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Nutritional and Pharmaceutical applications of bioactive compounds in plants

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Abbreviations

AA	antioxidant activity
AC	antioxidant capacity
ATR	attenuated total reflectance
CAA	cellular antioxidant activity
CDA	canonical discrimination analysis
CE	catechin equivalents
CGE	cyanidin-3glucoside equivalent
CUPRAC	Cupric reducing antioxidant capacity
DMACA	*p*-dimethylaminocinnamaldehyde
EGCG	epigallocatechin gallate
ESI	electrospray ionoization
FI	fluorescence intensity
FRAP	Ferric-reducing/antioxidant power
FTIR	Fourier transform infrared
GAE	gallic acid equivalents
HSA	Human serum albumin
ORAC	Oxygen radical absorbance capacity
PASS	Prediction of Activity Spectra for Substances
RFI	relative fluorescence intensity
TAC	Total antioxidant capacity
TPTZ	Tripyridyl Triazine
IUPAC	Union of Pure and Applied Chemistry committee
WE	water extracts

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Among the phytochemicals, berries, kiwi fruit are known as the important factor in health improvement (Duttoroy & Jorgensen 2004; El Grarras 2009; Kujazanosky et al., 2010). Berry fruits contain a lot of phenolic compounds, and flavonoids are parts of those phenolics which include mostly of flavonols, anthocyanidins, proanthecyanidins, catechins, flavons, and their glycosides. Flavonoids (or bioflavonoids) are benzopyrone derivatives which created spontaneously. They are also the big part of low molecular weight polyphenolic secondary metabolites that spread all over the kingdom of plants and they are the crucial factor in human diet (Parr and Bowell, 2000). Flavonoids have been defined for over 6000 kinds and most of them have been continuously described (Harborne and Williams, 1992).

Their skeleton is made of flaven nucleus which consists of two aromatic rings called ring A and ring B. They also have ring C assembled from three carbon atoms in the heterocycle that connected with six carbon atoms and modified central C ring and can be separated into different structural classes. The examples of those classes are flavanones, isoflavones, flavones, flavonols, and anthocyanins. It has been found that the large varieties of flavonoid structures are occurred by the work of enzymes like glycosyltransferases, methyl transferases, and acyl transferases which modified the basic skeleton of flavonoids (Harborne and Williams, 1992; Forkmann and Heller, 1999). Different flavonoids can be found in one plant species and majority of them are coupled with many sugar moieties (Forkmann and Heller, 1999).

Acidic compounds in the group of phenolics are associated with active antioxidants. For the natural antioxidants, the phenolic acids including caffeic, chlorogenic, p-coumaric, and vanallic acids are found to be vastly circulated in berry crops. In the molecular structure of hydroxyl groups, their antioxidant activities are concerned to a certain extent (Rice-Evans and Miller, 1998).

The possibility of antioxidant potency enhancement is high with dihydroxylation in the 3,4 position by increasing hydrogen donors availability. The most plentiful chlorogenic acid found in berry fruit extracts is chlorogenic acid (phonolic derivative), which also the most active antioxidant. It has been discovered that more than 80% of peroxide formation is inhibited by Chlorogenic acid ($1.2x10^{-5}$ in a linoleic acid test system) (Larson, 1988). Other derivatives of pheonolics like benzoic acid, caffeic acid, catechol, p-cresol, gallic acid. In the daily diet, large portion of berry fruits have other phenolic derivatives like rutin and vanillic acid. Their antioxidant activities are high and they also operate as natural antimicrobial agents (Sofos et al., 1998).

p-Coumaroyl glucose, dihydroflavonol, quercetin 3-glucoside, quercetin 3-glucuronide, kaempferol 3 glucoside, and kaempferol 3-glucurinide are the flavonols which has been detected in berry fruits. These flavonols interacting as effective antioxidants to free radicals by interfering with the propagation of new free radicals or chelating metal ions with their reduction-oxidation (redox) potentials are modified by catalyze lipid oxidation (Macheix et al., 1990; Bakker et al., 1994; Gil et al., 1997; Pratt, 1992; Satue-Gracia et al., 1997).

Anthocyanins are known to be powerful antioxidants broadly allocated in different berry fruits and they are recognized as important dietary constituents which have health benefits (Lanson et al., 2007; Lu et al., 2011; Macedo et al., 2013). Moreover, their antioxidant capacity is higher than many important micronutrients like ascorbate and tocopherols. Anthocyanins are glycosides and acyl

glycosides of anthocyanidins as well. Not only they have low first oxidation potential, they appear to have many oxidation waves, and have become pro-oxidants by recox cycling with their low oxidation potential (Fukumoto and Mazza, 2000; Matty-Riihinen et al., 2004; Pardo et al., 2008; Proteggente et al., 2002).

Some of common anthocyanins have hydroxyl (methyl substitutions) in their flavylium or their basic structure. Anthocyanins which occurred naturally for more than 250 kinds can be distinguished by their o-glycosylation with distinct substitutes of sugar (Francis, 1989). On the aglycon (anthocyanidin), glucose, rhamnose, xylose, galactose, arabinose, and fructose are its most common substitution of sugar and either 3- or 3,5 glycosylated are the common anthocyanins. The strong antioxidant properties of anthocyanins occurred from its ring structures which attached by the group of phenolic hydroxyl that has free radical scavenging properties (Rice-Evans and Miller, 1998; Wang et al., 1997; Wolfe et al., 2008; Yadav et al, 2012).

Proanthocyanidins are the natural polyflavonoid. They have the chains of flavan-3-ol units and they are widely distributed among berry fruits (Macheix et al., 1990). Those proanthicyanidins have comparatively high molecular weights. They also have the ability to bind solidly with carbohydrates and proteins. Proanthocyanidins are relatively stronger than the powerful antioxidant vitamin E and have shown a wide scope of pharmacological activity (Kahkonen et al., 2001; Maatta-Riihinen et al., 2005; Sun et al., 2002).

Catechin is a powerful polyphenol antioxidant that is water-soluble and can be oxidized easily. More than thousand types can be found in the world of plant and they are considered to have an ability to deal with tumors and immune system enhancing function (Maatta-Riihinen et al., 2005). These phytochemicals shown their additive and synergistic effects on antioxidant activity when they are contained in the mixture conditions. Lately, researchers have found that a whole food have better health functionality than a single active compound which hinting a synergistic interaction of phenolic phytochemicals in diet (Liu, R, 2003; Lila and Raskin, 2005). Thus, the phenolic phytochemicals can help improves synergistic activity, enriching the functionality of whole foods.

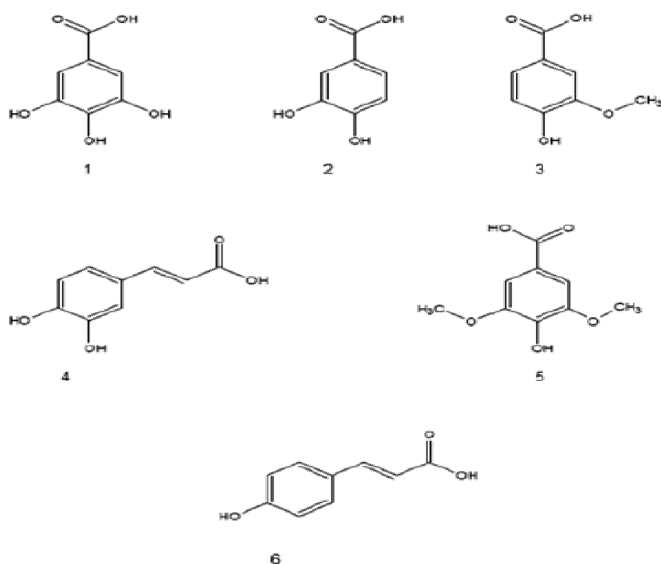


Figure 1: The structure of phenolic acids, (1) gallic acid; (2) protocatechuic acid; (3) vanillic acid; (4) caffeic acid; (5) syringic acid; and (6) p-coumaric acid.

Berries contain powerful antioxidants, potential allergens, and other bioactive compounds. Genetic and environmental factors affect production and storage of such compounds, where the anthocyanin (water-soluble plant pigments) have important functions in plant physiology as well in health effects (Wu et al., 2006). The high anthocyanin contents and biological activities of these fruits indicate that their consumption would be beneficial to health. It was revealed that the fruits contained superior levels of anthocyanins (146–2,199 mg/100 g fresh weight) to those previously reported for other raspberry and blackberry species, and their hexane, EtOAc, and MeOH extracts showed good antioxidant activity.

The majority of the extracts exhibited over 50 % lipid peroxidation inhibitory activity at 50 mg/mL. This may be useful in the production of functional foods containing an efficacious dose of anthocyanins (Bowen et al., 2010). The presence of predominantly phenolic compounds (ellagic and gallic acids, and corilagin) demonstrated varying degrees of antioxidative efficacy (Poltanov et al., 2009). Cranberries with high content of polyphenols have been associated with several cardiovascular health benefits (Basu et al., 2011). Borges et al. (2010) identified the content of bioactive compounds in different berries. A complex spectrum of anthocyanins was the major contributor to the total antioxidant capacities (TAC) of blueberries, whereas the lower TAC of cranberries was due mainly to reduced anthocyanin content. Vitamin C was responsible for 18–23 % of the TAC of cranberries and did not contribute to the TAC of the blueberry extract (Borges et al., 2010). Puente et al. (2011) studied the physicochemical and nutritional properties of the *Physalis peruviana* L. fruit and their relation of active components with beneficial effects on human health.

Oxidative stress and hypogonadism are linked to the increased incidence of cardiovascular disease (Deyhim et al., 2007). Recent studies *in vitro* and *in vivo* have improved the scientific understanding of how berries promote human health and prevent chronic illnesses such as some cancers, heart and neurodegenerative diseases (Prior et al., 2008; Seeram, 2010). Administration of a freeze-dried powder of mulberry (*Morus alba L.*) fruit to rats on a high-fat diet resulted in a significant decline in levels of serum and liver triglyceride, total cholesterol and serum low-density lipoprotein cholesterol, and a decrease in the atherogenic index (Yang et al., 2010). Oppositely, the serum high-density lipoprotein cholesterol was significantly increased (Yang et al., 2010). Berry phenolics may also act as antimicrobials which may be of help in the control of the wild spectra of pathogens, in view of recent problems associated with antibiotic resistance (Paredes et al., 2010).

The food industry has used cape gooseberry in different products including beverages, yogurts and jams, nutraceutical, and pharmaceutical industries (Hassanien, 2011; Ramadan, 2011). All the evaluated gooseberries extracts presented detectable amounts of phenolic, flavonoid, and tannin. Different extraction procedures reported in the literature and used to extract antioxidants in fruit were compared and analyzed (Arancibia et al., 2011; Chanda and Kaneria, 2012; Khoo et al., 2012).

The various health benefits of berries are well documented and have been attributed mainly to their antioxidant capacity. There is a growing public interest for cranberry, blueberry, and relatively new gooseberry as a functional food because of the potential health benefits linked to phytochemical compounds (Cote et al., 2010) responsible for secondary plant metabolites (flavonols, flavan-3-ols, proanthocyanidins, and phenolic acid derivatives). Several different mechanisms have been proposed to explain the possible role of cranberries, blueberries, and gooseberries in the prevention of atherosclerosis (Cote et al., 2010; Gua et al., 2007; Lin et al., 2010).

Fractions responsible for the antioxidant action were identified and seem promising for phytomedicinal development (Cao et al., 2013). In fact, 90-day and 48-h stability of the blackberry extract in biologically relevant buffers has been investigated in studies (Dai et al., 2009). Blackberry administration could minimize the toxic effects of fluoride, indicating its free radical scavenging and potent antioxidant activities. The induced oxidative stress and the alterations in antioxidant system were normalized by the oral administration of 1.6 g/kg body weight of blackberry juice (Hassan and Abdel, 2010).

Most of the used fruits have many cultivars (Fukuda et al., 2007; Toledo et al., 2008; Wall et al., 2008). It was shown that even cultivars grown in the same geographic and climatic conditions differ significantly (Ercisli et al., 2007; Toledo et al., 2008). So,Toledo et al. (2008) studied the bioactivity of durian cultivars such as Mon Thong, Chani, Kan Yao, Pung Manee and Kradum at the same stage of ripening from the same geographic region grown in the same climatic conditions in order to find the best among them for human consumption. It was concluded that among the studied durian cultivar Mon Thong is preferable (Haruenkit et al., 2010).

Plants are a source of compounds that may be used as pharmacologically active products. Cytisus multiflorus, Filipendula ulmaria and Sambucus nigra have been used as important medicinal plants in the Iberian Peninsula for many years and are claimed to have various health benefits as indigo plant (Barros et al., 2011). It was shown using chemical, biochemical, and electrochemical assays that these wild plants are source of phytochemicals and antioxidant potential (Barros et al., 2011). Also Dall'Acqua, Cervellati, Loi, and Innocenti (2008) examined the antioxidant capacities of 11 botanical species used in the tradition of Sardinia as tea beverages or as decoction for medicinal purposes.

Indigo (*Polygonum tinctorium Ait.*) is an herbaceous subtropical annual plant, belonging to the family Polygonaceae. Within the cells of its leaves, *P. tinctorium* accumulates large amounts of a colorless glycoside, indican (*indoxyl beta-D-glucoside*), from which the blue dye indigo is synthesized (Mantzouris et al., 2011; Selvius and Armitage, 2011). The composition of the natural dyes was determined after the extraction procedures with different solvent systems (Manhita et al., 2011). Indigo naturalis is used by traditional Chinese medicine to treat various inflammatory diseases (Lin et al., 2009).

Some wild indigo species as herbal drugs were evaluated (Barros and Teixeira, 2008). The data concerning the anticancer activity of indigo are very limited (Iwaki and Kurimoto, 2002). There are still few data on indigo plant; therefore, it is possible to compare it with other better investigated medicinal plants.

The anti-metastasis and immune stimulating activities of EtOH extracts of fermented Korean red ginseng (FRG-E) in animal and human subjects was investigated (Kim et al., 2012). The antioxidant properties of phenolic compounds from olive pulp of chamlal variety and those of individual phenolic compounds were evaluated and compared with that of vitamin C (Nadour et al., 2012). Generalic et al. (2011) studied the phenolic profile and antioxidant properties of Dalmatian sage. Another plant as Lithospermum erythrorhizon could be a promising rich source of natural antioxidants (Han et al., 2008).

Potential benefits of polyphenolic compounds from raspberry seeds of three different extracts as efficient antioxidants was studied (Gođevac et al., 2009). The influence of water content in the extraction system was evaluated. A 90-day stability study of the extract and a 48-h stability study of

the extract in biologically relevant buffers were completed (Dai et al., 2009).

The use of blackberry showed also its different properties: blackberry administration minimized the toxic effects of fluoride (Hassan and Abdel, 2010). Antioxidant capacity and phenolic compounds (phenolic acids and anthocyanins) of four berry fruits (strawberry, Saskatoon berry, raspberry, and wild blueberry), chokecherry, and seabuckthorn were compared (Li et al., 2009). Different fractions of mature wild blackberry Aristoteliachilensis (Mol) Stuntz (Elaeocarpaceae) were analyzed.

Cranberry was investigated as chemotherapeutic agent (Cuevas et al., 2010; Elberry et al., 2010). Some wild Jamaicagrown species and the Michigan-grown Rubusacuminatus, Rubusidaeus cv. Heritage, and Rubusidaeus cv. Golden were analyzed for their anthocyanin contents, and lipid peroxidation, cyclooxygenase enzyme, and human tumor cell proliferation inhibitory activities. The subject of different berries was investigated intensively, and it was shown in the cited literature, including the studies of Chilean berries (Céspedes et al., 2010). The effect of particle size, use of infrared radiation and type of freeze-drying (vacuum or atmospheric) on some nutritional properties of blueberries was investigated (Reyes et al., 2012).

Consumption of cranberries is known to exert positive health effects, especially against urinary tract infections. Cranberry was investigated as a chemotherapeutic agent (Elberry et al., 2010). For this reason, presumably, they are used in folk medicine (Wojnicz et al., 2012). Kusznierewicz et al. (2012) analyzed different Polish cultivars of blue-berried honeysuckles and wild and bog bilberry for bioactive compounds.

Some studies investigated and compared the composition, stability and antioxidant properties of berry extracts from selected cultivars using some extraction methods (Chanda and Kaneria, 2012; Khoo et al., 2012). *Physalis peruviana* (PP) commonly known as cape gooseberry, is an Andean Solanaceae fruit with high nutritional value and interesting medicinal properties. *Physalis peruviana* has been used in folk medicine for its medicinal properties including anticancer, treating malaria, asthma, hepatitis, dermatitis ,rheumatism, antimycobacterial, antipyretic, diuretic, immunomodulatory and anti-inflammatory properties (Franco et al., 2007; Helvaci et al., 2010; Martinez et al., 2010; Wu et al., 2005; Wu et al., 2009). Three species of Physalis fruit (*Physalis ixocarpa* Brot, *Physalis pruinosa* L. and *Physalis peruviana* L.) from Colombia, Egypt, Uganda and Madagascar were analyzed by multivariate analysis (El Sheikha et al., 2012).

Ugni molinae Turcz, also known as “Murtilla”, is a plant that grows in the south of Chile. Infusions of their leaves have long been used in traditional native herbal medicine (Rubilar et al., 2006; Suwalsky et al., 2006). The bioactivity of “Murta” (“Murtilla”) was investigated by Rufino et al. (2010). In our recent research, the methanol extracts from different berries were investigated and compared (Arancibia-Avila et al., 2011; 2012). Different aspects of berries phenolics activity were studied in individual papers (Ramadan, 2011; Hassanien, 2011; Chanda and Kaneria, 2012; Khoo et al., 2012; Xiao et al., 2011), but complex study in this matter is missing.

There are a number of investigations on the plants which were screened, however, the mechanisms behind the functions of berries, kiwi fruits and indigo plants with proteins are poorly understood. The interactions between polyphenols, especially flavonoids and plasma proteins, have attracted great interest among researchers. Few papers, however, have focused on the structure–affinity relationship of polyphenols on their affinities for plasma proteins (Cao et al., 2013; Xiao et al., 2011; Xiao et al., 2012; Zhang et al., 2009). It was interesting to compare different extraction

procedures in some fruits and plants (Chanda and Kaneria, 2012; Khoo et al., 2012). The water extracts of fruits are important from the point of tea consumption all year around, outside of the season of growing. The effect of particle size based on the reviewed reference (Reyes et al., 2012), solvent extraction and type of freeze-drying on some nutritional properties of the studied plants was investigated. Therefore it was interesting to investigate relatively new kind of Cape gooseberries (*P. peruviana*) in water, acetone, hexane, methanol, ethanol, ethyl acetate solvents, and to compare its composition with that of the widely consumed blueberries and cranberries; Chilean 'Murtilla' and 'Myrteola' berries, Chilean and Polish blueberries, Chilean raspberries and Polish black chokeberry. To meet this aim, the contents of bioactive compounds (polyphenols, flavonoids, flavanols, tannins, carotenoids, chlorophylls, anthocyanins, and ascorbic acid) and the level of antioxidant activity (AA) were determined and compared. In order to receive reliable data, AA was determined by five assays: 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), FRAP, cupric reducing antioxidant capacity (CUPRAC), 1,1-diphenyl-2picrylhydrazyl (DPPH), and β-carotene linoleate model system (Apak et al., 2004; Brand et al., 1995; Singh et al., 2002) (Benzie and Strain, 1996; Re et al., 1999), (Ozgen et al., 2006).

Widely consumed kiwi fruit has many cultivars (Ercisli et al., 2007). Which of them is preferable for human consumption? In order to answer this question it was decided to investigate seven kiwi fruit cultivars ('Hayward', 'Daheung', 'Haenam', 'Bidan', 'Hort16A', 'Hwamei' and 'SKK12') and to divide them to groups. The content of the bioactive compounds and the level of antioxidant capacity (AC) were determined and compared. The functional properties of new kiwi fruit cultivars were studied by the interaction of ethanol and water polyphenol extracts with a small protein such as HSA, using 3D-FL. The indigo plant was subjected to the same analyses as other investigated berries and fruits and compared with a mixture of other medicinal plants such as prolipid.

