalence of stunting in the *LNS* group was lower compared to the aforementioned groups (Adu-Afarwuah et al., 2016). In another study maternal fish-oil supplementation from 22nd gestational week until delivery significantly increased maternal *PUFA* stores (*DHA* and *EPA*) after adjustment for maternal baseline *DHA* and *EPA*. In addition, it increased cord blood *DHA* thus improving fetal *PUFA* status. Folate supplementation of pregnant women was significantly associated with increased maternal *DHA*. Andrés Catena et al. (2016) demonstrated that children born to mothers supplemented during pregnancy with *FA* alone solved the response conflict more quickly than those on the placebo or on supplementation with FA and long-chain polyunsaturated fatty acid.

Regarding birth defects, Wehby et al. (2013), investigated effects of different doses of *FA* supplementation (0.4 and 4 mcg per day) before pregnancy and throughout the first trimester on oral cleft recurrence and fetal growth. Cleft rates were compared to historic recurrence rates. The recurrence rates in both treated groups were similar (2.9 and 2.5% in the 0.4 and 4 mg groups) and significantly lower compared to historic rate (6.3%). In addition, there was no difference in fetal growth complications between the two FA groups. Based on the above results, mostly from low-income countries, prenatal

and/ or antenatal *MMN*, *IFA* supplementation with or without lipid-based nutrient supplementation improved perinatal outcomes mainly in malnourished and/ or primiparous pregnant women except in one study. Due to the increased dietary needs of the mother and the fetus during pregnancy, adequate energy and macro and micronutrients, including folic acid and iron, intake through food and supplements are required for normal fetal growth and development. Some studies have shown cumulative effect of those nutrients on fetal growth.

Conclusion

Pregnancy is characterized by significant changes in the hormonal and metabolic status of the pregnant woman, but also by increased needs for macro and micronutrients necessary for normal fetal growth and development. Adequate prenatal and antenatal nutrition and supplementation can improve perinatal outcome and prevent the development of chronic diseases of the mother and offspring later in life.

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THE POTENTIAL OF *Cannabis* sp. IN PAIN MEDICINE: A PERSPECTIVE

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review paper

Summary

Long before they were even properly named, plants form *Cannabis* genus (*C. indica* L., *C. sativa* L. and *C. ruderalis* L.) had their usefulness in the folk medicine. Recently, it has been scientifically proven that cannabinoids mainly act through two types of endocannabinoid receptors in the central nervous system (CB1) and immune cells (CB2). The usage can be either from recreational usage in glaucoma or in research for acute and chronic pain therapy. Some clinical studies support the use of hemp oil and hemp seed in the pain medicine. However, until today, there is still no evidence to suggest that medical cannabis can be used solely as cure, but the use of hemp seed and oil from hemp seeds or flowers in practice, refers to symptomatic treatment as adjunctive therapy. Treatment consists of an individual dose titration phase (with patient supervision) of delta-9-tetrahidrocannabinol (THC) or cannabidiol (CBD) and a maintenance phase. The main goal of this is study was the overview of therapeutic benefit to the familiar and wildly used hemp plant in acute and chronic pain, mostly because of its cost-effectiveness. Overall, this review paper highlights more possibilities of hemp plant usage in further symptomatic pain treatments.

Keywords: hemp oil, hemp seed, acute pain, chronic pain, humans, THC, CBD

Introduction

There is a growing interest of using plants due to their nutritional value of heling properties (Klir et al., 2019; Moses et al., 2017; Vinković et al. 2018; Grubišić et al., 2019; Singh, 2020; Šain et al., 2020). There is around 80% of the global population that still uses botanical drugs and natural substances as a source of therapeutic drugs against various pharmacological targets including cancer, brain performance, cardiovascular function, microbial infection, inflammation, pain, etc. (Sen et al., 2015; Martin et al., 2017; Tucak et al., 2019). Even with the modern medicine of Western primary health-care services, in rural areas health care often coexist with traditional medicine which include medicinal plants and traditional healers (Vandeborek et al., 2004).

There are three most important species of genus *Cannabis* that have a potential in medicinal use. They are the Indian hemp *Cannabis indica* L. (cannabis), the industrial hemp *Cannabis sativa* L. and *Cannabis ruderalis* L., which is growing wildly in Central and Eastern Europe and Russia. The industrial hemp, *C. sativa* L., is, as its name suggests, mostly used for industrial purposes. Industrial hemp stem was used for decades as a raw material in fiber production, but also by product – hemp hurds slices, which are woody core of the hemp plant is often considered to be waste, even though it can valuable and used in eg. as ecofriendly and innovative material in construction industry, in and paper industry, etc. (Cigasova et al., 2015, 2016; Zelca et al., 2017; Momeni et al., 2021).

There are more than 500 different chemical compounds that have been detected in the hemp plant, about one hundred of them are cannabinoids, compounds not detected in any other plant (Beaulieu, 2006). Beside the cannabinoids hemp plant contain flavonoids, terpenes, fatty acids which among others have potential medicinal use.

Pain is the bodily experience of suffering, the feeling of transmitting sensory nerves through the left brain and to the sensitive area of the brain, where the experience is felt. There are several pain signals and transmissions that give specific message to the brain, which then produces different pain sensations: somatic pain (imagine), visceral pain (tissues or organs become stretched or due injury or disease) and neuropathic pain (nerves themselves sustain injury) (Watson et al., 2000). Looking at the way of life and job requirements in the 21st century it is no surprise that there is an increase in chronic pain problems. Given that all approved medication has some unwanted side-effects and can be costly, it has made our mines turn to the nature's solutions, such as potential use of cannabis in acute and chronic pain treatment. So, the aim of this review is to discuss and overview of potential uses of *Cannabis* sp. in pain therapy.