The functional properties of investigated plants were studied by the interaction of polyphenol extracts with a small protein such as HSA, using 3D-FL. Human serum albumin is the drug carrier's protein and serves to greatly amplify the capacity of plasma for transporting drugs. In order to compare the fluorescence properties of the extracted bioactive compounds, *in vitro* studies were performed by interaction of proteins with polyphenols. The investigation also *in vitro* how this protein interacts with polyphenols extracted from plant samples in order to get useful information of the properties of polyphenol–protein complex. To determine the fluorescence properties of the extracted bioactive compounds, *in vitro* studies were performed by interaction of proteins with flavonoids.

This research aimed to evaluate the bioactivity of relatively new plants in comparison with widely consumed ones. The advanced analytical methods such as 3D-FL and FTIR spectroscopy were used for characterization of the phytochemicals in the studied plants, extracted with different solvents, especially with water and ethanol, which can be applicable in every day human consumption and pharmaceutical use.

Materials and Methods

Reagents

Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid); 2,2^{0} -azobis-2-methyl-propanimidamide; 1,1-diphenyl-2-picrylhydrazyl (DPPH), $FeCl_3$ $6H_2O$; Folin–Ciocalteu reagent

(FCR); Tris, tris(hydroxymethy1)aminomethane; β-carotene, linoleic acid, quercetin, human serum albumin, lanthanum (III) chloride heptahydrate; $CuCl_2$ $2H_2O$; and 2,9-dimethyl-1,10-phenanthroline (neocuproine), potassium persulfate, quercetin, human serum albumin, were obtained from Sigma Chemical Co., St. Louis, MO, USA. 2, 4, 6-tripyridyl-s-triazine (TPTZ) was purchased from FlukaChemie, Buchs, Switzerland. All reagents were of analytical grade. Deionised and distilled water were used throughout.

Samples

Cape gooseberries (Physalis peruviana), blueberries (Vaccinium corymbosum), and cranberries (Vaccinium macrocarpon) were investigated. Arandano (blueberries) and raspberries were purchased at the local market in Chillan, Chile; and blueberries and chokeberries were purchased at the local market in Warsaw, Poland. For the investigation, five replicates of five berries each were used. Their edible parts were prepared manually without using steel knives. The prepared berries were weighed, chopped, and homogenized under liquid nitrogen in a high-speed blender (Hamilton Beach Silex professional model) for 1 min. A weighed portion (50–100 g) was then lyophilized for 48 h (Virtis model 10–324), and the dry weight was determined. The samples were ground to pass through a 0.5 mm sieve and stored at -20 °C until the bioactive substances were analyzed.

Chilean “Murtilla”, “Murta” (*U. molinae* Turcz) and “Myrteola” berries (Myrtaceae, Myrteola nummularia (Poiret) Berg.), Chilean and Polish blueberries (Vaccinium corymbosum), raspberries (R. idaeus), and black chokeberry (Aronia melanocarpa) were investigated. “Myrteola” nummularia (Poiret) Berg. Myrtaceae, (Daudapo) is distributed geographically from Valdivia to Magallanes. The fruit is edible. The fruits were harvested at their maturity stage and “Murtilla” and “Myrteola” berries were in two stages of ripening. “Myrteola” ripe was harvested in May 2008. “Myrteola” non-ripe was harvested in February 2010, in Chiloé. “Murtilla” non-ripe was collected in Puerto Varas, Chile, in February 2010

Kiwi fruits of seven cultivars were harvested at the optimal stage in orchard, located in Haenam county (longitude 126° 15^{00} and latitude 34° 18^{00}), Jeonnam province, Korea, 2012. All cultivars, except ‘Hort 16A’, were bred in Korea and classified as ‘Hort’. ‘Hort 16A’ is a New Zealand gold kiwi fruit and was purchased in 2012 from farmer, located in Jeju Island. ‘Hwaemi’ and ‘SKK-12’ are green kiwi fruit cultivars of 100 g size as ‘Hayward’. ‘Bidan’ has a smaller size of 20 g and its skin is white (flesh is green).

Three samples of indigo plant (*P. tinctorium*): seeds and two samples of leaves. The leaves with slight green color (immature leaves) were harvested on April 10, 2010, and the leaves with green brown color (mature leaves) were harvested on July 20, 2010, from the same place. Two samples of leaves were used because one was mature and the other— immature. The most important is to find exact data when the leaves can be harvested. The leaves were dried for 5 days under sunlight. The leaves were pulverized in the laboratory conditions. The particle size was 200 mesh. For comparison, prolipid was used (Jastrzębski et al., 2007). Prolipid is a mixture of the following plants: Sonchus 532 Z. arvensis L. from the Compositae (Asteraceae) family, Guazumaulmifolia L. from the Sterculiaceae family and Murrayapaniculata L. from the Rutaceae family. Prolipid contains extracts of G. ulmifolia (20 g/100 g dry weight (dw)), M. paniculata (10 g/100 g dw), and S. arvensis (10 g/100 g dw). Prolipid capsules were obtained as a gift from the drug importer COWIK (Warsaw, Poland).

Extraction of Polyphenols

The extracts from all samples prepared by the same way for all tests (bioactive compounds, antioxidant, and anticancer assays). The phenols were extracted with methanol and ethyl (concentration 25 mg/mL) at room temperature twice for 3 h (Haruenkit et al., 2010). The prolipid capsules were opened and the content was dissolved in the same solvents at the same conditions.

Total Phenolic Content

The polyphenols were determined by Folin–Ciocalteu method with measurement at 750 nm with a spectrophotometer (Hewlett-Packard, model 8452A, Rockville, USA). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram DW (Singleton et al., 1999).

Extraction of Phenolic Compounds for MS

All lyophilized samples (1 g) were extracted with 100 mL of methanol/water (1:1) at room temperature and in darkness for 24 h. The extracts were filtered in a Buchner funnel. After removal of the methanol in a rotary evaporator at a temperature below 40 °C, the aqueous solution was extracted with diethyl ether and ethyl acetate, and then, the remainder of the aqueous solution was freeze-dried. The organic fractions were dried and redissolved in methanol.These extracts were used for MS, for determination of bioactive compounds and Fourier transform infrared (FTIR) analyses (Sanz et al., 2010).

Total Flavonoid Content

Total flavonoid content was determined by an aluminum chloride colorimetric method (Bener et al., 2010; Liu et al., 2002). 0.25 mL of the sample extract was diluted with 1.25 mL of distilled water. Then 75 µL of a 5 % $NaNO_2$ solution was added to the mixture. After 6 min, 150 µL of a 10 % $AlCl_3 \cdot 6H_2O$ solution was added, and the mixture was allowed to stand for another 5 min. Half of a milliliter of 1 M NaOH was added, and the total was made up to 2.5 mL with distilled water. The solution was well mixed, and the absorbance was measured immediately against the prepared blank at 510 nm in comparison with the standards prepared similarly with known (+)-catechin concentrations. The results are expressed as milligrams of catechin equivalents.

Total Flavanols Content

The total flavanols amount was estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method, and then the absorbance at 640 nm was read. To ensure the presence of flavanols on the nuclei, subsequent staining with the DMACA reagent resulted in an intense blue coloration in plant extract (Feucht and Polster, 2001). As it was mentioned previously, (+)-catechin served as a standard for flavonoids and flavanols, and the results were expressed as catechin equivalents (CE).

Total Tannins Content

To 50 µL of methanol extract of the plant sample, 3 mL of a 4 % methanol vanillin solution and 1.5 mL of concentrated hydrochloric acid were added. The mixture was allowed to stand for 15 min. The absorption was measured at 500 nm against methanol as a blank. The amount of total condensed tannins is expressed as (+)-catechin equivalents per gram of the sample (Broadhurst and Jones, 1978).

Total chlorophyll

Chlorophylls a and b, and total carotenoids were extracted with 100 % acetone from plants samples and determined spectrophotometrically at different absorbances (nm) such as at 661.6, 644.8, and 470, respectively (Boyer, 1990).

Total ascorbic acid

Total ascorbic acid was determined by CUPRAC assay (Ozyurek *et al*. 2007) in water extract (100 mg of lyophilized sample and 5 ml of water). The absorbance of the formed bis (Nc)-copper (I) chelate was measured at 450 nm.

Total anthocyanins were measured by a pH differential method. Absorbance was measured in a Beckman spectrophotometer at 510 nm and at 700 nm in buffers at pH 1.0 and 4.5, using the following equation: A 0 $((A_{510}–A_{700})_{pH1.0}—(A_{510}–A_{700})_{pH4.5})$. Results were expressed as milligrams of cyanidin-3glucoside equivalent (CGE) per gram of DW (Cheng and Breen 1991).

MS analysis

MS Analysis A mass spectrometer, TSQ Quantum Access Max (Thermo Fisher Scientific, Basel, Switzerland), was used.

In order to compare the extracted phenolics in additional to the used solvents 50% methanol in water acidified with 1% formic acid; and 50% methanol in water were used. Different extractions were carried out in order to achieve the better phenols recovery using variable ratio of water and methanol, with and without formic acid in mass-spectra profiles (Fracassetti, Costa, Moulay, &Tomás-Barberán, 2013) (Fracassetti et al., 2013). These extracts were submitted to MS analysis for determination of bioactive compounds (Sanz et al., 2010)

Analytes were ionised by electrospray ionoization (ESI) in negative mode. Vaporizer temperature was kept at 100 °C. All samples were done by direct infusion in the mass spectrometer by use ESI source at negative ion mode, full scan analysis, range of 100–900 m/z. For optimisation of the acquisition parameters and for identity confirmation only a part of standards was employed, not for all compounds that were found in the investigated samples. Settings for the ion source were as follows: spray voltage 3000 V, sheath gas pressure 35 AU; ion sweep gas pressure 0 AU; auxiliary gas pressure at 30 AU; capillary temperature at 200 °C, skimmer offset 0 V (Gómez-Romero et al., 2011;

Mikulic-Petkovsek, Slatnar, Stampar, &Veberic, 2012).

The Total Antioxidant Capacity was determined by following assays:

(1) 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) method for the screening of antioxidant capacity is reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant capacity. ABTS radical cation was generated by the interaction of ABTS (7 mM/L) and $K_2S_2O_8$ (2.45 mM/L). This solution was diluted with methanol until the absorbance in the samples reached 0.7 at 734 nm (Re et al., 1999).

(2) Cupric reducing antioxidant capacity (CUPRAC): This assay is based on utilising the copper (II)-neocuproine (Cu (II)-Nc) reagent as the chromogenic oxidising agent. To the mixture of 1 ml of copper (II)-neocuproine and NH_4A_C buffer solution, acidified and non acidified methanol extracts of fruits (or standard) solution (x, in ml) and H_2O ((1.1-x) ml) were added to make the final volume of 4.1 ml. The absorbance at 450 nm was recorded against a reagent blank (Apak et al., 2004).

(3) Scavenging free radical potentials were tested in solution of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH). In its radical form, DPPH has an absorption band at 515 nm which disappears upon reduction by an antiradical compounds. DPPH solution (3.9 mL, 25 mg/L) in methanol was mixed with the samples extracts (0.1 mL), then the reaction progress was monitored at 515 nm until the absorbance was stable (Brand et al., 1995).

(4) Ferric-reducing/antioxidant power (FRAP) assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripiridyltriazine (Fe (III)-TPTZ) to a ferrous form ((Fe (II)). FRAP reagent (2.5 mL of a 10 mmol ferric-tripiridyltriazine solution in 40 mmolHCl plus 2.5 mL of 20 mmol $FeCl_3xH_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6) of 900 lL was mixed with 90 lL of distilled water and 30 lL of kiwi fruit extract samples as the appropriate reagent blank. The absorbance was measured at 595 nm (Benzie and Strain, 1996)

(5) β-Carotene linoleate model system: A mixture of β-carotene (0.2 mg), linoleic acid (200 mg), and Tween-40 (200 mg) was prepared. Chloroform was removed at 40 °C under vacuum. The resulting mixture was diluted with 10 mL of water. To this emulsion was added 40 mL of oxygenated water. Four-milliliter aliquots of the emulsion were added to 0.2 mL of berry extracts (50 and 100 ppm). The absorbance at 470 nm was measured. The AA of the extracts was evaluated in terms of bleaching of the β-carotene: $AA=100\ (1-(A_0-A_t)/(A_0^\circ-A_t^\circ))$, where A_0 and A_0° are the absorbance values measured at zero time of the incubation for test sample and control, respectively, and A_t and A_t° are the absorbance values measured in the test sample and control, respectively, after incubation for 180 min (Singh et al., 2002).

Fluorometry and Fourier Transform Infrared (FT-IR) Spectra Studies

Fluorometric Measurements

Fluorometric measurements were used for the evaluation of the antioxidant activity of plant extracts and their *in vitro* binding properties to human serum albumin. Two-dimensional (2DFL) and three-dimensional (3D-FL) fluorescence measurements for all berry extracts at a concentration of 0.01 mg/mL were recorded on a model FP-6500, Jascospectrofluorometer, serial N261332, Japan, equipped with 1.0 cm quartz cells and a thermostat bath. The 2D-FL was taken at emission wavelengths from 310 to 500 nm and at excitation of 295 nm (Arancibia et al., 2011, 2012).

The 3D-FL spectra were collected with subsequent scanning emission spectra from 250 to 500 nm at 1.0-nm increments by varying the excitation wavelength from 200 to 350 nm at 10nm increments (Gorinstein et al., 2009). Quercetin (QUE) was used as a standard. All solutions for protein interaction were prepared in 0.05 mol/l Tris-HCl buffer (pH 7.4), containing 0.1 mol/l NaCl. The final concentration of HSA was 2.0×10^{-6} mol/l. The HSA was mixed with quercetin in the proportion HSA/extract=1:1.

The scanning speed was set at 1,000 nm/min for all measurements. All measurements were performed with emission mode and with intensity up to 1,000 (Wulf et al. 2005; Xiao et al., 2011; Xiao and Kai, 2014; Zhang et al. 2009). All solutions for protein interaction were prepared in 0.05 mol/L Tris–HCl buffer (pH 7.4), containing 0.1 mol/l NaCl. The final concentration of BSA was 2.0×10^{-4} mol/L. All solutions were kept in dark at 0–4 °C. The BSA was mixed with catechin. The samples were mixed in the proportion of BSA: extract 0 1:1. Denaturation with 2.4 M and 4.8 M urea was carried out as well. The samples after the interaction with BSA were lyophilized and subjected to FTIR.

The presence of polyphenols in the investigated berries ,indigosamples and the interaction between polyphenols and bovine serum albumin was studied by Fourier transform infrared spectroscopy. A Nicolet iS 10 FT-IR Spectrometer (Thermo Scientific Instruments LLC, Madison, WI, USA), with the smart iTR™ ATR (attenuated total reflectance) accessory was used to record IR spectra (Sinelli et al. 2008).

Chemometrical Processing

Samples with different concentrations of plant extracts (1, 2.5, 5, 10, 15, 20, and 30 mg/mL) were analyzed by DPPH antioxidant activity assay (Ozgen et al. 2006) (120). In the kinetic studies two variables were used: the change in the concentration of the samples and the change in time of the reaction with scavenging radical: 1, 10, 30, 60, and 90 min. Basic chemometric characterization of the investigated samples according to their ability to reduce the DPPH was carried out by summary, descriptive (normal probability, box/whisker, and dot plots) statistics and multisample median testing using the statistical program Unistat® (London, UK).

MTT Assay

Anticancer activity of water extracts of the studied samples on human cancer cell lines (Calu-6 for human pulmonary carcinoma and SNU-601 for human gastric carcinoma) were measured using

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5diphenyltetrazolium bromide) assay. The cell lines were purchased from Korean Cell Line Bank (KCLB) for MTT assay. Cells were grown in RPMI-1640 medium at 37 °C under 5 % CO_2 in a humidified incubator. Cells were harvested, counted (3×10^4 cells/mL), and transferred into a 96 well plate, and incubated for 24 h prior to the addition of test compounds.

Serial dilutions of test samples were prepared by dissolving compounds in water followed by dilution with RPMI-1640 medium to give final concentration at 25, 50, 100, 200, 400, and 800 and 1,000 μg mL^{-1}. Stock solutions of samples were prepared for cell lines at 90 μL and samplesat 10 μL, and incubated for 72 h. MTT solution at 5 mg mL^{-1} was dissolved in 1 mL of phosphate buffer solution, and 10 μL of it was added to each of the 96 wells. The wells were wrapped with aluminum foil and incubated at 37 °C for 4 h. The solution in each well containing media, unbound MTT, and dead cells were removed by suction and 150 μLof DMSO was added to each well.

The plates were then shaken and optical density was recorded using a micro plate reader at 540 nm. Distilled water was used as positive control and DMSO as solvent control. Controls and samples were assayed in duplicate for each concentration and replicated three times for each cell line. The cytotoxicity was obtained by comparing the absorbance between the samples and the control (Heo et al. 2007).

Statistical Analyses

To verify the statistical significance, mean ± SD of five independent measurements were calculated. Data groups' distribution character was tested by Shapiro–Wilk normality test and the homogeneity of variance by Levene's F test, both at 0.95 confidence level. Multiple comparisons also known as post hoc tests to compare all possible pairs of means of a group of berries extracts were performed by Student–Newman–Keuls method based on the studentised data range. P-values of <0.05 were considered significant. Linear regressions were also calculated and Pearson correlation coefficients (R) were used.

RESULTS AND DISCUSSION

I. BERRIES

A. Antioxidant activities and bioactive components in some berries

Bioactive compounds

The amounts of bioactive compounds in all studied samples are summarized in Table 1 and Fig.1. As can be seen, the significant highest content (P 0.05) of bioactive compounds was in blueberries water extract. Gooseberries showed average results in water extracts for polyphenols, tannins, anthocyanins, and ascorbic acid (Table1, Fig.1).

Total antioxidant capacity (TAC)

The results of the determination of the level of TAC of all studied samples are shown in Table 1. As can be seen, the TAC (µm TE/g) by ABTS and FRAP assays forgooseberrieswas 15.53 ± 1.6 and 6.51 ± 0.7, respectively. The TAC of blueberries was higher than that of gooseberries and cranberries. A very good correlation was found between the TAC and the contents of total polyphenols (R^2 from 0.96 to 0.83) in water extracts. The correlation between the antioxidant capacity and ascorbic acid (Fig. 1) was lower than with polyphenols (R^2 from 0.84 to 0.50, Table 1).

Table 1 Bioactive compounds and antioxidant capacities in water, ethyl acetate, and diethyl ether extracts of gooseberries (*Physalis peruviana*), cranberries (*Vaccinium macrocarpon*), and blueberries (*Vaccinium corymbosum*)

Extracts	Indices			
	POLYPHEN, mg GAE	TANNINS, mg CE	ABTS, µM TE	FRAP, µM TE
Goberry, H_2O	5.37 ± 0.6^c	0.71 ± 0.2^c	15.53 ± 1.6^c	6.51 ± 0.7^c
Crberry, H_2O	22.13 ± 2.5^b	5.12 ± 0.7^b	72.76 ± 6.5^b	26.97 ± 2.7^b
Blberry, H_2O	46.56 ± 4.2^a	13.04 ± 1.3^a	199.41 ± 18.6^a	94.10 ± 9.3^a
Goberry, EtOAc	0.29 ± 0.1^e	0.31 ± 0.1^d	1.47 ± 0.3^e	0.42 ± 0.1^d
Crberry, EtOAc	3.14 ± 0.4^c	0.51 ± 0.1^c	13.50 ± 1.3^c	5.10 ± 0.6^c
Blberry, EtOAc	3.87 ± 0.4^c	0.62 ± 0.2^c	17.73 ± 1.8^c	7.53 ± 0.8^c
Goberry, DEE	0.14 ± 0.01^e	0.30 ± 0.1^d	0.88 ± 0.1^e	0.084 ± 0.01^e
Crberry, DEE	2.11 ± 0.2^d	0.32 ± 0.1^d	10.72 ± 1.8^d	3.28 ± 0.4^d
Blberry, DEE	4.13 ± 0.4^c	0.62 ± 0.3^c	20.42 ± 2.3^c	9.59 ± 0.9^c

Value are mean ± SD of 5 measurements, Values in columns for every bioactive compound bearing different superscript letters are significantly different ($P < 0.005$)
Per g dry weight

POLYPHEN polyphenols, *CE* catechin equivalent, *GAE* gallic acid equivalent, *ABTS* 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt, *FRAP* Ferric-reducing/antioxidant power, *Goberry* gooseberries (*Physalis peruviana*), *Crberry* cranberries (*Vaccinium macrocarpon*), *Blberry* blueberries (*Vaccinium corymbosum*), *EtOAc* ethyl acetate, *DEE* diethyl ether.

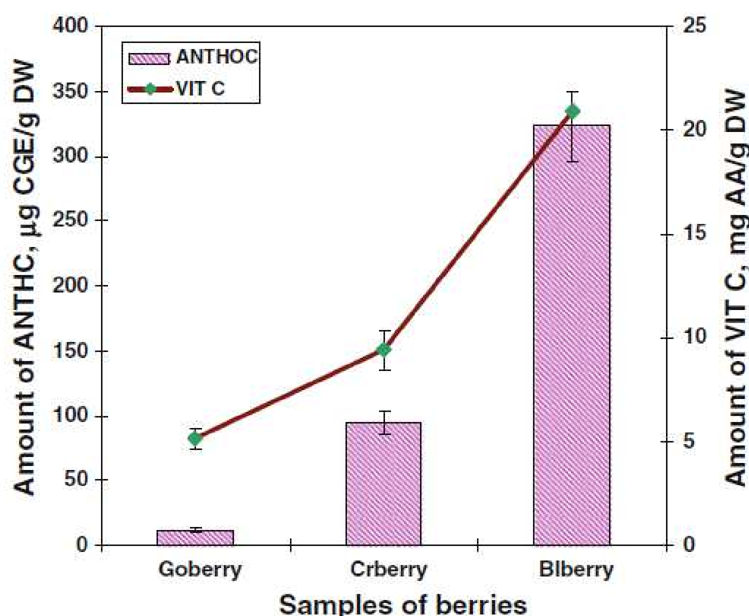


Fig. 2 Total anthocyanins (µg CGE/g DW) and vitamin C (mg AA/g DW) in gooseberries, blueberries and cranberries. *CGE* cyanidin-3-glucoside equivalent, *AA* ascorbic acid, *Goberry* gooseberries, *Crberry* cranberries, *Blberry* blueberries, *ANTHOC* anthocyanins, *VIT* C, vitamin C

Mass spectra data

The spectrum shows the main m/z peaks found (Fig. 2a, b, c) in water extract of berries with relative abundance (RA %) from 5 to 100 %. The main peaks were about 393, 381, and 290 for cranberries, gooseberries, and blueberries, respectively (Barroso et al., 2011; Mikulic-Petkovsek et al., 2012). Other peaks appeared for cranberry at 335 (87 %), for blueberries at 241 (70 %), for cranberry and blueberry were found at 104 with RA = 40 %, and RA=66%. Common peaks at 266 of 50% and 32% were in gooseberries and cranberries, respectively. The peaks of 116 (45 %), of 146 (25 %), and 219 (32 %) were estimated only in gooseberries. The RA of the obtained peaks corresponded with the amount of total polyphenols contents in these samples.

Fluorometry spectra studies and FTIR

Water extracts showed the highest antioxidant properties; therefore, only water extracts were subjected to binding studies with HSA. The scavenging properties of the berries samples in comparison with caffeic acid are shown in two-dimensional fluorescence spectra (2D–FL). One of the main peaks for HSA was found at λ *ex*/em of 220/360 nm. The second main peak appeared for these samples at λ *ex*/ em of 280/350 nm (Fig. 3b–d). The interaction of HSA and the water extracts of berries, HSA, water extracts and caffeic acid (Fig. 3b–d) showed slight change in the position of the main peak at the wavelength of 360 nm and the decrease in the relative fluorescence intensity (RFI).The following changes appeared when the water extracts of berries were added to HSA (initially the main peak at emission 360 nm and FI of 904.26 (Figs. 3a, b, and 4a, theupper line is HSA).

The addition of blueberry extracts and caffeic acid decreased the RFI of HSA (Fig. 3b, lines from the top to the bottom). The decrease in the RFI (%) was proportional to the concentration of the polyphenols and showed 23.3, 58.3, and 67.5 during interaction of 20, 100, and 200 μg/mL blueberry water extract with HSA. Oppositely, in the case of addition of caffeic acid, the decrease was of 29.0, 71.1, and 73 %, respectively. Cranberry extracts showed the following results (Fig. 3c): HSA with 20 μg/mL decreased the RFI on 12.5 % and CaA -23.1 %; with 100 μg/mL 28.5 % and CaA -42.4 %; and with 200 μg/mL of extract decreased the RFI on 35.9 % and with CaA on 47.7 %. Gooseberry extracts showed decrease in fluorescence (Fig. 3d): with 20 μg/mL on 3.1 % and addition of CaA -16.2 %; with 100 μg/mL on 10.8 % and 22.0 %, respectively; and with 200 μg/mL on 16.5 % and with CaA - 27.6 %. The decrease in the RFI of HSA with 200 μg/mL gooseberry, cranberry, and blueberry extracts was 16.5, 35.9, and 67.5 %, and when caffeic acid was added, the decrease was 27.6, 47.7, and 73 %, respectively (Fig 3b–d).

FTIR spectra of water extracts of gooseberries, blueberries, and cranberries (A), ethyl acetate extracts of blueberries, gooseberries, and cranberries (B), and diethyl ether extracts (C) of

gooseberries, blueberries, and cranberries are presented in Fig.4 (lines from the top to the bottom). The comparison between the berries, the extracts, and some standards in the range of common peaks is shown in Table 2 A, B, C. The best matching in the common range of the peaks was in water extracts of the berries samples in the range of 3,300–3,000 cm^{-1} (Table 2 A) of 75 % with hesperidin and 85 % with tannic acid. Caffeic acid showed the matching in the range of 2,500–2,000 cm^{-1} (Table 2 A) of 42 %. In ethyl acetate extract, similar matching in the range of 3,500–3,200 cm^{-1} of the peaks was found with tannic acid and quercetin (Table 2 B). In the range of 2,400–2,300 cm^{-1} (Table 2 B), gallic acid, fisetin, tannic, and caffeic acids showed about 70–78 % of common peaks.

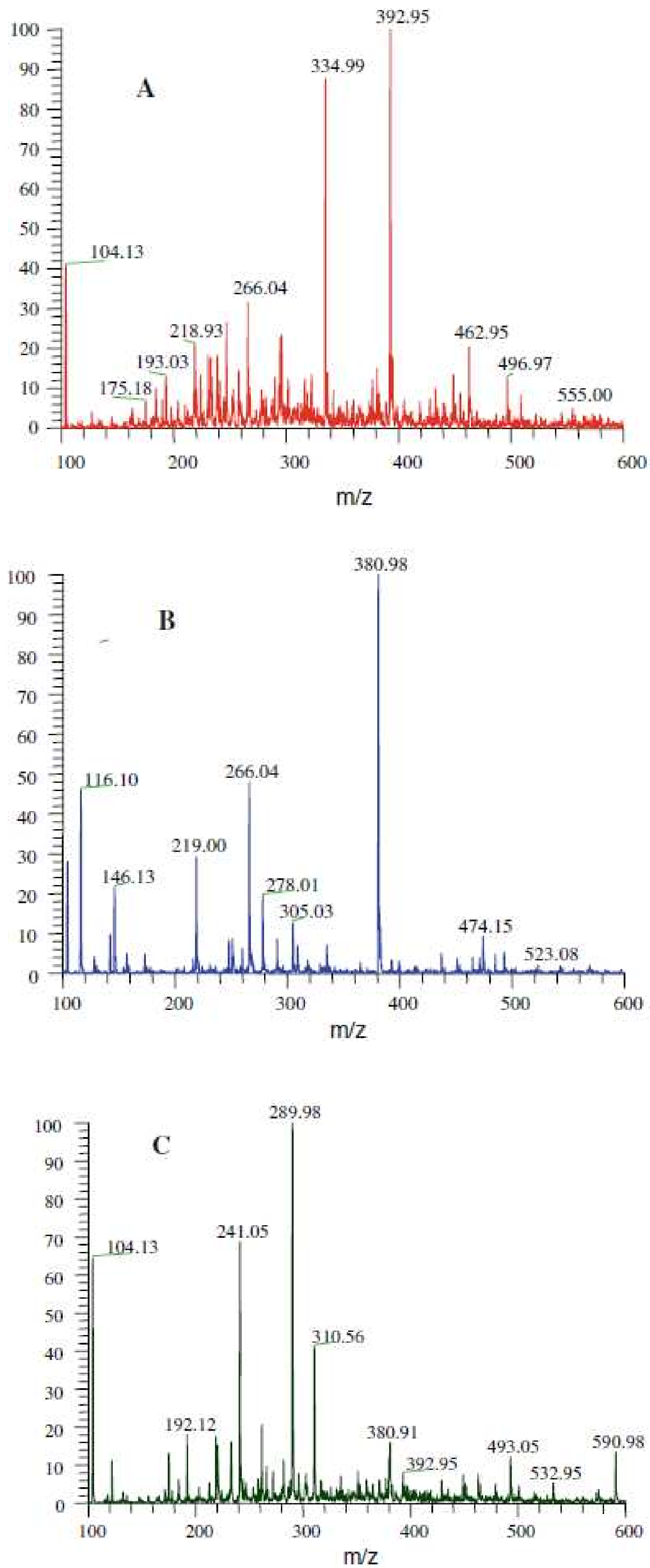


Fig. 3 ESI–MS spectra in positive ion mode of water fractions of the following berries: **a** cranberries; **b** gooseberries; **c** blueberries

Discussion

It was of great interest to compare gooseberries in order to find out if their bioactivity is on the same level as in other widely consumed berries and to use this kind of berries as a daily diet supplement. Therefore, the contents of the bioactive compounds and TAC were determined and compared with the widely consumed blueberries and cranberries.

As it was declared in Results, the contents of bioactive compounds (polyphenols, tannins, anthocyanins, and ascorbic acid) in three extracts were determined and compared. The significantly highest amounts of bioactive compounds were in water extract of all investigated berries, and the highest between the investigated berries was in blueberries. Also, the TAC according to ABTS and FRAP was significantly higher in water extract of blueberries.

Our results correspond also with the data of Wu et al. (Wu et al., 2006; Andersson et al., 2009), where concentrations of total anthocyanins varied considerably from 0.7 to 1,480 mg/ 100 g FW in gooseberry ('Careless' variety) and chokeberry, respectively. Total phenolic content and total anthocyanin content of four berry fruits (strawberry, Saskatoon berry, raspberry and wild blueberry), chokecherry, and seabuckthorn ranged from 22.83 to 131.88 g/kg and 3.51 to 13.13 g/kg, respectively, which corresponds with our results.

A number of reviewed articles showed that the main bioactive compounds determining the nutritional quality of berries are polyphenols, anthocyanins, and flavonoids (Bowen et al., 2006; Basu et al., 2011; Sinelli et al., 2008; Burdulis et al., 2009; Erkaya et al., 2012; Duen et al., 2011; Zhang et al., 2012; Valcheva et al., 2005). The high anthocyanin content and biological activities of these fruits indicate that their consumption would be beneficial to health. The berries may be useful in the production of functional foods containing an efficacious dose of anthocyanins (Bowen et al., 2010; Battino et al., 2009; Borges et al., 2010).

Our results were in accordance with the studies of Basu et al. (2011), based on the high amount of phenolics in cranberries. As it was mentioned above, Borges et al. (2010) showed that FRAP, vitamin C, and polyphenolic compounds have similar results, especially in the relationship between the anthocyanins which were the major contributor to the antioxidant capacity of blueberries, whereas the lower TAC of cranberries was due mainly to a reduced anthocyanin content. Vitamin C was responsible for 18–23 % of the TAC of cranberries, but did not contribute to the TAC of the blueberry extract. Our results on antioxidant capacity of gooseberry correspond with Puente et al. (2011) and Erkaya et al. (2012), who showed that addition of gooseberry in the concentration of 15 % to ice cream positively influenced the chemical, sensory, and mineral characteristics of the mixture.

The comparison of the results of different solvents in Dabai fruit parts (methanol, ethanol, ethyl acetate, acetone, and water) and total phenolics, total flavonoids, total anthocyanins, and antioxidant capacity (ABTS and FRAP assays) were in accordance with our data (Khoo et al., 2012). The acetone extract had maximum phenol and flavonoid content and showed best DPPH free radical scavenging activity and reducing capacity assessment. Ethyl acetate extract showed best superoxide radical scavenging activity, while aqueous extract showed best hydroxyl radical scavenging activity (Chanda and Kaneria, 2012). Our present results correspond with the previous ones where the amount of polyphenol compounds and their antioxidant capacities of Murtilla berries were significantly higher than in other studied berries and are comparable with blueberries (Arancibia-Avila et al., 2011).

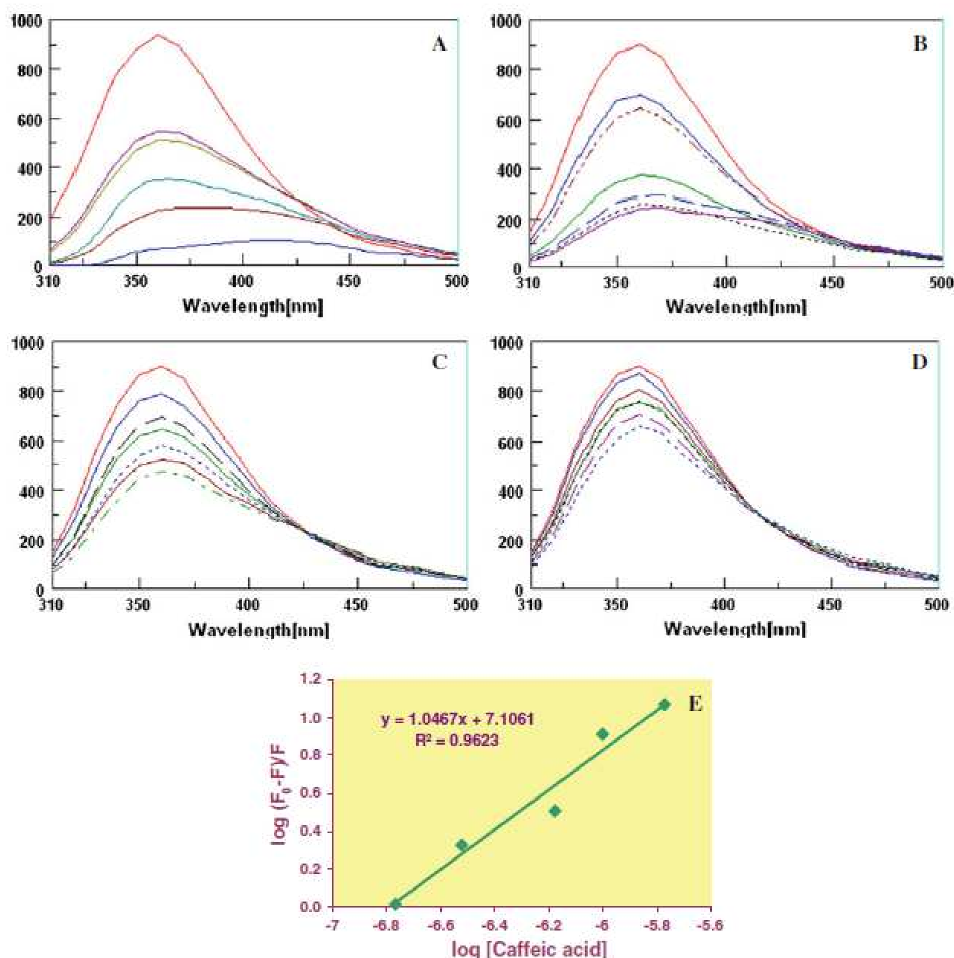


Fig. 4 Change in the relative fluorescence intensity (RFI) in two-dimensional fluorescence (2D-FL) spectra as a result of binding affinity of HSA, caffeic acid (CaA, 0.79 x 10^{-6} mol/L), and water extracts of berries: **a** fluorescence spectra of water extract of HSA (2.0 x 10^{-6} mol/L) in the presence of different concentrations of CaA 0, 0.17, 0.30, 0.67, 1.0, 1.7 x (10^{-6}) mol/L at pH 7.4 at excitation wavelength of 290 nm (lines from the top to the bottom with RFI of 947.37, 545.49, 509.67, 352.50, 237.02, 107.70) **b** Lines from the top to bottom with RFI of 904.26, 693.69, 640.57, 376.89, 294.08, 261.65, and 244.51 of HSA, HS and 20 µg/mL Blberry, HSA and 20 µg/mL Blberry and CaA, HAS and 100 µg/mL Blberry, HSA and 200 µg/mL Bilberry, HSA and 100 µg/mL Blberry and CaA, HSA and 200 µg/mL Blberry and CaA. **c** Lines from the top to bottom with RFI of 904.26, 791.65, 695.64, 646.56, 579.72, 520.84, and 472.82 for HSA; HSA and 20 µg/mL Crberry, HSA and 20 µg/mL Crberry, and CaA, HSA and 100 µg/mL Crberry, HSA and 200 µg/mL Crberry, HSA and 100 µg/mL Crberry and CaA, HSA and 200 µg/mL Crberry and CaA. **d** Lines from the top to the bottom with RFI of 904.26, 876.48, 806.60, 757.74, 755.29, 705.17, and 654.84 for HSA, HSA and 20 µg/mL Goberry, HSA and 100 µg/mL Goberry, HSA and 20 µg/mL Goberry and CaA, HSA and 200 µg/mL Goberry; HSA and 100 µg/mL Goberry and CaA, HSA and 200 µg/mL Goberry and CaA. **e** The linear plot for log (F_0-F)/F versus log [caffeic acid], where F_0, and F represent the fluorescence intensity of HSA in the absence and in the present of caffeic acid. Abbreviations: HSA, human serum albumin; Go, gooseberry, Crberry, cranberry and Blberry, blueberry.