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Historical uses of hemp in anesthesia

Although throughout the history there were many attempts in producing a state of general anaesthesia, more specifically in finding a way to undertake a surgical procedure without feeling pain, they remained individual efforts (Mixa et al., 2016; Sicgin et al., 2016). Those can be traced throughout the history in the writings of Sumerians, Babylonians, Assyrians, Egyptians, Greeks, Romans, Indians and Chinese. Most of those writings show use of a particular plant such as poppy or cannabis. One of the most used plants in the history of anaesthesia is cannabis. Its use dates back to ancient times. Ancient physicians in vary parts of the world mixed cannabis to treat pain and other ailments. For example, in ancient China the use of cannabis in medicine was a very early development. It is described in Shennong Bencaojin, the oldest Chinese pharmacopeia (RCOA, 2020). Hua Tuo was the early Chinese surgeon who is credited with the first use of cannabis as an aesthetic. He reduced plant to powder and mixed it with wine for administration prior to conducting surgery. In ancient Egypt there was use of cannabis as suppositories for relieving the pain of haemorrhoids. Furthermore, in ancient India it was a major religious and medicinal component, frequently used to relieve the pain of childbirth. Additionally, Islamic world used properties of *C. sativa* L. extensively for ten centuries, while ancient Greeks did not use cannabis only for humans, but also in veterinary medicine, to dress wounds and sores on their horses (RCOA, 2020).

The usage of *C. indica* L. as a potential analgesic is described in many ancient texts and traditional practices for pain relief. During Middle Ages there was an Islamic Golden Age, in which scientist and scholars made significant progress in science and medicine. But, even though it was rigorously researched in was not until the 19th century that there was a breakthrough in anaesthesia (WFSA, 2020). The two most memorable events together allowed the transition to modern anaesthesia and surgery. The first public demonstration of ether anaesthesia that took place at Massachussetts General Hospital in Boston on 16th October 1846 and the use of nitrous oxide for extraction of teeth by Horace Wells in 1844. It was an official beginning of what is now known as modern anaesthesia and analgesia (RCOA, 2020).

In the 19th century cannabis was introduced to Western medicine. Initially, it was reduced tussle powder and mixed with wine for administration. The drug Marinol was created in 1970s, when THC had been synthetized. The main mode for administrating cannabis is smoking, because it is almost immediately effective (WFSA, 2020).

Legislative and main species of genus *Cannabis*

C. sativa L., the industrial hemp, is mostly produced in Europe, over 70% (Fig. 1), whereas more than 40% of that production is located in France, estimating that Europe cultivates up to 25% of the world's industrial hemp known as "Cannabis Europe", which refers only to European production. Industrial hemp cultivation area in the EU is monitored by the European Industrial Hemp Association (EIHA).

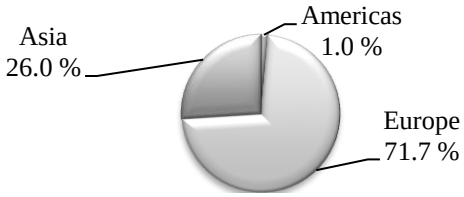

Fig. 1. Production of *Cannabis sativa* L. - industrial hemp seed by region (Average 1994 – 2018) (FAOSTAT, 2020)

The main difference between the *C. sativa* L. and *C. indica* L. plants is that Indian hemp has been overtaken by the phytocannabinoid delta-9-tetrahidrocannabinol (THC), while in industrial hemp THC is present in negligible traces, without psychoactive influence (Jakešević, 2015).

According to European Monitoring Centre for Drugs and Drug Addiction (2017), in most European countries a permitted level of THC for industrial hemp, which is grown for fiber is 0.2% (Italy, Denmark, Belgium, Slovakia), or 0.3 % (Luxemburg), up to 1 % (Switzerland, Czech Republic, Malta). As a consequence, the EIHA is advocating to re-establish this benchmark in Europe to the globally prevalent 0.3% limit.

Asian continent is still to reach its full potential with production of only 26%, this could be due to the fact that usage of *Cannabis sativa* L., is still illegal in most countries (Taiwan, Singapore, Malaysia, People's Republic of China). Moreover, Americas have production of just 1%, which is a great omission on their part considering most countries had legalized THC (Canada, Mexico, Peru, United states of America, Uruguay).

The popularity of the industrial hemp is increasing every day. This is because of their multi-purpose uses. The stem of industrial hemp can be used for fiber, while the seed is used for oil extraction and inflorescence is used for phytocannabinoids extraction. The top 5 hemp products for the EU are clothing, bags or back pack, hemp seed oil, soaps and hemp seed (according to EIHA). Except for humans

use, the seeds, oil, cake and meal can be used for animal fed (Klir et al., 2019).

Indian hemp (or cannabis) have 5 – 20 % THC (Pospišil, 2013). The British government of India prohibited consumption of *C. indica* L. in the 1930's (Bapat et al., 2015). European countries set their own national drug laws, although all of them are parties of the UN Single Convention (Bifulco and Pisanti, 2015). Nowadays, this plant is known as narcotics and it has several names as ganja, marijuana, weed, hasish, etc. In most of the countries the production, manufacture, consume, import or export is illegal.

The use of *C. indica* L. in medicinal purposes is legalized in the Netherlands, Austria, Spain, Portugal, Germany, Finland, Italy, Poland, Sweden, the United Kingdom, Switzerland, Canada, Bangladesh, Israel, Columbia, Uruguay, much of the United States (EIHA; Piper et al., 2017). Most countries legalized medicinal usage, however, recently the recreational purposes have been increasingly popularized.

C. rudealis L. is traditionally used in Russian and Mongolian folk medicine for treating depression. Because of its lowest producing biotypes of THC it is rarely used for recreational purposes. Due to its transition from vegetative to flowering stage with age, as opposed to the light cycle it is bred with *C. sativa* L. and *C.s indica* L. to create "auto-flowering cannabis strains" that exhibit the hardiness of *C. rudealis* L. plant while maintaining the medicinal effects of *C. sativa* L. and *C. indica* L. (EIHA; Piper et al., 2017).