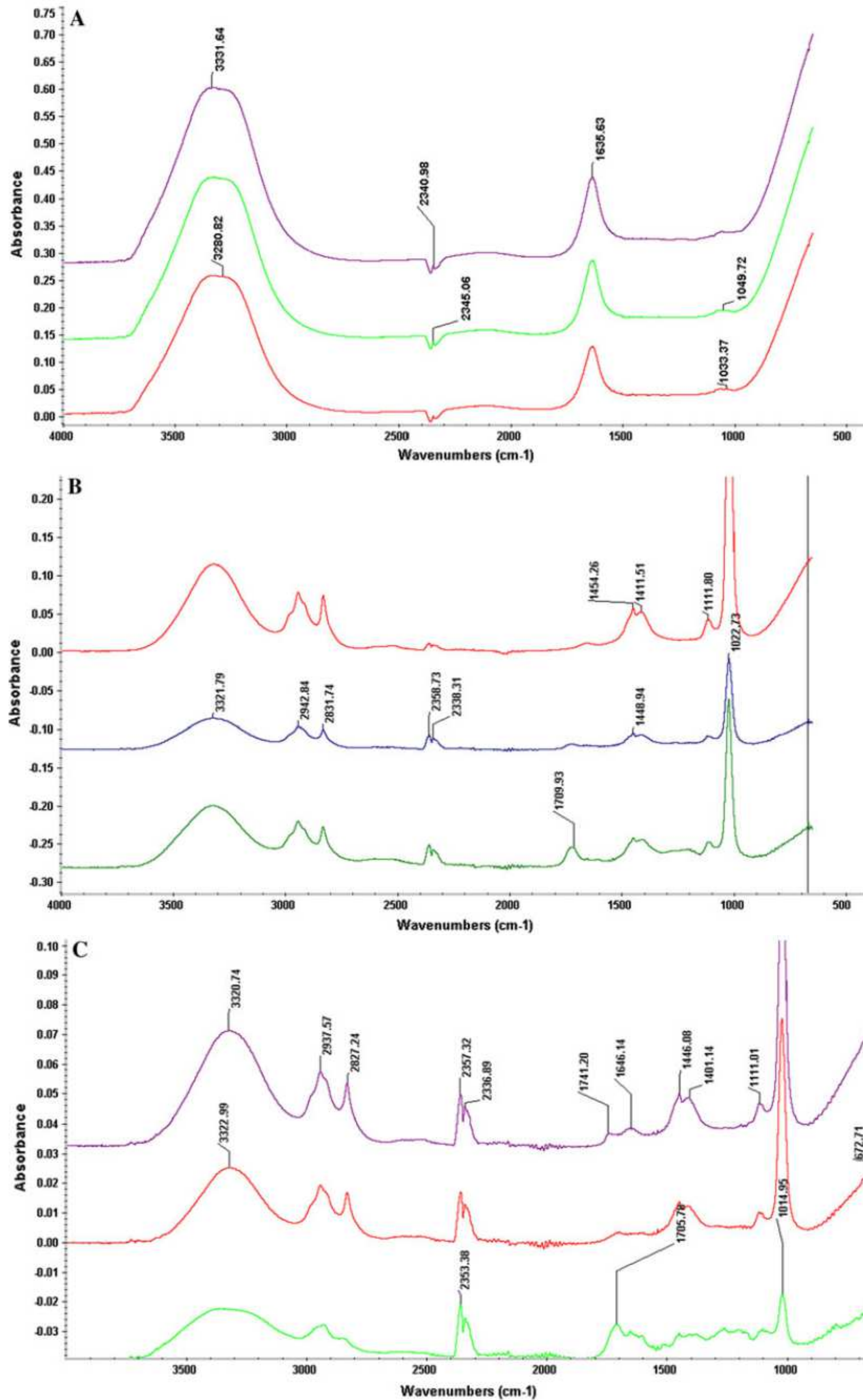


Fig. 5 FTIR spectra of: **a** water extracts of gooseberries, blueberries, and cranberries from the *top* to the *bottom*. **b** ethyl acetate extracts of blueberries, gooseberries, and cranberries from the *top* to the *bottom*. **c** diethyl ether extracts of gooseberries, blueberries, and cranberries from the *top* to the *bottom*

Table 2 Matching of the peaks (%) in the FTIR spectra of polyphenols and standards in water (A), ethyl acetate (B) and diethyl ether (C) extracts from

Range of bands	3,300–3,000 cm^{-1}			2,500–2,000 cm^{-1}			1,800–1,500 cm^{-1}			1,200–900 cm^{-1}		
Standards	Matching of standards/samples (%)											
	Gob	Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb
A												
Gallic acid	42	44	42	37	38	38	0	1	1	23	18	20
Ferulic acid	21	22	22	26	28	28	2	2	2	2	0	0
Fisetin	20	20	25	35	37	37	4	4	4	6	6	6
Hesperedin	75	75	75	5	7	7	29	29	29	19	15	15
Tannic acid	85	85	85	41	44	44	6	6	6	17	12	12
Caffeic acid	26	26	25	41	42	42	26	26	26	8	3	7
Quercetin	73	73	73	18	19	19	4	4	4	7	5	5
Range of bands	3,500–3,200 cm^{-1}			3,000-2,800 cm^{-1}			2,400-2,300 cm^{-1}			1,800-900 cm^{-1}		
Range of bands	3,500–3,200 cm^{-1}			3,000-2,800 cm^{-1}			2,400-2,300 cm^{-1}			1,800-900 cm^{-1}		
Standards	Matching of standards/samples (%)											
	Gob	Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb
B												
Gallic acid	48	51	46	8	9	11	75	73	72	45	46	43
Ferulic acid	22	19	22	15	15	16	57	55	57	15	13	19
Fisetin	17	26	16	10	11	15	71	70	70	17	13	8
Hesperedin	57	55	61	28	23	24	1	1	0	5	4	5
Tannic acid	79	77	78	13	13	15	77	77	78	51	48	57
Caffeic acid	18	15	17	36	39	39	77	75	76	12	16	10
Quercetin	73	72	71	0	6	9	36	34	36	4	6	9
Range of bands	3,300-3,100 cm^{-1}			3,000-2,800 cm^{-1}			2,500-2,200 cm^{-1}			1,800-600 cm^{-1}		
Standards	Matching of standards/samples (%)											
	Gob	Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb
C												
Gallic acid	50	47	48	12	10	26	61	59	61	30	29	22
Ferulic acid	15	9	31	15	16	4	38	37	37	6	37	9
Fisetin	25	25	22	9	9	4	51	49	51	3	2	3
Hesperedin	90	87	73	30	27	31	5	5	5	4	4	6
Tannic acid	85	78	75	16	14	27	67	66	67	41	42	32
Caffeic acid	8	3	21	36	37	9	58	58	58	2	3	9
Quercetin	69	65	59	3	3	4	29	28	29	3	1	12

Gob gooseberries, *Blb* blueberries, *Crb* cranberries

It was evaluated that the ability to inhibit LDL oxidation and total polyphenol content were consistent in classifying the antioxidant capacity of the polyphenolrich beverages in the following order: blueberry juice, black cherry juice, acai juice, cranberry juice, orange juice (Seeram et al., 2008). This order is in agreement with the polyphenol and antioxidant ability of blueberry and cranberry data in our investigation. Some studies contribute to the pharmacologic knowledge of Physalisperuviana regarding a remedy commonly used in Colombian traditional medicine (Pardo et al., 2008). Our results *in vitro* studies were compared with Faria et al. (2005) and Hurst et al. (2010), where anthocyanin-derived blueberry extracts were analyzed for the contents of polyphenols, flavonoids, anthocyanins, and anthocyanin-derived pigments. All of the extracts provided the protection of membranes against peroxyl radicals by increasing the induction time of oxidation (Faria et al., 2005; Flis et al., 2012, Gil et al., 1997).

This effect increased with the polyphenol content and with the structural complexity of the

anthocyanin-derived pigments of the extracts. Our results are in correspondence with Burdulis et al. (2009), where it was shown that the strongest antioxidant capacity possesses blueberry cultivar ‘‘Berkeley’’ (82.13 ± 0.51 %). Our results about the investigated berries like cranberries are in full correspondence with other reports that it is an excellent source of high-quality antioxidants and should be examined in human supplementation studies (Vinson et al., 2008).

Bog bilberry water extracts contained polyphenol, anthocyanin-rich (pigment), and sugar/acid fractions by using ethyl acetate, acidic methanol (MeOH) and 0.01 N HCl. The crude extract and fractions containing polyphenol and pigment exhibited the greatest antioxidantactivities with 50 % inhibitory concentration IC(50) values of 85.8, 33.2, and 16.7 µg/mL, respectively, for the DPPH assay, and 48.1, 83.8, and 51.9 µg/mL for the nonenzymatic superoxide radical assay.

In our case, the highest antioxidant capacity was shown in water; therefore, for binding properties were used only these extracts (Kim et al., 2009). The amount of total phenolics, anthocyanins, and ascorbic acid varied in berries depending on their maturity and varieties. Our results were in agreement with others (Viskelis et al., 2009), who showed the variation in their composition. The phenolic compounds were about 504 mg/100 g. The biggest quantities of ascorbic acid were found in the ripe berries of ‘‘Ben Lear’’ cultivar (15.8 mg/ 100 g).

Based on the data reported by Wolfe and Liu (2007), quercetin had the highest cellular antioxidant activity (CAA) value, followed by kaempferol, epigallocatechin gallate (EGCG), myricetin, and luteolin among the pure compounds tested. Among the selected fruits tested, blueberry had the highest CAA value, followed by cranberry > apple = red grape > green grape. Our results are similar to Kusznierewicz et al. (2012), where the antioxidant activities of different blue-berried honeysuckle cultivars were similar to that of wild-growing bilberries (ranging from 170 to 417 µmol TE/g DW in ABTS and from 93 to 166 µmol TE/g DW in DPPH and Folin-Ciocalteu tests).

The major anthocyanin in the blue-berried honeysuckle was cyanidin-3-glucoside, which constituted 84–92 % of the total anthocyanins. Our data can be comparable with another report (Cuevas et al., 2010), where the proanthocyanidins (condensed tannins) were present in the blackberry fruits. The average anthocyanin concentration was 49.2 mg/g in the commercial cultivar ‘Tupy’ while in the wild genotypes and the breeding line, the range was 361.3–494.9 mg/g (cyanidin 3-O-glucoside equivalent). The proanthocyanidin concentration varied widely among wild genotypes (417.5–1,343.6 mg/g CE). Comparison of different fractions of water extracts from wild blackberry Aristoteliachilensis (Mol) Stuntz (Elaeocarpaceae) corresponded with our results. Also, other authors reported similar results (Li et al., 2009). Total phenolics, flavonoids, and anthocyanins (mg/g FW) were in blueberry 261–585, 50, 25–495; raspberry 121, 6, 99; antioxidant activity (µmolTrolox/g FW for blueberry 14 by ABTS and 25.3 by DPPH) (Li et al., 2009). The result from this study indicated that blueberries had very high ORAC values and higher antioxidant capacity than other selected fruits and vegetables.

In the present report, the best binding ability to the HSA was with water extracts of berries. It is interesting that in Faria et al. (2005), the antiradical properties and the reducing power of the extracts by using DPPH and FRAP methods, respectively, were in agreement with those obtained with the liposome membranes. This is in accordance with our present data that the binding properties and the antioxidant capacities are in correlation. A blueberry fruit extract displayed a potent and significant dose-dependent protectivecapacity as it was shown in fluorescence studies with References binding with HSA (Hurst et al., 2010).

The obtained results by fluorescence are in direct relationship with the antioxidant properties of the berries extracts. The synergism of bioactive compounds is shown when to the mixture of HSA and berries extracts caffeic acid was added. Our very recent results showed that the fluorescence is significantly quenched, because of the conformation of the HSA changes in the presence of phenolic acids and berries extracts. This interaction between phenolic acids and HSA was investigated using tryptophan fluorescence scavenging. Other results (Xiao et al., 2011; Zhang et al., 2008) differ from the reported by us, probably because of the variety of antioxidant abilities of pure phenolic acids and different ranges of fluorometry scanning used in a similar study.

In vitro results of interaction of HSA and caffeic acid shown in the present study can be compared with other reports (Zhang et al., 2008). The displacement experiments confirmed that caffeic acid could bind to the site I of HSA, which was in agreement with the result of the molecular modeling study (Zhang et al., 2008). There are not too many applications of 3D fluorescence spectra; therefore, our present conclusions that 3-D fluorescence can be used as an additional tool for the characterization of the extracts of berries cultivars correspond with the previous data (Gorinstein et al., 2010). The matching results of common peaks for the first time showed that FTIR spectra can be used for a rapid estimation of extracted bioactive compounds. Quercetin and hesperidin exhibited the highest matching of the peaks in the investigated berries extracts in comparison with fisetin, caffeic, and gallic acids.

In our previous study, the FTIR spectra data showed that the main bands in the berries samples slightly shifted (Gorinstein et al., 2010). A shift in the difference between the standards and the investigated samples can be explained by the extraction procedures of the main polyphenols.

B. Comparative assessment of two extraction procedures for determination of bioactive compounds in some berries used for daily food consumption

Results

Bioactive compounds

The results of the determination of the contents of the bioactive compounds in all studied samples are summarised in Table 1. Water and methanol extracts of gooseberries showed lower amounts of polyphenols, flavonoids, flavanols and tannins (6.24–3.77 mg GAE g^{-1}; 0.29–0.45 mg CE g^{-1}; 6–8 lg CE g^{-1}; and 1.01–1.24 mg CE g^{-1}, respectively, Table 1) than blueberries and cranberries.

Antioxidant activity

As can be seen from Table 2, the AA (µm TE g^{-1}) for gooseberries by DPPH, FRAP, ABTS and CUPRAC assays was 6.05–4.61; 8.07–7.61; 18.70–19.13; and 13.44–12.71, respectively. The antioxidant activity of blueberries was higher than that of gooseberries and cranberries. As was calculated, a very good correlation was found between the antioxidant activity and the contents of total polyphenols in water and methanol extracts. The correlation between the antioxidant activity and polyphenols was between 0.87 and 0.78.

Fluorometry spectra studies and FTIR spectra

The quenching properties of the berry samples are shown in two-dimensional fluorescence spectra (2D FL) and also their comparison with quercetin (Q). One of the main peaks for HSA was found at μ ex/em of 220/360 nm. The second main peak appeared forthese samples at μ ex/em of 280/350 nm (Fig. 1). Water phenolic extracts showed slightly higher antioxidant properties than the methanol ones, but the differences were not always significant in all extracts. The interaction between HSA and the water extracts (WE) of berries, HSA, WE and Q (Fig. 1a), showed slight change in the position of the main peak at the wavelength of 360 nm and the decrease in the relative fluorescence intensity (RFI).

The following changes appeared when the water extracts of berries were added to HSA (initially the main peak was at emission of 360 nm and FI of 890.21 (Fig. 1a, the upper line is HSA). The reaction of blueberry water extracts (BLUEBWE) with HSA (second line from the top) and with BLUEBWE, HSA and Q (fifth line from the top) decreased the RFI of HSA by 28.1% and 41.7%, respectively. The reaction of cranberry water extracts (CRANBWE) with HSA (third line from the top) and with CRANWE, HSA and Q (sixth line from the top) decreased the RFI of HSA by 13% and 29.9%, respectively. The reaction of gooseberry water extracts (GOOSEBWE) with HSA (fourth line from the top) and with GOOSEBWE, HSA and Q (seventh line from the top) decreased the RFI of HSA by 3.9% and 27.8%, respectively. These results showed that the binding properties of gooseberries were 7.2 and 1.5 times and 3.3 and 1.1 times lower than that of blueberries and cranberries, respectively.

The following changes appeared when the methanol extracts of berries were added to HSA (initially the main peak was at emission of 360 nm and FI of 890.21, Fig. 1b, the upper line is HSA). The reaction of blueberry methanol extracts (BLUEBMeOHE) with HSA (second line from the top) and with BLUEBMeOHE, HSA and Q (fifth line from the top) decreased the RFI of HSA by 13.9% and 31.3%, respectively. The reaction of cranberry methanol extracts (CRANBMeOHE) with HSA (fourth line from the top) and with CRANBMeOHE, HAS and Q (seventh line from the top) decreased the RFI of HSA by 8.0% and 24.0%, respectively. The reaction of gooseberry methanol extracts (GOOSEBMeOHE) with HSA (third line from the top) and with GOOSEBMeOHE, HSA and Q (sixth line from the top) decreased the RFI of HSA by 1.3% and 18.9%, respectively. The lowest decrease was with GOOSEBMeOHE without quercetin, but the synergism of quercetin with cranberries and gooseberries showed similar results. The water extracts showed higher binding properties to berries than the methanol, and the difference was significant in all berries.

Table 3 Bioactive compounds in water (H_2O) and methanol (MeOH) polyphenol extracts of gooseberries (GOOSEB, *Physalis peruviana*), cranberries (CRAN, *Vaccinium macrocarpon*) and blueberries (BLUEB, *Vaccinium corymbosum*)*,†,‡

	Indices, g^{-1} DW			
Extracts of berries	POLYPHEN, mg GAE	FLAVON, mg CE	FLAVAN, μg CE	TANNINS, mg CE
GOOSEB, H_2O	6.24 ± 0.6^c	0.29 ± 0.01^c	6 ± 0.8^d	1.01 ± 0.2^c
CRAN, H_2O	15.32 ± 2.5^b	3.06 ± 0.4^b	249 ± 14.5^c	2.30 ± 0.7^c
BLUEB, H_2O	57.47 ± 4.2^a	6.68 ± 0.6^a	1762 ± 25.6^b	5.00 ± 0.6^b
GOOSEB, MeOH	3.77 ± 0.1^c	0.45 ± 0.01^c	8 ± 1.1^d	1.24 ± 0.1^c
CRAN, MeOH	20.25 ± 0.4^b	2.20 ± 0.1^b	393 ± 20.3^c	1.76 ± 0.1^c
BLUEB, MeOH	57.96 ± 0.4^a	6.68 ± 0.7^a	3210 ± 40.4^a	24.80 ± 2.5^a

POLYPHEN, polyphenols; CE, catechin equivalent; GAE, gallic acid equivalent; FLAVON, flavonoids; FLAVAN, flavanols; nd, not determined; DPPH, 2,2-diphenyl-1-picrylhydrazyl; CUPRAC, cupric reducing antioxidant capacity; ABTS, 2, 2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; FRAP, ferric reducing antioxidant power.
*Values are means ± SD of five measurements.
†Values in columns for every bioactive compound bearing different superscript letters are significantly different ($P < 0.05$).
‡Per gram dry weight.

Table 4 Antioxidant activities in water (H_2O) and methanol (MeOH) extracts of gooseberries (GOOSEB, *Physalis peruviana*), cranberries (CRAN, *Vaccinium macrocarpon*) and blueberries (BLUEB, *Vaccinium corymbosum*)*,†,‡

	Indices, μM TE g^{-1} DW			
Extracts of berries	DPPH	FRAP	ABTS	CUPRAC
GOOSEB, H_2O	6.05 ± 0.6^e	8.07 ± 0.9^c	18.70 ± 1.8^d	13.44 ± 1.2^c
CRAN, H_2O	44.23 ± 4.5^c	22.45 ± 2.4^b	64.83 ± 6.5^c	28.45 ± 2.7^b
BLUEB, H_2O	75.09 ± 6.2^b	177.25 ± 14.6^a	254.83 ± 25.6^b	250.95 ± 18.6^a
GOOSEB, MeOH	4.61 ± 0.4^e	7.61 ± 0.9^c	19.13 ± 2.1^d	12.71 ± 1.1^c
CRAN, MeOH	23.25 ± 2.4^d	26.11 ± 2.1^b	68.40 ± 6.3^c	32.67 ± 3.1^b
BLUEB, MeOH	142.03 ± 11.4^a	149.00 ± 11.7^a	265.92 ± 25.4^a	265.76 ± 20.5^a

POLYPHEN, polyphenols; CE, catechin equivalent; GAE, gallic acid equivalent; FLAVON, flavonoids; FLAVAN, flavanols; nd, not determined; DPPH, 2,2-diphenyl-1-picrylhydrazyl; CUPRAC, cupric reducing antioxidant capacity; ABTS, 2, 2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; FRAP, ferric reducing antioxidant power.

*Values are means ± SD of five measurements.

†Values in columns for every bioactive compound bearing different superscript letters are significantly different ($P < 0.05$).

‡Per gram dry weight.

Our most recent results showed that the fluorescence is significantly quenched, because of the conformation of the HSA changes in the presence of quercetin and berry extracts. This interaction between quercetin and HSA was investigated using tryptophan fluorescence quenching. Other results (Xiao *et al*., 2011a,b; Zhang *et al*., 2009) differ from that reported by us, probably because of the variety of antioxidant abilities of pure flavonoids and different ranges of fluorometry scanning used in a similar study. Our *in vitro* results of interaction between HSA and quercetin can be compared with other reports (Zhang *et al*., 2009).

There are not too many applications of 3D fluorescence spectra; therefore, our present conclusions – that 3D fluorescence can be used as an additional tool for the characterisation of the polyphenol extracts of berries cultivars – correspond with the previous data (Gorinstein et al., 2009; 2010; 2013) and can be applied to any food analysis.

FTIR spectra of water (a) and methanol (b) extracts of gooseberries, blueberries and cranberries are presented in Fig. 2 (lines from the bottom to the top). The FTIR wave numbers in polyphenol water extracts showed a broad band at 3273 cm^{-1} for gooseberries and blueberries, but for cranberries, there was a shift to 3332 cm^{-1} (phenolic OH band). Other bands were detected at 2342, 2349 and 2345 cm^{-1} for gooseberry, blueberry and cranberry, respectively. At 1642 cm^{-1} (C=O stretching phenyl ring amino acid-1), this band was detected for gooseberry and blueberry and at 1636 cm^{-1} only for cranberry (Fig. 2a). The methanol polyphenol extracts (Fig. 2b) showed similar bands at 3313, 2943 and 2834 cm^{-1} for three berries. At 1652 cm^{-1} (characteristic CO stretching), bands appeared for gooseberry and blueberry and at 1715 cm^{-1} for cranberry. In the range of 1445 cm^{-1}, a band was found for gooseberry. At 1410 cm^{-1}, a band was found for blueberry and at 1391 cm^{-1} (–OH phenolic bending) for cranberry. The common bands at 1115 cm^{-1} (aromatic bending and stretching) and at821 cm^{-1} were estimated for all berries.

The comparison between the berries, their extracts and some standards in the range of common peaks is shown in Tables 3–4. The best matching in the common range of the peaks was in water extracts of the berries between 3200 and 3000 cm^{-1} (Table 3) of 87% with tannic acid, 78% with hesperidin and 64% with gallic acid. Caffeic and tannic acids showed the matching in the range of 2500–2000 cm^{-1} (Table 3) of 40%. In phenolic extracts with methanol, similar matching of the peaks was found in comparison with tannic acid (84%) and hesperidin (70%). Quercetin in the range of 3500–3100 cm^{-1} (Table 4) showed similarity with the same bands of 70%, which was three times higher than that in water phenolic extract. In the range of 3000–1600 cm^{-1} (Table 4), caffeic, gallic,

tannic and ferulic acids showed matching with the investigated berries from 30 to 12%.

These matching results for the first time show that FTIR spectra can be used for the rapid estimation of extracted bioactive compounds. Quercetin exhibited the highest matching in the investigated fruit extracts in comparison with fisetin, and caffeic and gallic acids in methanol extracts of investigated berries. Difference between the standards and the investigated samples can be explained by the extraction procedures of the main polyphenols.

Discussion

Our results, connected with the bioactive compounds and AAs, are in correspondence with others, showing that water extracts of blackberries contain high amounts of bioactive compounds (Dai et al., 2009). Our results correspond also with the research of Wu et al. (2006), where concentrations of total anthocyanins varied considerably from 0.7 to 1480 mg per 100 g FW in gooseberry ('Careless' variety) and chokeberry, respectively. DPPH radical scavenging activity of currant varied from 12.67 to 31.18 mmol TE kg^{-1} (Wojdyo et al., 2013), and it was similar to the results obtained in this research.

Total phenolic content of four berry fruits (strawberry, saskatoon berry, raspberry and wild blueberry), chokecherry and seabuckthorn ranged from 22.83 to 131.88 g kg^{-1}, which corresponds with our results as well. Conclusions made in the report of Elberry et al. (2010) are in line with our results about the high antioxidant activity of berries.

Our results are in accordance with You et al. (2011), where four rabbiteye blueberry cultivars (Powderblue, Climax, Tifblue and Woodward) grown organically and conventionally were compared regarding their chemical profiles and antioxidant capacity in terms of total phenolic contentand antioxidant values determined by ABTS, DPPH, FRAP and CUPRAC assays. The comparison of the results of different solvents in dabai fruit parts (methanol, ethanol, ethyl acetate, acetone and water) ($ABTS^+$ and FRAP assays) were in accordance with our data (Khoo et al., 2012).

The acetone extract had maximum phenol and flavonoid content and showed the best DPPH free radical scavenging activity and reducing capacity assessment. Ethyl acetate extract showed best superoxide radical scavenging activity, while aqueous extract showed best hydroxyl radical scavenging activity (Chanda & Kaneria, 2012). Rop et al. (2012) showed that gooseberry (*Physalis peruviana*) fruit is one of the less used raw materials of plant origin, which can be used for human nutrition and can be promoted as a food additive in fresh and processed food, as an extract from fresh or frozen fruits. The methanolic extracts of three cultivars expressed high antioxidant activity and correlated with the amount of polyphenols.

We have investigated the binding properties of quercetin in aqueous and methanol media, using UV/vis and fluorometry, which is one of themajor phenolic compounds found in berries. Our results were in accordance with Guo et al. (2007), who demonstrated that quercetin and other phenolic compounds can effectively modulate iron biochemistry under physiologically relevant conditions, providing insight into the mechanism of action of bioactive phenolics (Guo et al., 2007). Our results are in agreement with Xiao et al. (2011) as well that dietary flavonoids are important polyphenols in berries as they are of great interest for their bioactivities, which are related to the antioxidative-

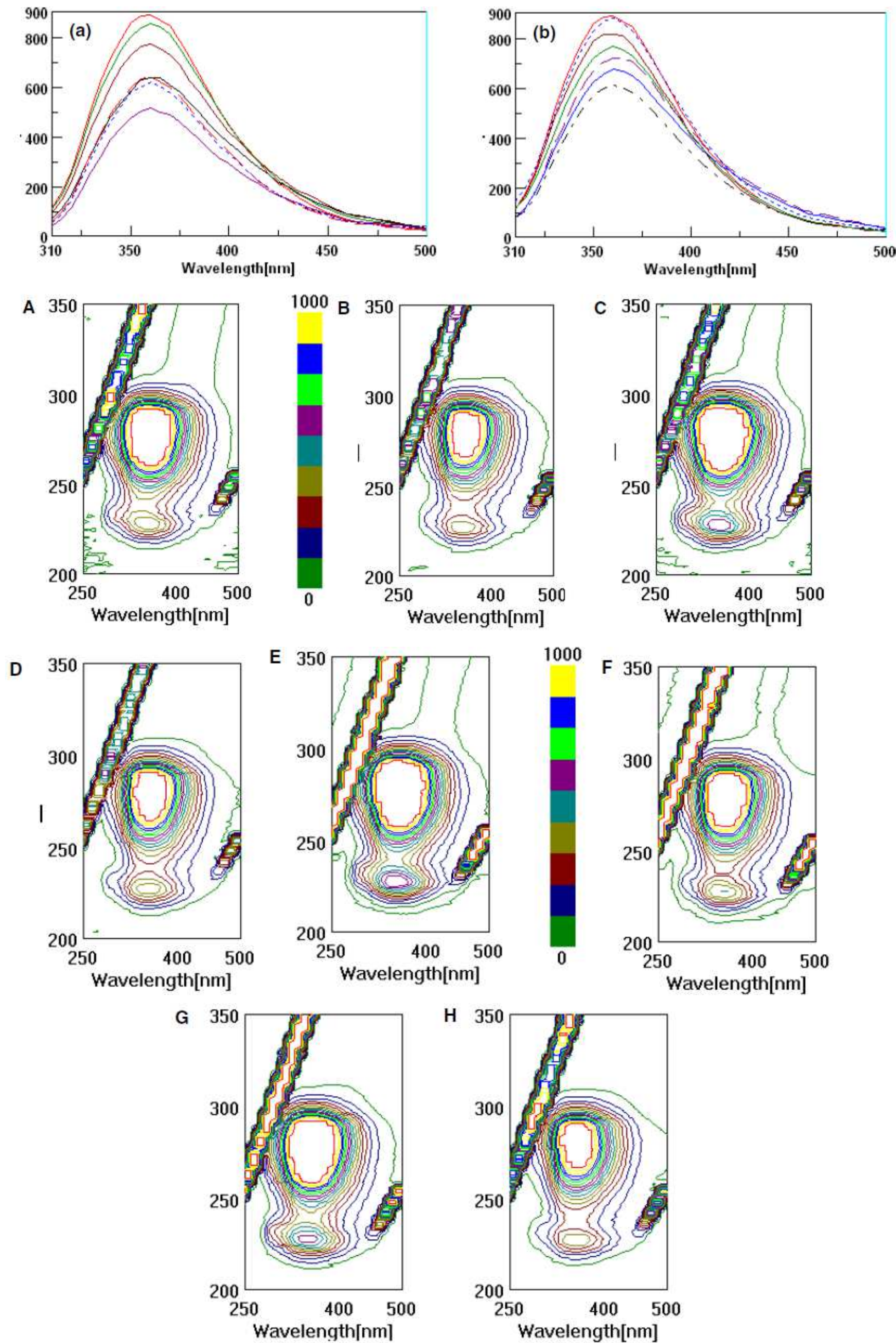


Figure 6 Change in the relative fluorescence intensity (RFI) in two-dimensional fluorescence (2D FL) spectra as a result of binding affinity of HSA, quercetin (Q, 0.79×10^{-6} M), water and methanol extracts of berries: (a) fluorescence spectra of HSA (2.0×10^{-6} M), HSA and water extracts (WE) of blueberries (BLUEB), WE of cranberries (CRAN), WE of gooseberries (GOOSEB), HSA and WE BLUEB and quercetin (Q), HSA and WE CRAN and Q; HSA and WEGOOSEB and Q (lines from the top to the bottom with RFI of 890.21, 640.45, 774.65, 855.14, 518.74, 623.66 and 642.97). (b) fluorescence spectra of HSA (2.0×10^{-6} M), HSA and methanol extracts (MeOHE) of BLUEB; MeOHE of CRAN; MeOHE of GOOSEB; HSA and MeOHE BLUEB and Q, HSA and MeOHE CRAN and Q; HSA and MeOHE GOOSEB and Q (lines from the top to the bottom with RFI of 890.21, 767.52, 878.87, 818.87, 611.73, 676.80 and 722.15). A, B, C, D, E, F, G, H, three dimensional fluorescence spectra of WECRAN and HSA, WECRAN and HSA and Q; WEGOOSEB and HSA; WEGOOSEB and HSA and Q; MeOHECRAN and HSA, MeOHECRAN and HSA and Q; MeOHEGOOSEB and HSA; MeOHEGOOSEB and HSA and Q.

Table 5 Matching of the peaks (%) in the FTIR spectrum of extracted polyphenols in water from gooseberries (GB, *Physalis peruviana*), cranberries (CB, *Vaccinium macrocarpon*) and blueberries (BB, *Vaccinium corymbosum*) and standards

Range of bands	3200–3000 cm^{-1}			2500–2000 cm^{-1}			1800–1500 cm^{-1}		
	Matching of standards/samples (%)								
Standards	GB	BB	CB	GB	BB	CB	GB	BB	CB
Gallic acid	64	64	64	37	33	36	0	1	0
Ferulic acid	23	23	23	26	19	25	2	3	3
Fisetin	20	19	16	35	34	35	3	2	3
Hesperidin	78	77	78	5	5	5	28	28	28
Tannic acid	87	87	87	41	40	40	6	6	6
Caffeic acid	52	48	53	40	35	40	26	26	26
Quercetin	23	22	22	26	26	26	1	1	1

Table 6 Matching of the peaks (%) in the FTIR spectrum of extracted polyphenols in methanol from gooseberries (GB, *Physalis peruviana*), cranberries (CB, *Vaccinium macrocarpon*) and blueberries (BB, *Vaccinium corymbosum*) and standards

Range of bands	3500–3100 cm^{-1}			3000–2800 cm^{-1}			1800–1600 cm^{-1}			1500–700 cm^{-1}		
	Matching of standards/samples (%)											
Standards	GB	BB	CB	GB	BB	CB	GB	BB	CB	GB	BB	CB
Quercetin	70	71	70	1	5	6	1	3	3	5	8	5
Ferulic acid	17	17	18	14	12	11	18	8	18	6	5	5.5
Fisetin	10	17	17	10	15	12	3	22	3	7	7.5	7.5
Hesperidin	69	69	70	34	35	40	20	26	9	6	4	3.5
Tannic acid	84	84	84	14	16	14	9	35	2	35	35	37
Caffeic acid	34	38	35	27	30	24	16	17	3	11	10	16
Gallic acid	59	57	57	11	13	11	19	28	16	27	18	26

- property. The binding affinities with HSA were strongly influenced by the structural differences of dietary polyphenols from berries.

We have investigated the binding properties of quercetin in aqueous and methanol media, using UV/vis and fluorometry, which is one of themajor phenolic compounds found in berries. Our results were in accordance with Guo et al. (2007), who demonstrated that quercetin and other phenolic compounds can effectively modulate iron biochemistry under physiologically relevant conditions, providing insight into the mechanism of action of bioactive phenolics (Guo et al., 2007). Our results are in agreement with Xiao et al. (2011) as well that dietary flavonoids are important polyphenols in berries as they are of great interest for their bioactivities, which are related to the antioxidative property. The binding affinities with HSA were strongly influenced by the structural differences of dietary polyphenols from berries.

The HSA–polyphenol interaction weakened with the free radical scavenging potential of polyphenols. The structural difference of flavonoids strongly affects the binding process with plasma proteins. Flavonoids played as a hydrogen bond acceptor when bound to HSA (Xiao et al., 2011). The relatively high binding properties of gooseberries are important from the point of view of their incorporation in food products as an important ingredient. Our *in vitro* fluorometry studies are in agreement with others, who investigated the properties of berries *in vivo*.

So, the drinking of cranberry juice for 4 months affected antioxidant capacity and lipid profile in orchidectomised rats. Orchidectomy depressed plasma antioxidant capacity of plasma and increased triglyceride and cholesterol values of liver and plasma (Deyhim *et al*., 2007). Rats fed with goldenberry (*Physalis peruviana*) juice showed lower levels of total cholesterol, total triacylglycerol and total low-density lipoprotein cholesterol, as well as higher levels of high-density lipoprotein cholesterol in comparison with animals fed with HCD and cholesterol-free diet (Ramadan, 2012).

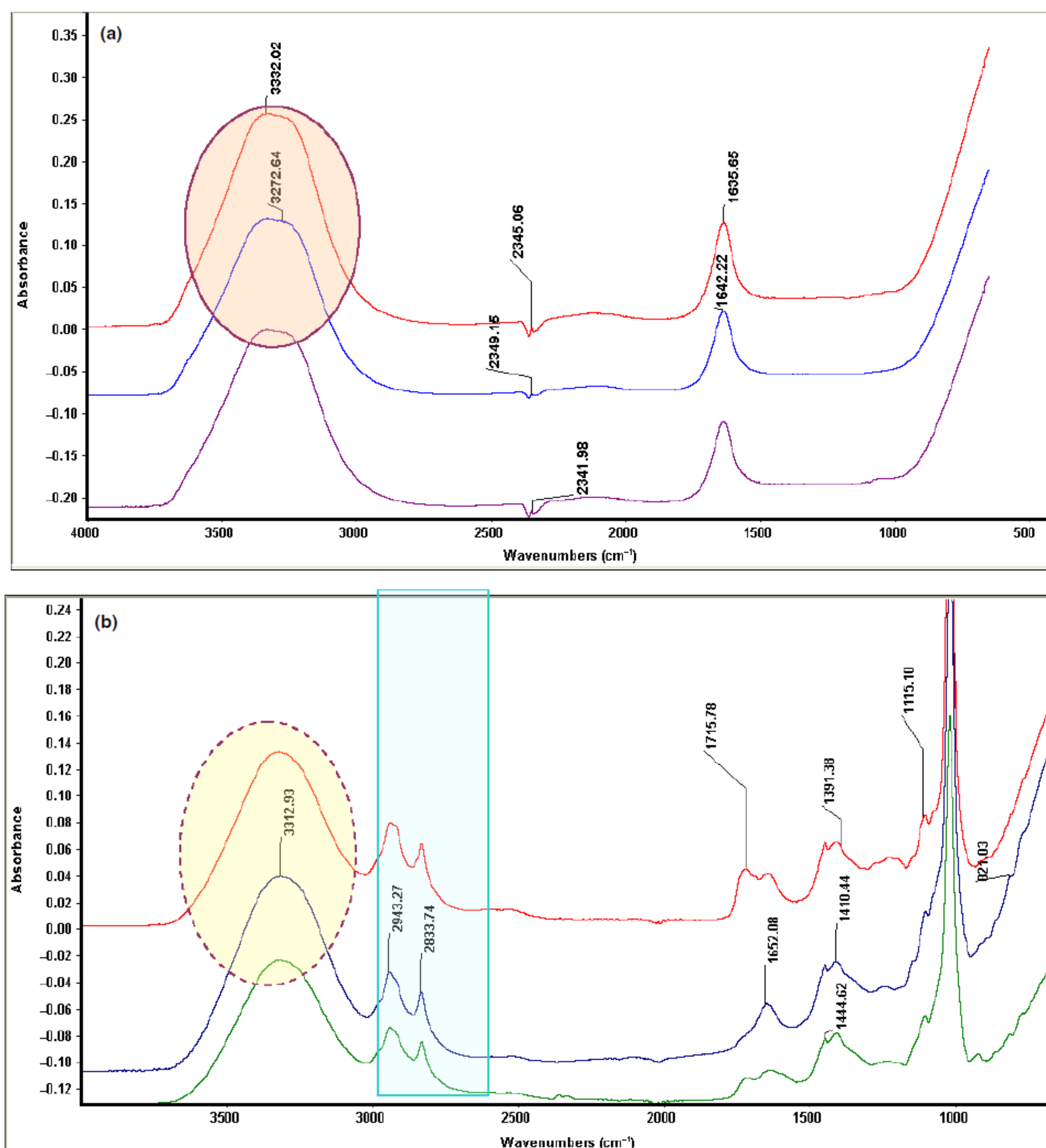


Figure 7 FTIR spectra of: (a) water extracts of gooseberries, blueberries and cranberries; (b) methanol extracts of gooseberries, blueberries and cranberries from the bottom to the top. The elliptical symbols showed the similar range of the spectra in two extracts.

It is possible to supplement food products with the extracts of the studied berries, as it was shown in the study by Lastawska (2010). The selected products were in the form of hard gelatin capsules. They contained the extracts from chokeberry, cranberry and blueberry. All studied preparations showed antioxidant properties and may provide substantial antioxidant protection. The *in vitro* antioxidant capacity varied considerably and was associated with the content of polyphenols in the capsule.

The studied gooseberry can be used as dry or fresh material or as water extracts. The most important aspect is the prevention of antioxidant properties during the food processing. Our present results are in correspondence with the previous results, where only aqueous extracts were used, with other kinds of berries. In our previous report, it was shown that aqueous extracts of investigated berries were subjected to different times of thermal processing. Only thermal treatment of studied berries influences their quality: berries after 10 and 20 min of thermal processing preserved their bioactivity (Arancibia-Avila et al., 2012).

This is in accordance with Reyes et a*l*. (2011), who showed that ascorbic acid content was decreased in freeze-dried blueberries compared with fresh fruit, while polyphenols were decreased in atmospheric freeze-drying unlike in vacuum freeze-drying, where this nutritional property was increased. The results show promising perspectives for the exploitation of berry species with considerable levels of nutrients and antioxidant capacity in foods. Our data add valuable information to current knowledge of the nutritional properties of berries, such as the considerable antioxidant and binding capacities that were found. In conclusion, the bioactivity of gooseberries is lower and comparable with blueberries and cranberries. Gooseberries are a promising exotic fruit that could be made into many novel dishes. 3D fluorescence spectroscopy and FTIR spectroscopy were used as additional tools for the characterisation of the polyphenol extracts in different berry cultivars. The analytical methods used in this study can be applied for any food analysis.

C. In Vitro Studies on the Relationship Between the Antioxidant Activities of Some Berry Extracts and Their Binding Properties to Serum Albumin

Bioactive Compounds and Antioxidant Activities

It was interesting to use different solvent systems such as diethyl ether, ethyl acetate, and water in order to find out the best extraction conditions and the maximum antioxidant activities of gooseberries in comparison with blueberries and cranberries. The results of the determination of the contents of the bioactive compounds in the extracts of three solvents of all studied samples are summarized in the Table 1. As can be seen, the significant highest contents ($P<0.05$) of polyphenols and flavanols were in the water fraction of blueberries (46.56± 4.2 mg GAE/g and 1.75±0.3 mg CE/g, respectively).

The contents of flavonoids are comparable with the data in cranberries. The contents of chlorophylls and carotenoids (Fig. 1) were the highest in blueberries as well ($P<0.05$). The weight ratio of Chla and Chlb is an indicator of the functional pigments. The ratios of chlorophylls a/b were the following: 0.68, 1.17, and 2.55 for gooseberries (GOOSEB), cranberries (CRAN), and blueberries (BLUEB), respectively. The ratio of total chlorophylls to total carotenoids is an indicator of the greenness of plants (Fig. 1).

Carotenoids and chlorophylls are important in the composition of berries. The ratio of total chlorophylls to total carotenoids was 2.15, 2.47, and 8.67 for gooseberries, cranberries, and blueberries, respectively. The two ratios were in the range which shows that the berries were grown and collected at optimal growing conditions (Lichtenthaler, 1987). The obtained contents of chlorophylls and carotenoids were in acceptable range, showing their sensitivity to seasonal variation in climatic conditions (Kamffer et al., 2010).

Our data can be compared with other reports (Andersson et al., 2009), where different carotenoids in seabuck thorn berries increased in concentration during ripening and comprised from 120 to 1,425 μg/g DW of total carotenoids (1.5–18.5 mg/100 g of FW), depending on the cultivar, harvest time, and year. The content of chlorophyll can act as a marker of the degree of ripening.