The influence on human

Cannabis is a psychoactive drug from *Cannabis* plant used primarily for medical or recreational purposes. Most common plants are *C. sativa* L., *C. indica* L. and *C. ruderalis* L. (Greydanus et al., 2013).

Beside the THC, the other important cannabinoid is cannabidiol CBD. Unlike THC, CBD does not have psychoactive effect. Both cannabinoids appear in the plant, they work synergistically and chemically they have the same molecular structure: 21 carbon atoms, 30 hydrogen atoms and two oxygen atoms (Fig. 2).

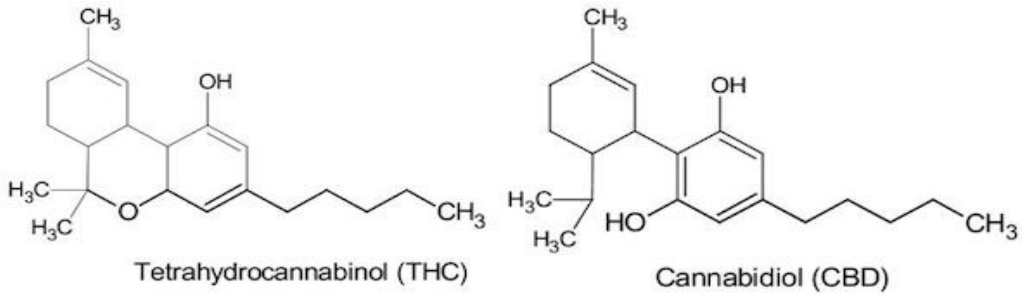

Fig. 2. Molecular structure of THC and CBD (Greydanus et al., 2013)

The difference is in the arrangement of the atoms, which explains different effects on the organism of the consumer. They also are chemically similar to human body endocannabinoids. It allows them to interact with endogenous cannabinoid receptors.

THC is the main psychoactive component of cannabis (Greydanus et al., 2013; Vulfsons et al., 2020). It can be used by smoking, vaporizing, as an extract or within food (Watson et al., 2000). Effects of cannabis are mental and physical. Some of the mental effects are a general change in thought and perception, short-term memory impairment, altered sense of time, impaired body movement, increase in appetite and relaxation (Vulfsons et al., 2020). Additionally, physical effects include increased heart rate, nausea and respiratory problems. The high lipid-solubility results in long persistence in the body. There are two types of cannabinoid receptors, CB1, and CB2, both of which are G protein-coupled receptors. CB1 receptor is primarily found in the brain and some peripheral tissues, while CB2 receptor is primarily found in peripheral tissues, but also in neuroglial cells (Zagožen et al., 2021). THC appears to alter cognition and mood through actions on the CB1 receptors, which inhibit a secondary messenger system, that can be blocked by selective CB1 receptor antagonist rimonabant, that was discontinued due to the dysphoric effect. It is metabolized by the liver cytochrome P450 system (Greydanus et al., 2013; Grichnik and Ferrante, 1991; Altun et al., 2015).

Both THC and CBD are metabolized by the liver cytochrome P450 (CYP-450) system and often the potential of drug-drug interaction of cannabinoids and other drugs or herbs is not recognised for the patients (Vulfons et al., 2020; Mikolašević et al., 2013).

Potential of medicinal use of cannabis

Medicinal cannabis is used not to treat but to alleviate the symptoms of the diseases. Scientific community is very much interested for *Cannabis* sp. pharmacological power, eg. for nausea, food intake, gastro protection, cancer patients, Chron's disease, multiple sclerosis, etc. (Waseem and Seymour, 2016). In recent years a number of nations put the specific laws and programmes to allow patients to use cannabis for relieve the symptoms of e.g. chronic pain, muscular cramps and spasticity in patients with multiple sclerosis or spinal cord damage, as well as for patients affected by neurogenic pain caused by nerve damage and other causes (Bifulco and Pisanti, 2015). It is also used in the treatments of patients with terminal cancer and AIDS, who use the cannabis to alleviate nausea and vomiting, and to stimulate appetite and weight increase of AIDS symptoms, glaucoma, neuropathy, nausea and relapse during chemotherapy in treatment malignant diseases, than pain gains structural psycho-physiological diseases (e.g. in the speech-hearing area), muscle spasticity and pain in the extremities (multiple sclerosis or spinal cord injury), symptoms of motion sickness as in Parkinson's disease, Huntington's disease (movement disorders and dementia), Tourette's syndrome (motor and vocal tics), stimulates appetite and migraine headache (Duraković, 2016; Markus Klarić et al., 2020).

The Ministry of Health of the Republic of Croatia (EMCDDA, 2017) has approved the use of medical hemp for: (i) symptomatic relief of spasticity in patients with multiple sclerosis which spasticity is not adequately controlled by conventional therapy; (ii) in patients with advanced / terminal malignancy and chronic measure to moderately severe pain; (iii) alleviation of nausea and return in patients with malignant diseases receiving emetogenic therapy (antitumor drugs, radiation); (iv) in the treatment of cachexia/anorexia in HV/AIDS patients; (v) in the treatment of Dravet's syndrome (childhood epileptic syndrome) - "cannabidiol Principle".

However, there is no unique list of pathologies that can be treated with cannabis-based drugs, since it is not a cure, but rather a palliative treatment.

The flowers and top leaves of the plant contains a number of chemical compounds which are most important. So, in recent years there is an increased interest of growing industrial hemp because of their flower and seed which can be used for oil production and oil with CBD content (Rozyczko and Brett, 2021). Scientists began to discover what all this interesting herb in its composition was back in 1895 (Shahbazi et al., 2020), when the first cannabinoid was discovered. Cannabidiol (abbreviated CBD), the third isolated cannabinoid compound, was discovered in 1940 (Shahbazi et al., 2020), some 20 years before the most famous THC. Cannabidiol accounts for 40% of industrial hemp extract, its main cannabinoid and is not psychotropic (Russo and Guy, 2006).

In the early 1990s, scientists were quite surprised to find that the human body produces its own cannabinoids (Shahbazi et al., 2020). One is anandamide, or colloquially, the "happiness molecule". This endocannabinoid name was derived from the Sanskrit word *Ananda* meaning happiness and bliss.

Harmful effect of cannabis

The harmful effects of THC in the human body are detrimental, in a sense abuses, hallucinations, feelings of euphoria, disorders in auditory, visual and spatial perception, dehydration, impaired motor skills, drowsiness and redness of the eyes (Markus Klarić et al., 2020). Moreover, influence is to the lungs such that it causes changes similar to those in the lung's chronic tobacco smoker - larynx inflammation, bronchitis, chronic cough. There is also a negative influence of the cannabis on humans. The authors from Croatia reported about two cases of Cannabis induces acute pancreatitis (Mikolašević et al., 2013). Also, there can be a several other like anxieties and loss of appetite (Russo et al., 2006).

Potential of uses cannabis in analgesia

While anaesthesia is defined as a state of controlled, temporary loss of awareness and sensation induced for medical purposes, analgesia is relief from or prevention of pain (ASA, 2020; Vučković et al., 2018). Today we differ an acute and chronic pain and approach to it (Grichnik and Ferrante, 1991). Acute pain is provoked by a specific injury or disease, it is associated with skeletal muscle spasm and sympathetic nervous system activation, it is self-limited and serves a useful biological purpose. Chronic pain, in contrast, may be considered a disease state. It outlasts a normal time of healing, it may arise from psychological state, and has no recognizable end-point. Acute pain treatment is aimed at underlying cause and interrupting the nociceptive signals, while treatment for chronic pain is a multidisciplinary approach and often involve more than one therapeutic modality. In a trial of 40 women undergoing abdominal hysterectomy and receiving a single dose of either a 5 mg of THC in capsule form or placebo no analgesic effect was observed in either group. Additionally, in an experimental trial of 18 healthy

volunteers, oral cannabis extract or placebo were administered after sunburn, no pain reduction was found. It appears that cannabis is not effective analgesic agent in the acute pain setting. However, in meta-analysis of cannabis-based treatments for neuropathic and multiple sclerosis related pain, an overall reduction in pain was found (Iskedjian et al., 2007). Furthermore, the evidence suggest cannabis may be effective for managing arthritis pain, back pain, and truma-related pain, although the quality of the evidence is poor (Niv and Devor, 2004).

In the study of 984 participants which are legal members of medicinal cannabis use in north-eastern US (Pipper et al., 2017), classified their pain as abdominal, back/neck, cancer, chronic pain following surgery, neuropathic, or trauma/injury (Tečić Vuger et al., 2016; Lončarić-Katušin et al., 2019). The participants used medicinal cannabis as joint, pipe, vaporizer, edibles or tincture.

Since chronic patients carry a high prevalence of accompanying symptoms such as depression, sleep disturbance, cognitive dysfunction, and more, a study was completed for cannabis treatment. There were mild improvements found in pain symptoms and severity. Secondly, it had mild beneficial effects for neuropathic pain (Cepeda et al., 2003). Another study was concluded on adults with chronic pain and benefits of cannabis use where it was shown that the benefits of cannabis night be outweighed by their potential harms. Lastly, a study between man and women showed that in men cannabis decreased pain sensitivity when immersing hand in cold water, while in women there was no decrease in pain sensitivity. These results indicate that in cannabis smokers, men exhibit greater cannabis induces analgesia relative to women, as such. sex-dependent differences in cannabis analgesic effects are an important consideration in potential therapeutic effects (Cooper and Haney, 2016). Also, cannabinoids combined with opioids produce synergistic introspective effect, decreasing the lowest effective opioid dose in laboratory animals. Even though, pain patients reported a greater analgesia when cannabis was used with opioids (Cepeda et al., 2003; Buggy et al., 2003; Niv and Devor, 2004), no study showed direct effects of opioids combined with cannabis in humans. In another randomized study it was shown that postoperatively, cannabis users required significantly more opioid rescue analgesia.

Table 1. Dosage in patients with malignant diseases pain relief in patients at the highest tolerable dose of opiates (Ministry of health of Republic of Croatia, 2020)

<table>
<tr><td>First dose</td><td>3 x 2.5 mg
(THC/CBD)</td><td>If patient well tolerate, this dose should be repeated over the next 2 days, followed by a 3 x 2.5 mg/day increase to 3 x 5.0 mg/day.

If there are problems with this initial dose that are not too pronounced, the dose should be repeated over the next 4-5 days because there is a possibility that the problems will subside.
If the problems are too pronounced - treatment is given up.</td></tr>
<tr><td rowspan="3">Further doses</td><td>3 x 5.0 mg
(THC/CBD)</td><td rowspan="3">If well tolerated, the dose should be repeated over the next 2 days, followed by a 3 x 2.5 mg/day dose increase.

If there are problems with this dose that are not too pronounced, the dose should be repeated over the next 4-5 days because there is a possibility that the problems will subside.
If the problems are too pronounced - give up treatment, or take one step.

In this way, a gradual increase of 3 x 2.5 mg/day or a possible decrease if the disturbances are too pronounced, the highest dose is reached which is still tolerated without major difficulties.