Table 7 Bioactive compounds in water, ethyl acetate, and diethyl ether extracts of gooseberries (*P. peruviana*), cranberries (*V. macrocarpon*), and blueberries (*V. corymbosum*) per gram dry weight

Extracts	Indices		
	POLYPHEN, mg GAE	FLAVON, mg CE	FLAVAN, μg CE
GOOSEB, H_2O	5.37±0.6	0.22±0.04	nd
CRAN, H_2O	22.13±2.5	3.83±0.4	467.36±14.5
BLUEB, H_2O	46.56±4.2	3.89±0.6	1,751.51±25.6
GOOSEB, EtOAc	0.29±0.1	0.11±0.01	nd
CRAN, EtOAc	3.14±0.4	0.66±0.1	44.14±4.3
BLUEB, EtOAc	3.87±0.4	0.74±0.1	112.06±7.4
GOOSEB, DETETHR	0.14±0.01	0.08±0.01	1.21±0.1
CRAN, DETETHR	2.11±0.2	0.10±0.01	7.66±0.8
BLUEB, DETETHR	4.13±0.4	0.39±0.1	32.55±3.9

Values are means ± SD of five measurements. All statistical data are presented in Table 4

POLYPHEN polyphenols, *CE* catechin equivalent, *GAE* gallic acid equivalent, *FLAVON* flavonoids, *FLAVAN* flavanols, *nd* not determined, *GOOSEB* gooseberries (*P. peruviana*), *CRAN* cranberries (*V. macrocarpon*), *BLUEB* blueberries (*V. corymbosum*), *EtOAc* ethyl acetate, *DETETHR* diethyl ether

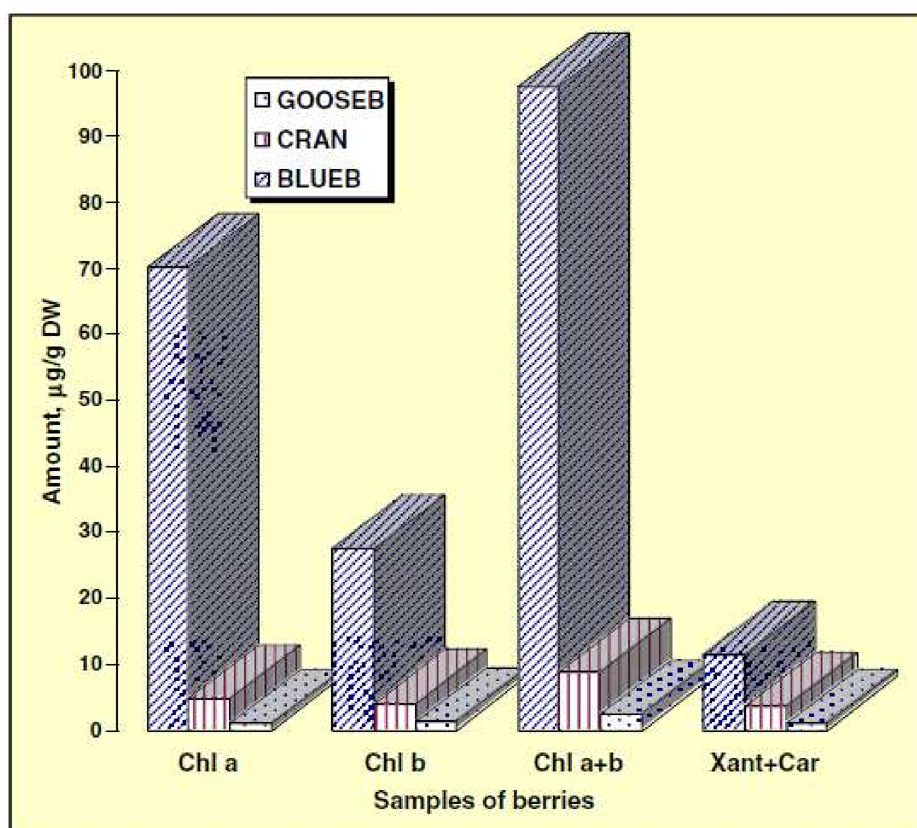


Fig. 8 Chlorophyll and carotenoid levels in berries. Values are means ± SD: ±7.15, ±0.48, and ±0.01 for Chl a in BLUEB, CRAN, and GOOSEB, respectively; ±2.45, ±0.43, and ±0.01 for Chl b in BLUEB, CRAN, and GOOSEB, respectively; ±10.08, ±0. 86, and ±0.12 for Chl a + b in BLUEB, CRAN, and GOOSEB, respectively; ±1.25, ±0. 34, and ±0.08 for Xant + Car in BLUEB, CRAN, and GOOSEB, respectively. *Chl* chlorophyll, *Xant* xanthophylls, *car* carotenes, *GOOSEB* gooseberries, *CRAN* cranberries, *BLUEB* blueberries

We investigated the properties of quercetin, the major phenolic phytochemical present in berries, in aqueous media using UV spectroscopy, fluorometry, and ESI-mass spectrometry. As was declared in "Results and Discussion", the contents of bioactive compounds (polyphenols, flavonoids, and flavanols) in three different extracts was determined and compared, and the significantly highest amounts were in water extract of blueberries. Gooseberries showed a moderate amount of bioactive compounds.

Table 8 Mass spectral data (molecular ion and the major fragment ions of polyphenols extracted from berries)

Extracts	Berries	$[M-H]^-$ and fragmentation in ESI, (% in MS)	Compound
Water	Gooseberries	190.79 (100)	Quinic acid
	Cranberries	352.77 (40), 190.79 (100)	Chlorogenic acid, quinic acid
		294.74 (15)	*p*-Coumaroyl tartaric acid
		212.6 (20)	2,3 Dihydroxy-*1*-guaiacyl propanone
	Blueberries	404.85 (60)	Piceatannol 3-*O*-glucoside
		346.68 (40), 190.93 (100)	5-Heptadecylresorcinol, quinic acid
Ethyl acetate	Gooseberries	444.40 (35)	Apigenin 7-*O*-glucuronide
		190.79 (30)	Quinic acid
		212.6 (100)	2,3 Dihydroxy-*1*-guaiacyl propanone
	Cranberries	444.5 (10)	Apigenin 7-*O*-glucuronide
		190.79 (100)	Quinic acid
		212.6 (50)	2,3 Dihydroxy-*1*-guaiacyl propanone
	Blueberries	346.68 (20)	5-Heptadecylresorcinol
		190.79 (100)	Quinic acid
Diethyl ether	Gooseberries	444.33 (40)	Apigenin 7-*O*-glucuronide
		212.6 (100)	2,3 Dihydroxy-*1*-guaiacyl propanone
		168.81 (30)	Gallic acid
	Cranberries	444.47 (40)	Apigenin 7-*O*-glucuronide
		300.83 (40)	quercitin
		212.6 (100)	2,3 Dihydroxy-*1*-guaiacyl Propanone
		190.7 (55)	Quinic acid
	Blueberries	366.9 (50), 190.8 (80)	3-Feruloylquinic acid, quinic acid
		212.7 (100)	2,3 Dihydroxy-*1*-guaiacyl propanone

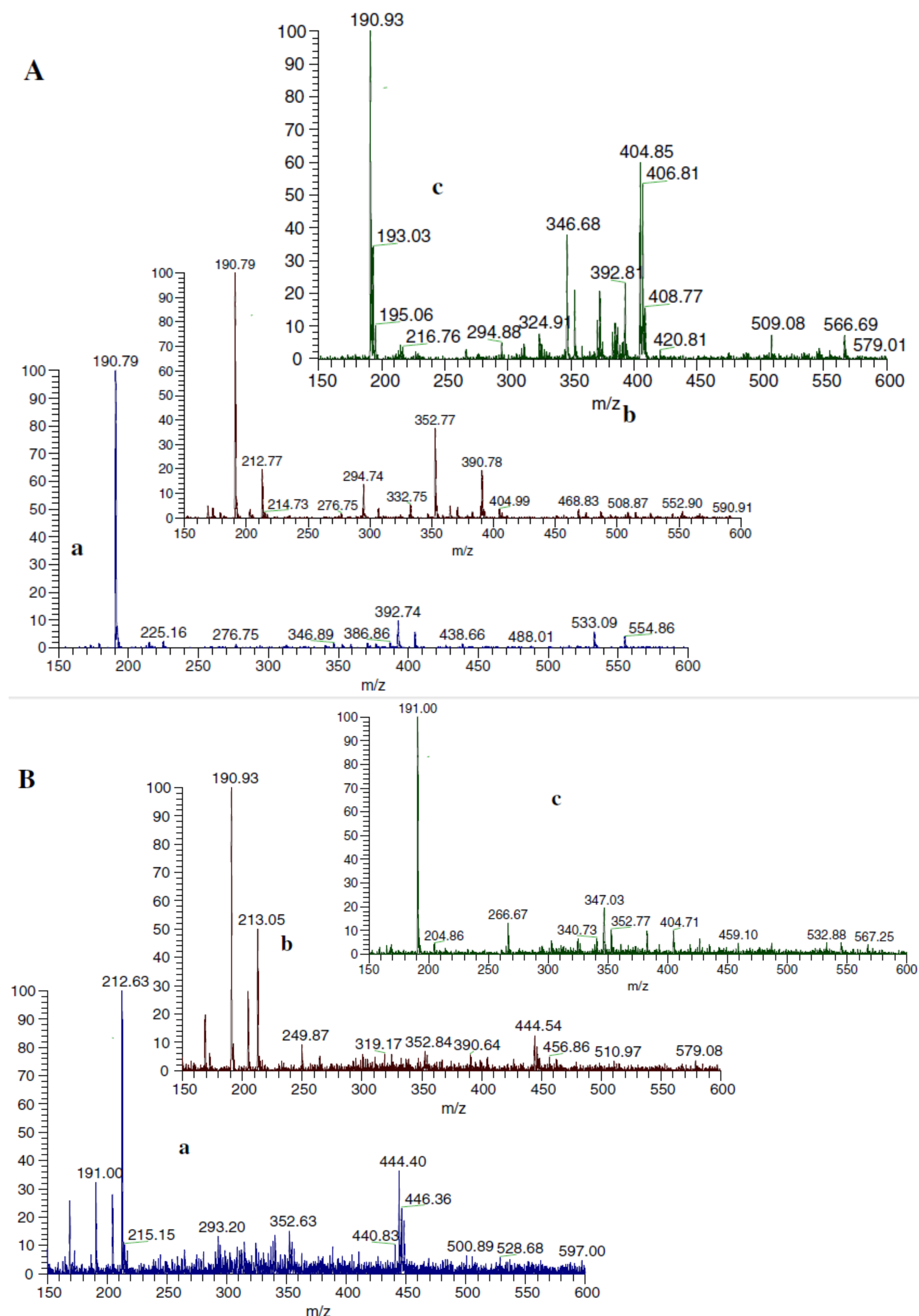


Fig. 9 ESI-MS spectra of extracted fractions from three studied berries. **a** Aqueous, **b** ethyl acetate, and **c** diethyl ether of *a* gooseberries, *b* cranberries, and *c* blueberries in negative ion mode. Phenolic compounds were identified at *m/z* based on the mass spectra data

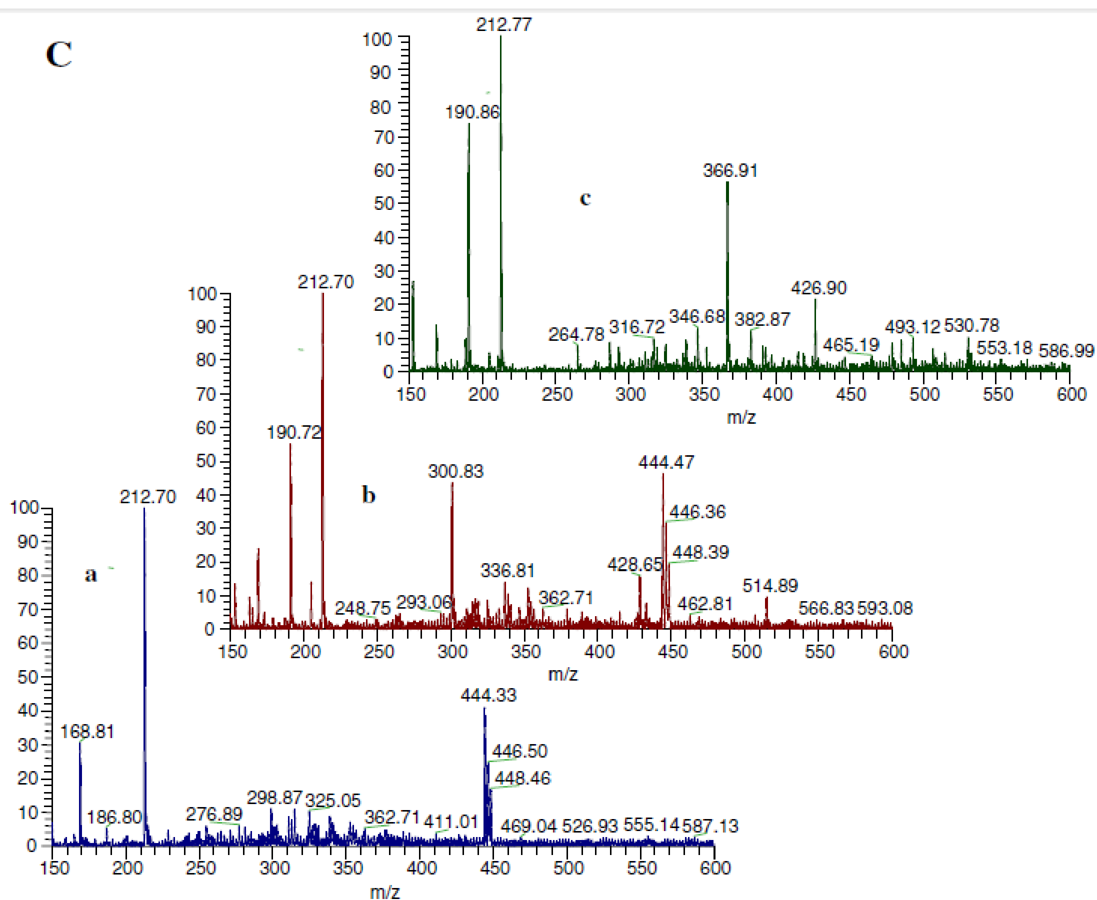


Fig. 9 (continued)

Our results were in agreement with others, showing that water extracts of blueberries contain high amounts of polyphenols (Dai et al., 2009). The amount of phenolics for blueberry and cranberry was reported as 261–585 and 315 mg/g FW and for flavonoids as 50 and 157 mg/g FW (Fernández et al., 2008; Matta-Riihinen et al., 2004) The ESI-MS in negative ion mode (Table 2; Fig. 2a) of water extracts differs between berries. The water extract of gooseberry (Table 2; Fig. 2—Aa) showed that the molecular ion at m/z 190.79 corresponded to quinic acid. Oppositely, cranberry (Table 2; Fig. 2—Ab) water extract was characterized by chlorogenic acid of the (M-H)– deprotonated molecule (m/z 353) and the ion corresponding to the deprotonated quinic acid (m/z 191), which was consistent with Sun et al., (2007).

Blueberry water extract (Table 2; Fig. 2c) demonstrated a peak at 404.85 (piceatannol 3-O-glucoside), 346.68, and 190.93 as a result of destroying 5-heptadecylresorcinol. Ethyl acetate extracts of berries showed similar spectral peaks. Gooseberry (Table 1; Fig. 2—Ba) and cranberry (Table 1; Fig. 2—Bb) were similar in molecular ions but differ in the percentage in MS. Blueberry ethyl acetate extract (Table 2; Fig. 2—Bc) and water extract (Table 2; Fig. 2—Ac) were similar. In the diethyl ether extracts (Table 2; Fig. 2c) of all berries, the main peak was of m/z 212.6. The spectra of blueberry differ from gooseberry and cranberry with one peak at m/z 366.9. In gooseberry and cranberry extracts, one common peak appeared at m/z 444.4, but gooseberry extract is characterized by the peak of gallic acid and in cranberry only quercetin is found.

The recorded spectra were in the same scale (in the range between 100 and 600m/z) for comparison. We choose negative mode as the MS method because in many publications it was

described that this mode is the best for analysis of low molecular weight phenolic compounds (Gómez-Romero et al., 2011; Dastmalchi et al., 2011; Zuo et al., 2002; Sun et al., 2007). All of the peaks were identified and the recorded MS spectra can be used as a fingerprint for characterization of different berry extracts based on the percentage of the main peaks.

Our obtained results by MS are similar to Zuo et al. (2002), where 15 benzoic and phenolic acids (benzoic, o-hydroxybenzoic, cinnamic, m-hydroxybenzoic, p-hydroxybenzoic, p-hydroxyphenyl acetic, phthalic, 2,3-dihydroxybenzoic, vanillic, o-hydroxycinnamic, 2,4dihydroxybenzoic, p-coumaric, ferulic, caffeic, and sinapic acid) were identified in cranberry fruit. The most abundant is benzoic and then p-coumaric and sinapic acids. The phenolic constituents in the berries were identified as chlorogenic acid, p-coumaric acid, hyperoside, quercetin-3-O-glucoside, isoorientin, isovitexin, orientin, and vitexin (Dastmalchi et al., 2011). The AA of blueberry in water extracts (Table 3) as determined by CUPRAC, DPPH, and β-carotene assays (131.09±12.9.3 μM TE/g DW, 108.09±7.2 μM TE/g DW, and 80.11±8.9 %, respectively) in all of the extracts used is significantly higher than that recorded for other berries studied ($P<0.05$).

The AA of gooseberry is lower by about nine times than in blueberries and four times than in cranberries. As was calculated, a very good correlation was found between the antioxidant activity and the contents of total polyphenols in water extracts. All groups of data (Tables 1 and 3) were tested for character of their distribution and homogeneity of variance at 0.95 confidence level. The Shapiro–Wilk normality test showed that all the data in groups are normally distributed, with the exception of flavanols in gooseberry water and ethyl acetate extracts with no quantified content. Levene's F test, which is widely accepted as the most powerful homogeneity of variance test, indicated extract types which have no the same variance tested at 0.95 confidence level.

Table 4 presents significant differences (with P values <0.05) between bioactive compounds contents and antioxidant activities in different extracts of berries found by multiple comparisons using the method of Student–Newman–Keuls. The method denotes significantly different pairs, and the group in the first position means that it is higher in the contents of bioactive substances. For example, the case of polyphenols in line G/W-G/D means a statistically different content of polyphenols between gooseberry water and diethyl ether extracts. Water extract is higher in the content of polyphenols of about 10.2 mg GAE/g DW. From Table 4, it is evident that in majority of the cases, water extraction yields the highest content of bioactive compounds and antioxidant activities.

The antioxidant activity of different extracts was evaluated by DPPH free radical scavenging activity, taking total phenolic content as an index (Reddy et al., 2008). Our obtained results correspond with the data of Kusznierewicz et al. (2012), where the DPPH antioxidant activity varied from 93 to 166 mol TE/g DW. The obtained phenolic compounds and DPPH values (Tables 1 and 2) were as well in the range of those reported by Li et al. (2009) of four berry fruits (strawberry, Saskatoon berry, raspberry, and wild blueberry), chokecherry, and seabuck thorn ranging from 22.83 to 131.88 g/kg and DPPH ranging from 29.97 to 78.86 %.

The bioactivity of blueberry is significantly higher than the bioactivity of other berries; however, this index in the gooseberry is comparable with the studied samples. According to the results of Table 4, the antioxidant activities of extracts, partitions, and fractions were strongly correlated with the highest polyphenol contents. Correlation between polyphenols and antioxidant properties exactly corresponded with our results: the highest phenolic content was found in walnut, which revealed the best antioxidant properties (Mishra et al., 2010). This corresponds with Seeram

(2010), who discussed also that phytonutrients ranged from fat-soluble/lipophilic to water-soluble/hydrophilic compounds. Our results about the high antioxidant activity of berries (Table 3) are in line with Elberry et al., (2010), showing a high antioxidant activity of cranberry extract.