When a dose is reached during titration within the maximum recommended daily dose, which is well tolerated and with a decrease in NRS score, treatment is continued with that dose.</td></tr>
<tr><td>3 x 7.5 mg
(THC/CBD)</td></tr>
<tr><td>3 x 10.0 mg
(THC/CBD)</td></tr>
<tr><td>Further treatment</td><td colspan="2">During treatment, the NRS score should be repeated every 1-2 weeks.

If the reduction in score from pre-treatment is <25%, an attempt should be made to increase the dose, as in the titration phase, by one step. If this is not possible (exceeding the maximum daily recommended dose), or if even increased doses (up to the maximum recommended) do not reduce the NRS score ≥25%, ie. it is <25% compared to the initial condition - treatment should be discontinued.</td></tr>
<tr><td>Maximum daily dose</td><td>3 x 12.5 mg</td><td>The maximum daily dose should not exceed 37.5 mg THC/37.5 mg CBD per day (3 x 12.5 mg/day).
Treatment should be discontinued if any maximum (≤37.5 mg/day) dose tolerated during the first 2 weeks does not result in a ≥25% reduction in NRS.</td></tr>
</table>

*NRS=numeric rating scale used pain scales in medicine

This agrees with retrospective study of postoperative period in patients undergoing major surgery, where cannabinoid use was associated with higher pain scores, and poorer quality of sleep (Holdcroft et al., 2006; Andre et al., 2016; Liu et al., 2019).

In Republic of Croatia the dosage for patients with malignant diseases pain relief is regulated with the law (Ministry of health of the Republic of Croatia, 2020). The treatment consists of a dose titration phase and a maintenance phase. Before starting treatment, it is necessary to assess the state of symptoms using NRS. The recommendation only applies to a situation where patients receive standard analgesic therapy that includes opiates, and despite the highest tolerable dose of opiates still suffer moderate to severe pain (NRS≥4, on a scale of 0-10), and THC/CBD preparation is added as an "add-on" therapy. Because of specificity of the indication, the titration phase should not last longer than 10-14 days and each dose increase is 3 x 2.5 mg/day.

There are several synthetic cannabinoids. One of them is Nabilon, which is an oral cannabinoid synthetic tetrahydrocannabinol analogue, which decreases morphine consumption, pain scores, nausea and vomiting following major surgery. For example, in two studies (Vulfsons et al., 2020; Wedman-St. Louis, 2019) did not find significant differences between groups of patients, undergoing major surgery, who were given 1 mg, 2 mg of nabilone, 50 mg ketoprofen or placebo, so they concluded that, with respect to episodes of nausea and vomiting, quality of sleep, sedation, euphoria, pruritus, no serious adverse event was recorded.

Ministry of health of Republic of Croatia published the highest dose of opiates for palliative and oncological patients (Table 1). We can see that the pain scales are used as indicators of effectiveness of given therapy, where numeric rating scale of THC/CBD doses are indicators for further increment, reduction or parity in pain treatment.

Conclusion

The scientific community has highlighted the risks that the acceptance of *C. indica* L. for medicinal purposes could have on the population, specifically on adolescents, due to frequency of the cannabis abuse and misuse in that age group. So, for that reasons, there will always be efforts to find the line between illegal or legal applications, due to its profitable nature. We should doubtlessly continue the research, but it should include scientific and empirical multidisciplinary approach, with utmost understanding for the patients and the causes for cannabis use. *C. sativa* L. is legal to grow and to use as the source of CBD, so the interest it has reached in the recent years is understandable. The range of use is vast and ever growing. From recreational usage in glaucoma to more recently researched use in acute and chronic pain therapy. Furthermore, forms of consumptions of cannabis are large, from vaporization to smoking and oil consuming, so it is available to huge number of consumers. To conclude, there is a lot more to be done with the hemp, cannabis and cannabinoid, and we have yet to discover all of its benefits and means of usage, but we should not be discouraged because finding the right way or right dose could help a majority of patients dealing with overpowering, everyday pain. And the pain is the most common disease of the 21st century.

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NEAR INFRARED SPECTROSCOPY (NIRS) COUPLED WITH CHEMOMETRIC TOOLS USED FOR FOOD PRODUCTS ADULTERATION DETECTION

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professional paper

Summary

The growing problem of food products adulteration requires a rapid and simple method for adulteration detection. Adulterants, which can range from plant material to natural and synthetic colours and compounds have a severe influence on customer trust as well as public health risks. Food safety regulations are being tightened and expanded, requiring a more thorough examination of hazards and risk management solutions. Due to its advantages, near infrared spectroscopy has been recognised as an effective method for food adulteration detection. In this work, an overview of the basics of NIR spectroscopy, NIR data analysis and the use of NIR for food adulteration detection are discussed.

Keywords: NIR spectroscopy, food adulteration detection, chemometrics

Introduction

Food is one of the basic human needs. This fact offers many opportunities in the field of food production, but, at the same, can lead to possible threats in the form of fraud, i.e. food adulteration. High-quality foods such as honey or extra virgin olive oil are increasingly becoming targets of counterfeiting. In practice, high-value foods are usually compromised by cheaper, commercially available foods or product ingredients of similar composition (Drabova et al., 2019). Adulterated foods are often labelled as natural and are priced the same as pure foods, which is wrong and unfair to consumers. Although the addition of cheaper components does not pose a health risk, such practices affect market growth and undermine consumer confidence (Downey, 2013). Due to the growing consumer and market demand for better food quality, there is a need for quick, easy and accurate analytical methods to assess the quality and authenticity of food products. Near infrared spectroscopy (NIRs) proved to be one of such method (Bázár et al. 2016). The advantage of NIR spectroscopy as a non-destructive method is that very little or no prior sample preparation is required, no reagents are needed and therefore no waste is generated. Due to the large amount of data obtained and various overlaps in the data, the results must be processed using methods of multivariate analysis. NIR spectroscopy is sensitive to changes in the chemical and physical properties of the sample under investigation and can therefore be used to verify the authenticity and origin of various products. In order to detect and prevent food manipulation and to question the authenticity of individual food products in a timely manner, several studies conclude that in the future it would be desirable to promote the development of rapid, simple and non-destructive methods and emphasize that the application of NIRs should be further explored to develop specific models for detection of adulteration. In this paper, some basic information about NIR spectroscopy, NIR data analysis and the application of NIR for food adulteration detection and analysis are presented.

NIR spectroscopy

Near-infrared spectroscopy is based on the absorption of electromagnetic radiation with a wavelength in the range of 780 - 2500 nm (Figure 1), where the absorption bands correspond mainly to the overtones and vibrational combinations of molecules. The infrared region was discovered in 1800 by F. W. Herschel when he projected light through a prism and measured the relative thermal effect of individual parts of the spectrum. In this way, he discovered that the maximum thermal effect is far outside the visible range.

The intensity of the NIR absorption band depends on the change in dipole moment that occurs during a vibration. The hydrogen atom is the lightest and therefore shows the strongest vibrations and the largest displacement of bonds (C-H, N-H, O-H, S-H).

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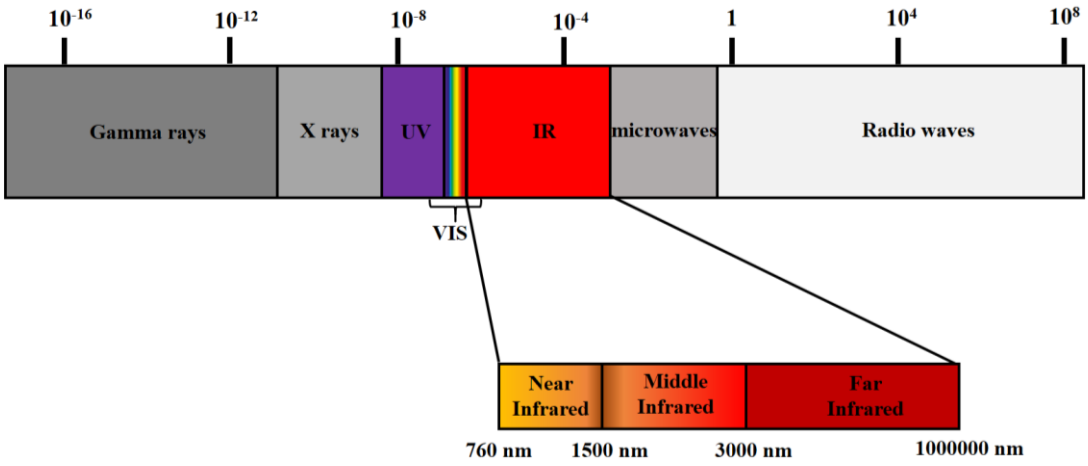

Fig. 1. Electromagnetic spectrum (according to AZO Optics, 2019)

Like any radiation, NIR behaves like a wave with the property of simple harmonic motion, which can be defined by two properties: vibrational frequency and wavelength (Ozaki et al., 2018).

The chemical bonds between atoms in molecules oscillate and to a first approximation, these oscillations behave like simple harmonic motion. The motion of each atom can be treated as an independent oscillation with respect to a fixed centre of mass of the molecule, rather than the mass of the corresponding source. The frequency of oscillation is a function of the mass of two atoms m_1 and m_2 and the bond strength k, and there is a parabolic relationship between potential energy and interatomic distance. Interactions between atoms in different molecules change the vibrational energy states, causing absorption bands to shift and new ones to appear due to differences in the crystal structure. This allows the detection of crystalline forms and the determination of the physical properties of the substance (e.g. density, viscosity and particle size in suspension) (Blanco and Villar, 2001). NIR spectroscopy is a unique tool for studying hydrogen bonding, inter- and intramolecular interactions and hydration, and also provides information on transition of electrons form one *d* orbital to another (*d*-*d* transition) and charge-transfer (CT) transitions. NIR spectroscopy is suitable for non-destructive and *in situ* analysis (Ozaki et al., 2018) and can be useful when it is necessary to acquire data in real time with a reduced possibility of contamination or destruction of the sample (Findlay and Bugay, 1998). NIR is used in monitoring the quality of fruit juices, butter, flour, bread, sugar, edible oils, fish, meat, i.e. in almost all technologies of the food industry. It is possible to perform non-destructive analysis using IR spectroscopy, but if attenuated total reflection or photoacoustic spectroscopy is used, there is no other choice than NIR spectroscopy if it is to be measured in a non-destructive way. NIR spectroscopy allows contactless analysis and analysis with optical fibre probes, which is why it can be used in hazardous environments and the probe can be manipulated remotely. This is one of the reasons why NIR spectroscopy is suitable for online analysis (Ozaki et al., 2018). NIR spectroscopy has been successfully used for qualitative and quantitative analysis. NIR spectra are characterized by a large signal to noise ratio, and the position in the intensity of the bands can be determined very accurately, which is very important for quantitative analysis. The bands in NIR spectra are less informative and may overlap in many cases. Therefore, the obtained data must be processed using multivariate analysis methods (Jednačak and Novak, 2013).

NIR spectra analysis

The analysis of NIR spectra is performed using chemometric methods. Chemometrics is defined as a chemical discipline that uses mathematical and statistical methods to design or select the optimal metrological procedure or experiment and to obtain maximum information by analyzing the obtained data. The results of spectroscopic measurements in complex systems such as food matrix are often ambiguous. Qualitative and quantitative analyses in such complex systems can be problematic, time-consuming, and complicated because of overlapping spectral

responses. The solutions before the implementation of chemometrics were the previous separation of components or increasing the resolution of the spectrum. With the implementation of chemometric techniques, the path of quantitative and qualitative analysis became easier and faster. Spectroscopic techniques in combination with chemometric methods are used for process monitoring and quality control management (Matijević and Blažić, 2008). The statistical methods often used in the analysis of the spectral data are: Principal Component Analysis (PCA), Canonical Correlation Analysis (CCA), Factorial Discriminant Analysis (FDA), Principal Component Regression (PCR), Common Components and Specific Weights Analysis (CCSWA), Partial Least Squares (PLS), and Artificial Neural Networks Method (ANNs) (Karoui et al., 2003).