Table 9 Antioxidant activities in water, ethyl acetate, and diethyl ether extracts of gooseberries (*P. peruviana*), cranberries (*V. macrocarpon*), and blueberries (*V. corymbosum*) per gram dry weight

Extracts	Indices		
	DPPH, μM TE/g DW	CUPRAC, μM TE/g DW	β-carotene, %
GOOSEB, H_2O	8.39±0.9	11.25±1.1	11.40±0.9
CRAN, H_2O	46.58±4.5	49.38±4.4	36.71±3.8
BLUEB, H_2O	108.09±7.2	131.09±9.6	80.10±6.6
GOOSEB, EtOAc	0.35±0.1	0.88±0.1	0.54±0.1
CRAN, EtOAc	3.02±0.4	9.20±1.1	6.09±0.6
BLUEB, EtOAc	8.83±4.4	12.40±1.1	8.13±0.9
GOOSEB, DETETHR	0.16±0.01	0.24±0.01	0.20±0.01
CRAN, DETETHR	3.42±0.4	5.77±0.6	3.48±0.3
BLUEB, DETETHR	10.97±0.9	14.87±1.1	6.79±0.7

Values are means ± SD of five measurements. All statistical data are shown in Table 4

DW dry weight, *DPPH* 2,2-diphenyl-*1*-picrylhydrazyl, *CUPRAC* cupric reducing antioxidant capacity, *β-carotene* β-carotene linoleate assay, *GOOSEB* gooseberries (*P. peruviana*), *CRAN* cranberries (*V. macrocarpon*), *BLUEB* blueberries (*V. corymbosum*), *EtOAc* ethyl acetate, *DETETHR* diethyl ether

Table 10 Statistically significant differences between the content of bioactive compounds in different extracts of berries by Student–Newman–Keuls multiple comparisons

Comparison between berries extracts	Difference	Standard error	*q* stat	Table *q*	Probability, $P<0.05$
Polyphenols					
G/W–G/D	10.2053	0.7071	14.4325	3.6332	0.0000
G/E–G/D	8.6337	0.7071	12.2099	2.7718	0.0000
B/W–B/D	4.3603	0.7071	6.1665	3.6332	0.0001
B/W–B/E	3.8084	0.7071	5.3860	3.3145	0.0004
Flavonoids					
G/W–G/E	2.7948	0.7071	3.9525	3.6332	0.0267
C/W–C/D	7.0963	0.7071	10.0357	4.0301	0.0000
C/E–C/D	4.3453	0.7071	6.1452	3.8577	0.0001
B/W–B/E	4.1482	0.7071	5.8665	3.8577	0.0003
B/W–B/D	4.1482	0.7071	5.8665	3.6332	0.0002
C/W–C/E	2.7510	0.7071	3.8905	2.7718	0.0059

Table 10 (continued)

Comparison between berries extracts	Difference	Standard error	*q* stat	Table *q*	Probability, $P<0.05$
Flavanols					
G/W–G/D	3.2040	0.7071	4.5311	3.3145	0.0039
G/E–G/D	3.2040	0.7071	4.5311	2.7718	0.0014
C/W–C/D	6.3189	0.7071	8.9363	3.8577	0.0000
C/E–C/D	3.9136	0.7071	5.5347	3.3145	0.0003
B/W–B/D	4.6555	0.7071	6.5839	3.8577	0.0000
C/W–C/E	2.4053	0.7071	3.4016	3.3145	0.0427
B/W–B/E	2.7159	0.7071	3.8409	3.3145	0.0181
DPPH					
G/W–G/D	12.0877	0.7071	17.0946	4.0301	0.0000
G/E–G/D	7.8126	0.7071	11.0486	2.7718	0.0000
G/W–G/E	4.2751	0.7071	6.0460	3.8577	0.0002
C/W–C/E	4.3824	0.7071	6.1976	4.0301	0.0002
C/W–C/D	4.3289	0.7071	6.1219	3.8577	0.0001
B/W–B/D	4.2085	0.7071	5.9517	3.8577	0.0002
B/W–B/E	2.8095	0.7071	3.9733	3.3145	0.0138
CUPRAC					
G/W–G/D	9.7648	0.7071	13.8095	4.0301	0.0000
G/E–G/D	4.4785	0.7071	6.3335	2.7718	0.0000
G/W–G/E	5.2863	0.7071	7.4760	3.8577	0.0000
C/W–C/D	4.8131	0.7071	6.8068	4.0301	0.0000
B/W–B/E	4.3484	0.7071	6.1495	4.0301	0.0002
B/W–B/D	4.1359	0.7071	5.8490	3.8577	0.0003
C/W–C/E	2.9609	0.7071	4.1874	2.7718	0.0031
β-CAROTENE					
G/W–G/D	8.5379	0.7071	12.0744	4.0301	0.0000
G/E–G/D	3.8783	0.7071	5.4847	2.7718	0.0001
G/W–G/E	4.6596	0.7071	6.5897	3.8577	0.0000
C/W–C/D	5.2270	0.7071	7.3921	4.0301	0.0000
C/W–C/E	3.6094	0.7071	5.1045	3.8577	0.0028
B/W–B/D	4.0614	0.7071	5.7437	3.8577	0.0005

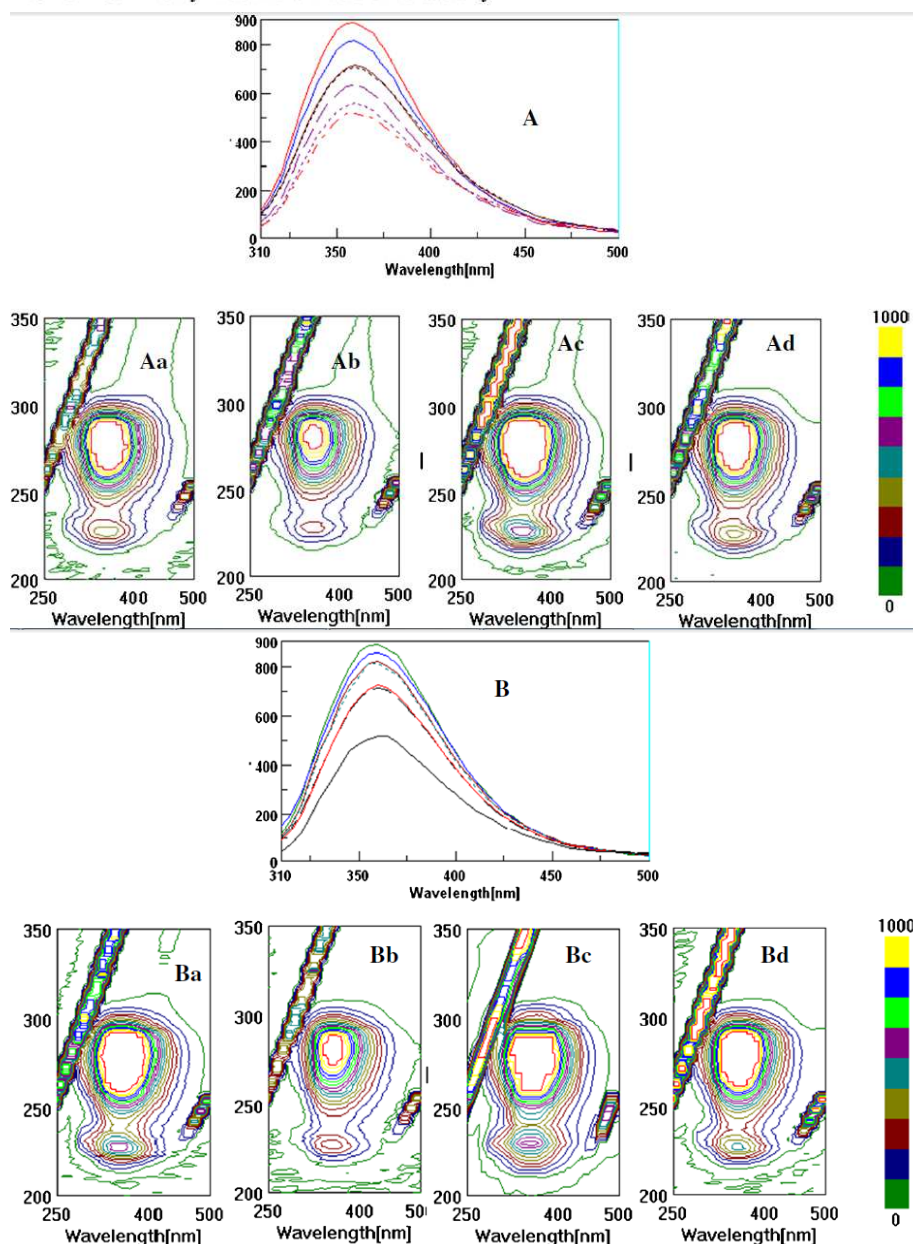
B berries, *G* gooseberries, *C* cranberries, *B* blueberries, *W* water, *E* ethyl acetate, *D* diethyl ether

Pronounced antioxidant and radical scavenging properties of cranberry was shown by Wojnicz et al., (2012). Ethanol-soluble acidic components were used in order to determine the bioactivity of natural novel sources against oxidation (Li et al., 2012). Our results are in accordance with You et al., (2011), where four Rabbiteye blueberry cultivars grown organically and conventionally were compared by their total phenolic content and antioxidant values by DPPH and CUPRAC. Our studies are not in full correspondence with others (Wu et al., 2005) based on the different extraction systems.

In our case, the most active was the water fraction of *P. peruviana* (PP) in comparison with ethyl acetate and diethyl ether. As was reported by Wu et al., (2005), supercritical carbon dioxide SCEPP-5 PP extracts in comparison with hot water and ethanol possessed the highest total flavonoid (226.19 mg/g) and phenol (100.82 mg/g) contents. Our results connected with other reports (Reddy et al., 2008; Victoria et al., 2012), where the methanol extract of leaves from some plants was more potent against Aspergillusfumigatus and Candida tropicana.

The lowest MIC values obtained for LM, LA, and LH were 78, 156, and 625 µg/mL against A. fumigatus, C. tropicana, and orange juice was significantly more potent in protecting the bladder against ischemia/reperfusion damage than either blueberry or cranberry juice. Thus, it is concluded that chemical tests for TAA do not necessarily correlate with their physiological activity (Bean et al., 2010). The obtained antioxidant activity by FRAP of blueberry and cranberry extracts was similar to other studies. Probably, a complex spectrum of anthocyanins was the major contributor to the antioxidant activity (Borges et al., 2010).

Fig. 10 Two-dimensional fluorescence (2D-FL) and three (3D-FL) spectra illustrate the interaction between HSA, quercetin, aqueous (positions *Aa*, *Ab*, *Ac*, and *Ad*), and ethyl acetate (positions *Ba*, *Bb*, *Bc*, and *Bd*) extracts of studied berries. **a** Change in the fluorescence intensity as a result of binding affinity with water extracts: HSA [*first line* from the *top* with FI of 890.21], HSA + WGOOSEB (*second line* from the *top* with FI=817.50), HSA + WCRAN (*third line*, FI=717.39), HSA + WBLUEB (*fourth line*, FI=709.75), HSA + WGOOSEB + QUE (*fifth line*, FI=635.24), HSA + WCRAN + QUE (*sixth line*, FI=560.83), and HSA + WBLUEB + QUE (seventh line, FI=518.96). *Aa–Ad* cross maps from the 3D-FL spectrum of HSA + WBLUEB, HSA + WBLUEB + QUE, HSA + WGOOSEB, and HSA + WGOOSEB + QUE. **b** Change in the fluorescence intensity as a result of binding affinity of HSA with ethyl acetate extracts: HSA [*first line* from the *top* with FI of 890.21], HSA + EtOAcGOOSEB (*second line*, FI=834.70), HSA + EtOAcCRAN (*third line*, FI=821.65), HSA + EtOAcBLUEB (*fourth line*, FI=811.70), HSA + EtOAcGOOSEB + QUE (*fifth line*, FI=724.76), HSA + EtOAcCRAN + QUE (*sixth line*, FI=713.41), and HSA + EtOAcBLUEB + QUE (*seventh line*, FI=618.96). *Ba–Bd* cross maps from the 3D-FL spectrum of HSA + EtOAcBLUEB, HSA + EtOAcBLUEB + QUE, HSA + EtOAcGOOSEB, and HSA + EtOAcGOOSEB + QUE. In all reactions, the following conditions were used: HSA (2.0×10^{-6} mol/L), quercetin (1.7×10^{-6} mol/L), and water and EtOAc extracts in concentration of 25 and 50 µg/ml, respectively. Binding was during 1 h at 25 °C. Fluorescence intensities are on *y*-axis and emission wavelengths are on *x*-axis. *HSA* human serum albumin, *QUE* quercetin, *EtOAc* ethyl acetate, *WGOOSEB* water extracts of gooseberry, *WCRAN* water extracts of cranberry, *WBLUEB* water extracts of blueberry, *EtOAcGOOSEB* ethyl acetate extracts of gooseberry, *EtOAcCRAN* ethyl acetate extracts of cranberry, *EtOAcBLUEB* ethyl acetate extracts of blueberry



Fluorometry Spectra Studies

The binding properties of the berry samples in comparison with the pure flavonoids such as quercetin are shown in 3DFL spectra, which illustrated the elliptical shape of the cross map. The results showed that the 3DFL cross maps of berries differed. One of the main peaks for HSA was found at λ ex/em of 220/360 nm. The second main peak appeared for these samples at λ ex/em of 280/350 nm (Fig. 3a, b). The interaction of HSA and the water and ethyl acetate extracts of berries (Fig. 3—Aa, Ac, Ba, and Bc), HSA, water, and ethyl acetate extracts, and quercetin (Fig. 3—Ab, Ad, Bb, and Bd) showed a slight change in the position of the main peak at the wavelength of 360 nm and a decrease in fluorescence intensity (FI). The following changes appeared when the water extracts of berries were added to HSA (initially the main peak at emission 360 nm and FI of 890.21) (Figs. 3a, b and 4a, b; the upper line is HSA).

The reaction with the berry extracts and quercetin decreased the FI of HSA (Fig. 3a, b; middle and low lines). The following decrease in the FI (%) occurred during the interaction of water extracts with HSA: HSA+WGOOSEB=8, HSA+WCRAN=19.4, and HSA+WBLUEB= 20.3. The decrease in the FI with ethyl acetate extracts was lower than with water extract: HSA+EtOAcGOOSEB=6.0, HSA+EtOAcCRAN=7.7, and HSA+EtOAcBLUEB=8.2. The diethyl ether extracts did not show any binding properties with HSA.

These results are in direct relationship with the antioxidant properties of the extracts. The synergism of bioactive compounds is shown when quercetin was added to the mixture of HSA and extracts of berries. The decrease in the FI of HSA with WGOOSEB, WCRAN, and WBLUEB was 28.6, 37.0, and 41.7, respectively (fifth, sixth, and seventh lines (Fig. 3a)). Therefore, the participation of quercetin in synergism was 20.6, 17.6, and 21.4 for WGOOSEB, WCRAN, and WBLUEB, respectively. With ethyl acetate extracts, the participation of quercetin was 13.9, 10.9, and 17.6 for GOOSEB, CRAN, and BLUEB, respectively (Fig. 3b).

The concentrations of water extracts of berries in the interaction with HSA are 3.01971, 5.12232, and 5.23493×10^{-8} QUE for GOOSEB, CRAN, and BLUEB, respectively. Ethyl acetate extracts showed lower concentrations at 2.5751, 2.90949, and 3.16139×10^{-8} for GOOSEB, CRAN, and BLUEB, respectively. Our very recent results showed that the fluorescence is significantly quenched because the conformation of the HSA changes in the presence of pure flavonoids and berry extracts.

This interaction between quercetin and HSA was investigated using tryptophan fluorescence quenching. Our result is in agreement with others that quercetin, as an aglycon, is more hydrophobic and demonstrates strong affinity toward HSA. Other results (Xiao et al. 2011; Xiao and Kai, 2012) differ from those reported by us, probably because of the variety of antioxidant abilities of pure flavonoids and different ranges of fluorometry scanning ranges used in a similar study. The biological relevance of quercetin interaction in human organism is important from the point of view that this molecule of polyphenolic type extensively binds to HSA, the most abundant carrier protein in the blood.

Our *in vitro* results of interaction of HSA and quercetin can be compared with other reports in vivo, showing the protective effects of quercetin on hepatic injury induced by different chemical reactions. Our results on BSA binding with other types of berry correspond with our present results with HSA and investigated berries. Results on water extracts of blueberries were similar to these samples (Gorinstein et al., 2013; Flis et al., 2012). Strong binding properties have been confirmed for

the compounds containing high bioactivity.

The strong binding properties of phenolics show that they may be effective in the prevention of atherosclerosis under physiological conditions. Quercetin can suppress HSA. These results demonstrate that quercetin and other phenolic compounds can effectively protect from atherosclerosis under physiologically relevant conditions, providing insight into the mechanism of action of bioactive phenolics. Our explanation of the binding affinity of berry polyphenols is similar to the description of Xiao et al., (2011) and Xiao and Kai, (2012) that one or more hydroxyl groups in the B-ring of flavonoids enhanced the binding affinities to proteins. Much of the bioactivities of citrus flavanones significantly appear to impact blood and microvascular endothelial cells (Cao et al., 2011)

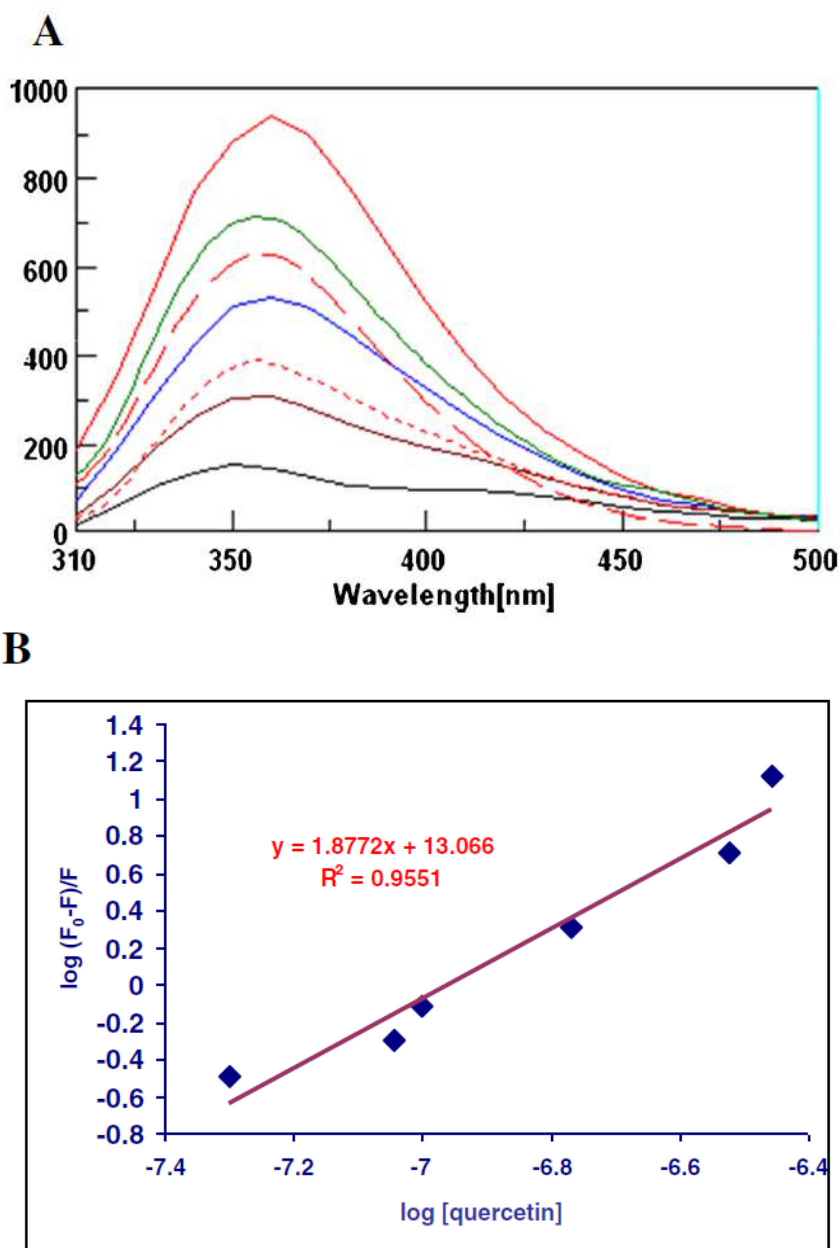


Fig. 11 a Fluorescence spectra of aqueous solutions of HSA (2.0×10^{-6} mol/L) in the presence of different concentrations of quercetin: 0, 0.17, 0.30, 1.0, and 1.7×10^{-6}) mol/L at pH 7.4 at excitation wavelength of 290 nm. **b** Linear plot for log $(F_0-F)/F$ vs log [quercetin], where F_0 and F represent the fluorescence intensity of HSA in the absence and in the presence of polyphenols, respectively

Therefore, it was essential to investigate the interaction between berry polyphenols and serum albumin. The binding constants ranked in the following order: quercetin>rutin>calycosin>calycosin-7-O-(sup)-D-glucoside (formononetin-7-O-(sup)D-glucoside (Liu et al., 2009). 3D fluorescence can be used as an additional tool for the characterization of the polyphenol extracts of berry cultivars and their binding properties.