Principal component analysis is probably the most widely used multivariate statistical method and is considered to be the technique that has changed the way data analysis is viewed (Brereton, 2007). Principal component analysis allows for qualitative analysis and grouping of data without a fixed physical model. It is also used to quickly assess data structure prior to detailed analysis or quantification of a physical or chemical process (Jednačak and Novak, 2013). PCA processes raw data representing samples described by several dependent variables, which are, in most cases, correlated with each other. This method is based on determining the correlations between individual variables by grouping the samples into principal components and describing the relationship between each variable and allowing visualization of their relationship, i.e. whether they are similar or different. If they are similar, they are grouped together while different samples are further apart (Abdi and Williams, 2010). The so-called factor plots are often used to visualize the data (Fig. 2).

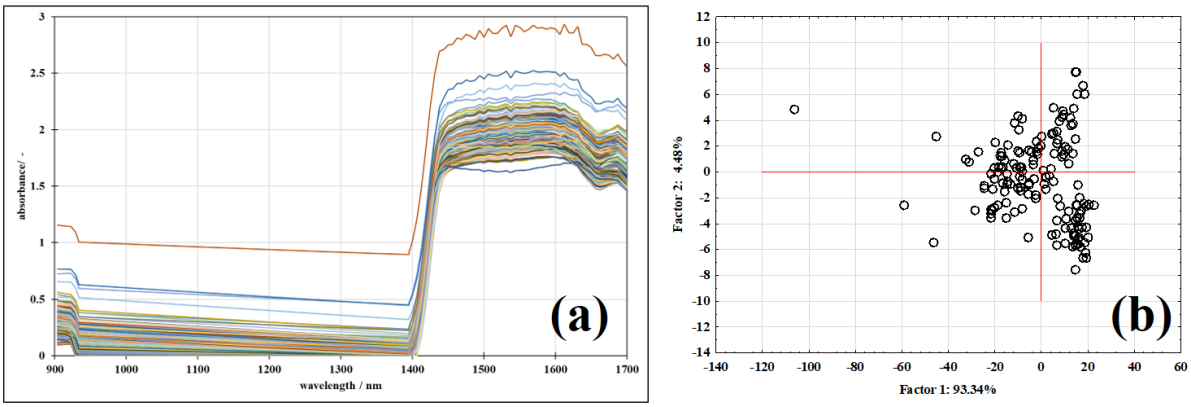

Fig. 2. (a) NIR spectra of honey samples (b) factor plot of NIR spectra samples

The values of the factor components for the spectra that differ are separated, while the values of the factor components for similar spectra are very close to each other, which allows their grouping (Jednačak and Novak, 2013). The goals of PCA are:

1. to extract the most important information from a set of data
2. to reduce the dimensionality of the data - by introducing principal components (PC), the dimensionality of the space is reduced, which simplifies the interpretation of the data. The first principal component (PC1) describes the largest variation in the data, while the remaining variation is described by other principal components (PC2, PC3, ...) (Jednačak and Novak, 2013).

Partial Least Squares (PLS) regression is a multivariate technique used to develop models for latent variables or factors (Djuris et al., 2013). The PLS method is used to describe the data using a model in which the smaller the number of variables, the better. Unlike PCA, where principal components are determined only by the variance of the measured variables and are independent of their correspondence with the desired property, in PLS the latent variables are selected to obtain the best correspondence between the property under study and the measured variable. PLS can be considered as a regressive form of PCA that combines data with a linear multivariate model located in two matrices, X and Y (Jednaček and Novak, 2013). The X and Y blocks (data sets) are modelled to find the variables in the X matrix that best describe the Y matrix. The information in the original X data is projected onto a small number of basic ("latent") variables to ensure that the first components are those that are most important in predicting the Y variables. The goal of PLS analysis is to describe the relationship between objects in X-space and their position in the corresponding Y-space with minimal deviations (Héberger, 2008).

Artificial neural networks (ANNs) are, in the broadest sense of the term, artificial replicas of the human brain that attempt to simulate the learning process and the role and function of the brain itself. Artificial neural networks consist of groups of interconnected elements called neurons. These neurons are organized into layers that form the "architecture" of the network. The first layer is called the "input layer", and each of its neurons receives information from the outside (generally independent variables are used as inputs). The last layer is the "output layer", which contains the neurons with the response or responses - depending on whether one or more parameters have been quantified. Layers of neurons between the input and output layers are called "hidden layers". Input and output data (X and Y matrices) are used to train the network, e.g. by changing the weights for each connection; the sum of all inputs for a given neuron transmits the information using an appropriate transfer function (e.g. sigmoidal function, tangens hyperbolic ...) and passes on the results (Héberger, 2008) (Fig. 3).

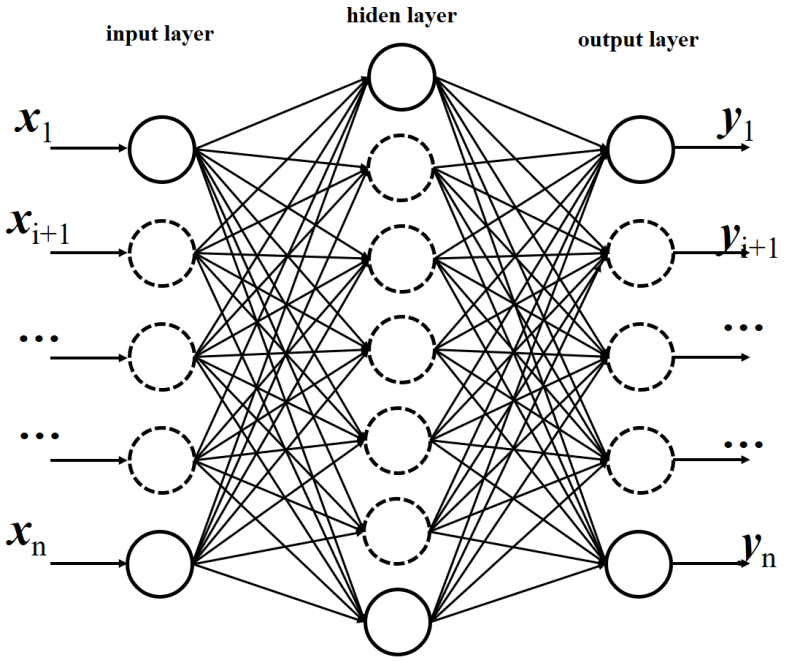

Fig. 3. General structure of ANN model

Neural networks with multilayer perceptrons consist of perceptron neurons organized in serially connected layers. The layers are usually described by numbers from 0 to N, where the zero layers only passes the vector of inputs to the network, while the N-th layer is also the output layer of the network. The layers between the zero and N-th layers are called inner or hidden layers. Multilayer perceptrons have the ability to learn. They also have an algorithm called the "Reverse Back Propagation Algorithm", with capabilities such as: initializing the weights of the network, displaying the first input vector, propagating the input vector through the network to obtain the output, calculating the error signal by comparing the actual output with the desired (target) output, propagating the error signals through the network, adjusting the weights to reduce the total error, repeating all steps with the next input vector as long as the total error is not satisfactorily small. Multilayer perceptrons are used for a variety of tasks that can be categorized as prediction, function approximation, or classification examples. Prediction is about predicting future trends in a time series of data given current and past conditions. Function approximation is about modelling relationships between variables. Sample classification is about classifying data into discrete classes.

Application of near-infrared spectroscopy in the detection of food adulteration

Authenticity of food and agricultural products is of significant importance to meet consumer demands and avoid unfair competition in the market (Downey, 2013). Food authenticity is an established area of research that involves the use of various analytical methods such as gas chromatography (GC), mass spectrometry (MS), nuclear magnetic resonance

(NMR), DNA fingerprinting and vibrational spectroscopy (NIR) (Downey, 2013). Although methods such as GC-MS can accurately detect the food adulteration, they have numerous shortcomings. They require highly skilled manpower, long and extensive instrumental analysis and complicated sample preparation. Therefore, there is an obvious need for the development of simple, rapid, non-destructive and cost-effective analytical methods for the detection and quantification of pathogens in honey. NIR spectroscopy is a rapid, very accurate, multi-analytical method based on the electromagnetic absorption of organic compounds in the short wavelength region of the infrared spectrum. It is used for qualitative or quantitative analysis of multiple sample constituents by a single measurement. Moreover, it does not require reagents and no hazardous waste is generated (Bázár et al., 2016).

Due to the growing problem of food adulteration, NIR as a simple, non-destructive method is everything that makes it more widely used in the chemical and food industries. Basri et al. (2017) in their study used MicroNIR (a palm-sized device) to find lard as a counterfeit component in palm oil. The results agreed with the mean square error of less than 1, which shows that the device has good repeatability. Vanstone et al. (2018) used NIR and related methods to detect adulteration of extra virgin olive oil with various vegetable oils. "Counterfeit" producers mainly use corn oil, sunflower oil, and soybean oil because they are the most accessible and economical. Oils like palm oil, however, are also much easier to detect. They have shown that NIR can distinguish the types of edible oil used for counterfeits and detect counterfeits in any sample with a low detection limit. Mendes et al. (2015) described the use of three different methods to detect counterfeit extra virgin olive oil with soybean oil. This work showed that NIR, in combination with certain chemometric methods, could be a useful alternative technique for the industry to identify possible adulteration in food products, as it is easy to apply and does not require specific chemical knowledge to perform measurements. Radman et al. (2018) applied NIR spectroscopy to detect gluten as a contaminant in food. The determination of gluten in food is very important because there are people who cannot tolerate it or are allergic to it. Two types of wheat flour were used to simulate cross contamination. Rice, rice flour, cornflour and corn semolina with a percentage of 5% to 30%. Based on the very satisfactory results of the study, the developed models could be used to predict possible cross-contamination with gluten. Mabood et al. (2016) tried to detect the adulteration of camel milk with goat milk using NIR. Camel milk is of great importance in desert countries as it contains high levels of vitamin C, up to six times more than cow's milk, and adulteration of this milk can human health. According to them, the newly developed NIR spectroscopic method combined with multivariate analysis is a suitable method to verify the detection and quantification of adulteration in camel milk.

Conclusion

From the above studies, it can be concluded that NIRs is a simple method in which there is no need for long-term sample preparation and which has excellent sensitivity and repeatability. In order to be on time observed and prevent food manipulations and questioning the authenticity of certain foodstuffs products, all the mentioned studies conclude that it would be desirable to encourage development in the future quick, simple and non-destructive methods. They emphasize that NIR could be one of the methods that should be further researched in order to develop as specific detection models as possible for their use in food adulteration detection.

Based on the findings of the above investigations, it can be stated that NIRs is a simple analytical method that requires no long-term sample preparation and has great sensitivity and reproducibility.

All of the research suggests that it would be beneficial to encourage the development of rapid, easy, and non-destructive procedures in order to be able to detect and avoid food manipulations. They emphasized that NIR is one of the technologies that should be investigated further in order to produce as specific detection models as feasible for application in food adulteration detection.

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