D. Application of Analytical Methods for the Determination of Bioactive Compounds in Some Berries

Bioactive Compounds

The results of the determination of the contents of the bioactive compounds in all studied samples are summarized in Fig. 2a. As can be seen, the significant highest content ($P< 0.05$) of polyphenols, flavonoids, anthocyanins, and ascorbic acid was in "Murtilla" non-ripe sample (84.81 ± 3.9 mg GAE/g, 11.47 ± 0.6 mg CE/g, 16.7 ± 0.9 mg CGE/g, and 9.12 ± 0.4 mg/g, respectively, Fig. 2a and b). Only the content of anthocyanins (Fig. 2b) was significantly higher ($P < 0.05$) in blueberries from Poland (323.2 ± 16.1 mg CGE/g). The following order of the value of polyphenols was obtained (Fig. 2a): "Murtilla" non-ripe (MNR) >Aronia (ARON) > Polish blueberry (POLBB) > Chilean blueberry (CHBB) > "Murtilla-like" non-ripe (M-LNR) > raspberry (RASB) > "Murtilla-like" ripe (M-LR).

Antioxidant Activity

The results of the determination of the level of antioxidant activity of all studied samples are shown in the Fig. 2c. As can be seen, the AA of Murtilla non-ripe as determined by ABTS and CUPRAC assays was 620.74 ± 30 and 600.52 ± 27 μmol TE/g, respectively) was significantly higher than in other studied berries ($P < 0.05$). The antioxidant activity of blueberries was higher than that of raspberries, and comparable with AA of "Murtilla" non-ripe (Fig. 2c). As was calculated, a very good correlation was found between the antioxidant activity and the contents of total polyphenols and other bioactive compounds (R^2 from 0.96 to 0.83) in water extracts. Flavonoids showed lower correlation. The correlation between the antioxidant activity and ascorbic acid (Fig. 1c and b) was lower than with polyphenols (R^2 from 0.84 to 0.50).

Anticancer Activity

It was observed that the percentage of proliferativity of the water extracts of berries samples on two cell lines (Fig. 2d, Calu-6 for human pulmonary carcinoma and Fig. 2e, SNU601 for human gastric carcinoma) were different. The proliferativity (%) for concentrations of 1,500 μg/mL for water extracts of "Murtilla" on Calu-6 and SNU-601 were 41.76 and 42.12 %, respectively, and for "Murtilla-like" were 73.43 % and 71.23 % on Calu-6 and SNU-601, showing the higher antiproliferative activity of "Murtilla" in comparison with all other samples. Our investigation showed that antioxidant activity of the studied samples was correlated with their antiproliferative activity directly: the highest antioxidant activity was matching the highest antiproliferative activity.

Fluorometric Data

Fluorometric data showed the characterization of bioactive compounds in different berries with their specific fluorescence intensity and the location of the main peak and its shift. In addition the quenching ability of bioactive compounds in extracts was compared with pure catechin by the interaction with BSA in the presence of urea. The 3-D FL was used to determine the peak situation and the picture of the full peak. The 2-D FL was used for the determination of the fluorescence properties and for the change in the fluorescence intensity.

In three-dimensional fluorescence spectra and contour maps of berries one main peak can easily be observed in water extracts at the location of λ em/ex 340/275 nm in “Murtilla-like” non-ripe with fluorescence intensity (FI) of 680 and the average second peak at em/ex 430/310 nm with FI = 480; and one very small peak at em/ex 620/280 nm with FI 80 (Fig. 3a). “Murtilla-like” ripe (Fig. 3b) showed nearly the same two peaks at em/ex 330/280 nm with intensity of 507; and the second peak at em/ex 420/310 nm with FI= 400; one very small peak at em/ex 620/280 nm with FI 60.

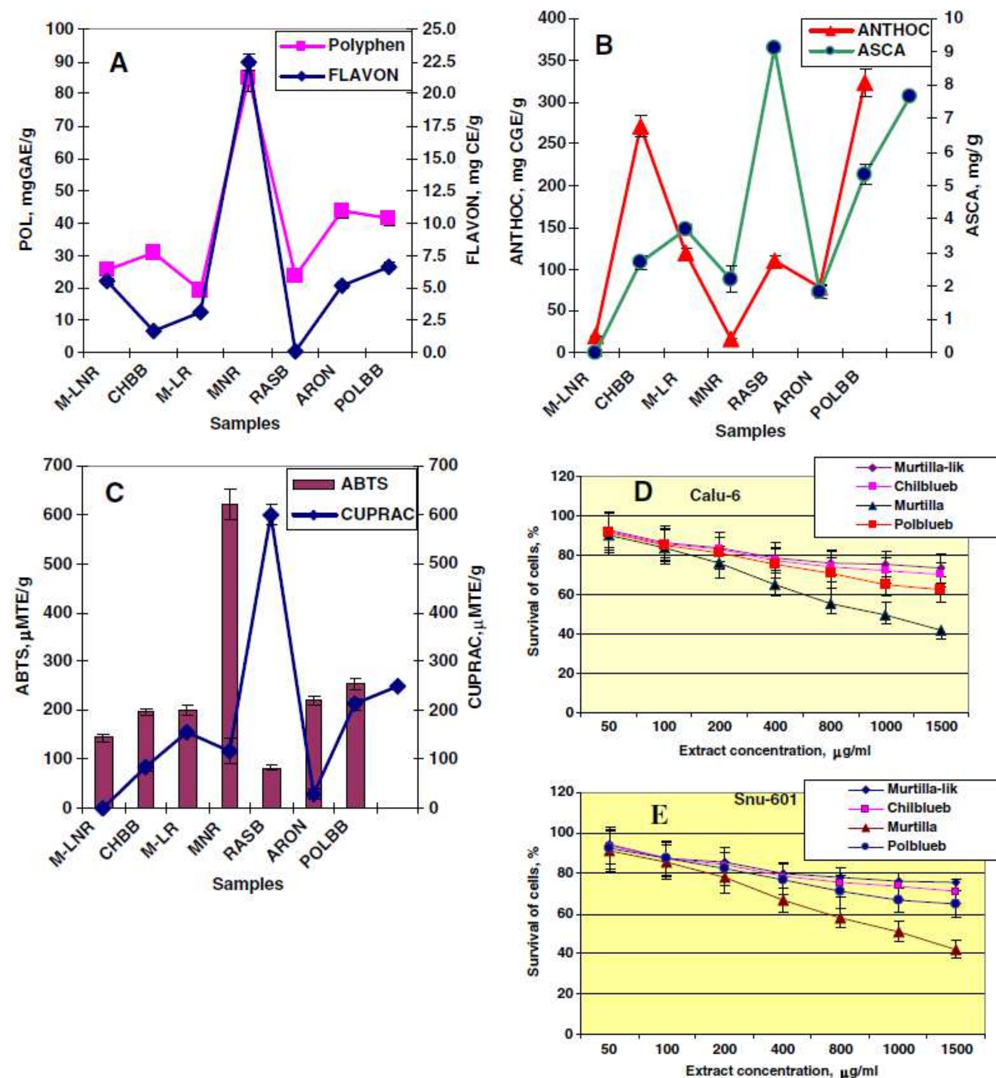
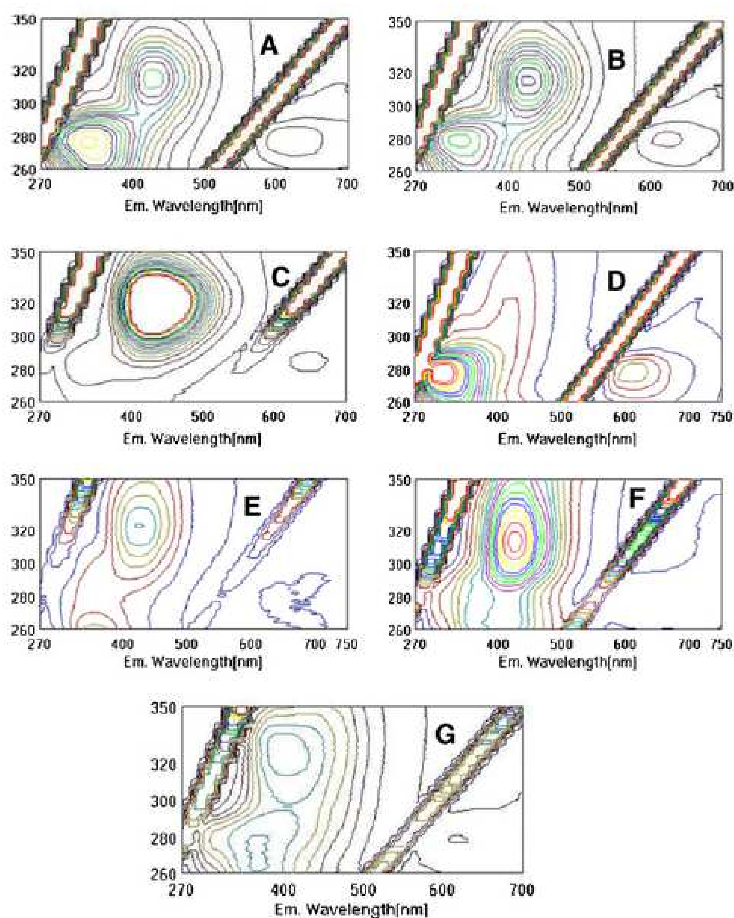


Fig. 12 **a** Total polyphenols (*Polyphen*, mg GAE/g) and flavonoids (*FLAVON*, mg CE/g); **b** anthocyanins (*ANTHOC*, mg CGE/g) and ascorbic acid (*ASCA*, mg/g); **c** antioxidant activities (µMTE/g) by *ABTS* and *CUPRAC* in the following berries: “Murtilla-like” non-ripe (*M-LNR*), Chilean blueberry (*CHBB*), “Murtilla-like” ripe (*M-LR*), “Murtilla” non-ripe (*MNR*), raspberry (*RASB*), *Aronia* (*ARON*), Polish blueberry (*POLBB*). *Abbreviations*: *GAE* gallic acid equivalent, *CE* catechin equivalent, *CGE* cyanidin-3-glucoside equivalent, *ABTS* 2, 2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diamonium salt, *CUPRAC* cupric-reducing antioxidant capacity, *TE* trolox equivalent. The survival of cells (%) of human cancer cells of the **d** Calu-6 and **e** SNU-601 in the presence of water extracts of “Murtilla-like” (*Murtilla-lik*); Chilean blueberries (*Chilblueb*); “Murtilla”, Polish blueberries (*Polblueb*). Each point represents the mean±SD (n=6)

Fig. 13 Contour maps in three-dimensional fluorescence of water extracts (2.5 mg/mL) of "Murtilla-like" non-ripe, "Murtilla-like" ripe, "Murtilla" non-ripe, raspberry, Chilean blueberries, *Aronia*, Polish blueberries (a–g). The 3D-FL were run emission mode and fluorescence intensity up to 1,000, emission wavelengths from 270 to 750 nm and excitation wavelengths from 260 to 350 nm; scanning speed was 1,000 nm/min, emission wavelength on *x*-axis and excitation wavelength on *y*-axis



The difference was only in a small shift in the case of the ripe sample and higher fluorescence intensity of "Murtilla" non-ripe sample. "Murtilla" non-ripe (Fig. 3c) showed only one main peak at *λ* em/ex 420/320 nm with FI=800; raspberry (Fig. 3d) showed the following peaks: an average one at *λ* em/ex 290/280 nm with FI=200, and a small one at em/ex 620/280 nm with FI=78. Chilean blueberries (Fig. 3e) showed one peak at *λ em*/ex 420/325 nm with FI=468, and a small one at λ em/ex 640/270 nm with FI=27. Aronia (Fig. 3f) showed one big peak at λ em/ex 420/310 nm with FI=580, and Polish blueberries (Fig. 3g) two peaks: one small at λ em/ex 380/275 nm with FI=11, and another bigger one at λ em/ex 400/330 nm.

There are not too many applications of 3D fluorescence spectra, therefore our present conclusions that 3-D fluorescence can be used as an additional tool for the characterization of the polyphenol extracts during different stages of ripening and different berries cultivars correspond with the previous data (Gorinstein et al. 2010). The interaction between BSA, urea, catechin, and berry extract is shown in Fig. 4 by the changing of fluorescence intensity and shift of the main peak. Two different concentrations of urea were used: 2.4 M at 37 °C during 1 h and the FI of BSA decreased from 878 to 605 (Fig. 4a and c). Oppositely at 4.8 M urea at the same conditions of time and temperature the FI of BSA decreased till 97, nearly full denaturation (Fig. 4e).

Partly the same binding was obtained with 2.4 M urea and addition of catechin (Fig. 4b) and water extract of "Murtilla" non-ripe (Fig. 4c). The binding of catechin was higher (Fig. 4d, FI = 646) than under the same conditions of the extract of "Murtilla" non-ripe (Fig. 4f, FI=731.2). The main peak has changed in the region of *λ* ex/em of 225–230/335 nm.The decrease of the intensity of the main peak of BSA with berry extract was about 16.7 % in comparison with catechin of 26.4 %. Other

berry samples showed the decrease from 15 to 8 %. The decrease in the fluorescence intensity is the indicator of the quenching of berries extracts in interaction with BSA.

Fourier Transform Infrared Spectra Studies

The FTIR spectra of BSA and catechin (Fig .5a, first line from the top) were compared with BSA and "Murtilla" non-ripe (Fig. 5a, second line from the top) and BSA (Fig. 5a, third line from the top). The amide I and amide II peaks of BSA (Fig. 5a, third line from the top) were shifted from 1,548 to 1,544 cm^{-1} and from 1,650 to 1,627 cm^{-1} upon interaction with catechin (Fig. 5a, first line from the top) and to 1,552 and 1,630 cm^{-1} upon interaction with "Murtilla" non-ripe extract (Fig. 6a, second line from the top). The FTIR wave numbers of catechin (Fig. 5a, third line from the top) shows broad phenolic OH band centered around 3,183 cm^{-1}, characteristic –CO stretching at 1,650 cm^{-1} aromatic bending and stretching around 1,040 and 1,650 cm^{-1}, –OH phenolic bending around 1,205 and 1,393 cm^{-1}.

The FTIR spectra of BSA (Fig. 5b, third line from the top) were compared with BSA–urea (Fig. 5b, third line from the top). The amide I and amide II peaks of BSA (Fig. 5b, third line from the top) disappeared under denaturation with urea and with urea and addition of "Murtilla" non-ripe extract (Fig. 5b, second line from the top). The phenolic OH corresponding to catechin appeared around 3,400 cm^{-1} for thecatechin–BSA complex was at 3,188 cm^{-1}. Matching between the peaks in the range from 4,000 to 400 cm^{-1} between (BSA + urea + "Murtilla" nonripe)/(BSA + urea) 0 99.8 %; (BSA + catechin)/(BSA + "Murtilla" non-ripe) = 98.05 %; (BSA + catechin)/BSA =47.38%;and (BSA + "Murtilla" non-ripe)/BSA=48%(Fig. 5a and b)

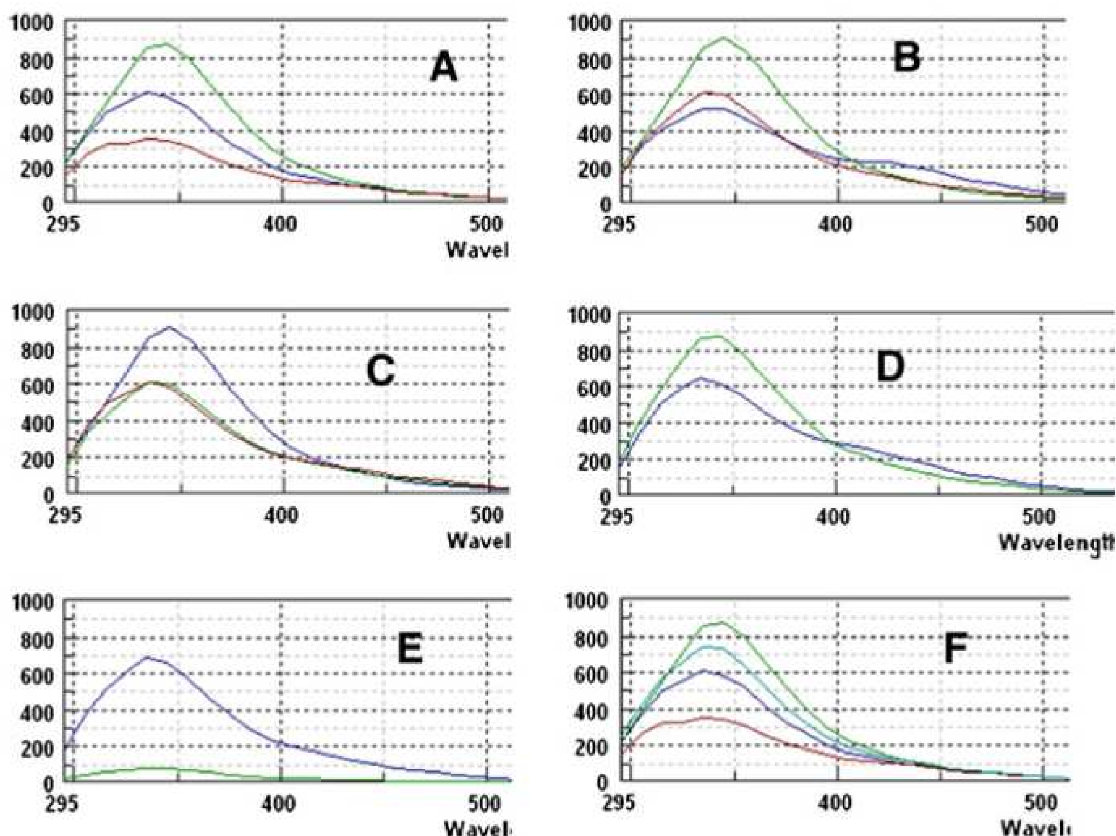


Fig. 14 Two-dimensional fluorescence spectra illustrate the interaction between BSA, catechin, urea, and water extracts of studied berries. **a** change in the fluorescence intensity as a result of binding affinity: 0.0132 μM BSA [*upper line* with fluorescence intensity of 877.8]; 0.0132 μM BSA+2.4 M urea during 1 h at 25 °C (*middle line* with FI=605); 0.0132 μM BSA+2.4 M urea+30 μM catechin during 1 h at 37 °C (*lower line* with FI=341); **b** 0.0132 μM BSA (*upper line* with FI of 877.8]; 0.0132 μM BSA+2.4 M urea during 1 h at 37 °C (*middle line* with FI=600); 0.0132 μM BSA+2.4 M urea+50 μg/ml of water extract of “Murtilla” non-ripe during 1 h at 37 °C (*lower line* with FI=525); **c** 0.0132 μM BSA (*upper line* with FI of 900), 0.0132 μM BSA+2.4 M urea during 1 h at 25 °C (*middle line* with FI=605.3); 0.0132 μM BSA+2.4 M urea+50 μg/ml of water extract of “Murtilla” non-ripe (*lower line* with FI=604.2) during 1 h at 25 °C; **d** 0.0132 μM BSA (*upper line* with FI of 878), 0.0132 μM BSA+30 μM catechin during 1 h at 25 °C (*lower line* with FI=646); **e** 0.0132 μM BSA+ 4.8 M urea at 0 time (*upper line* with FI of 686.4), 0.0132 μM BSA+ 4.8 M urea during 1 h at 25 °C (*lower line* with FI of 97); **f** 0.0132 μM BSA (*first line from the top* with FI=878), 0.0132 μM BSA+50 μg/ml of water extract of “Murtilla” non-ripe at 0 h time (*second line from the top* with FI=731.2), 0.0132 μM BSA+2.4 M urea+30 μM catechin during 1 h at 25 °C (*third line from the top* with FI=600); .0132 μM BSA+2.4 M urea+30 μM catechin during 1 h at 37 °C (*fourth line from the top* with FI=341). Fluorescence intensities are on *y*-axis and emission wavelengths on *x*-axis (*Wavel, Wavelength)*

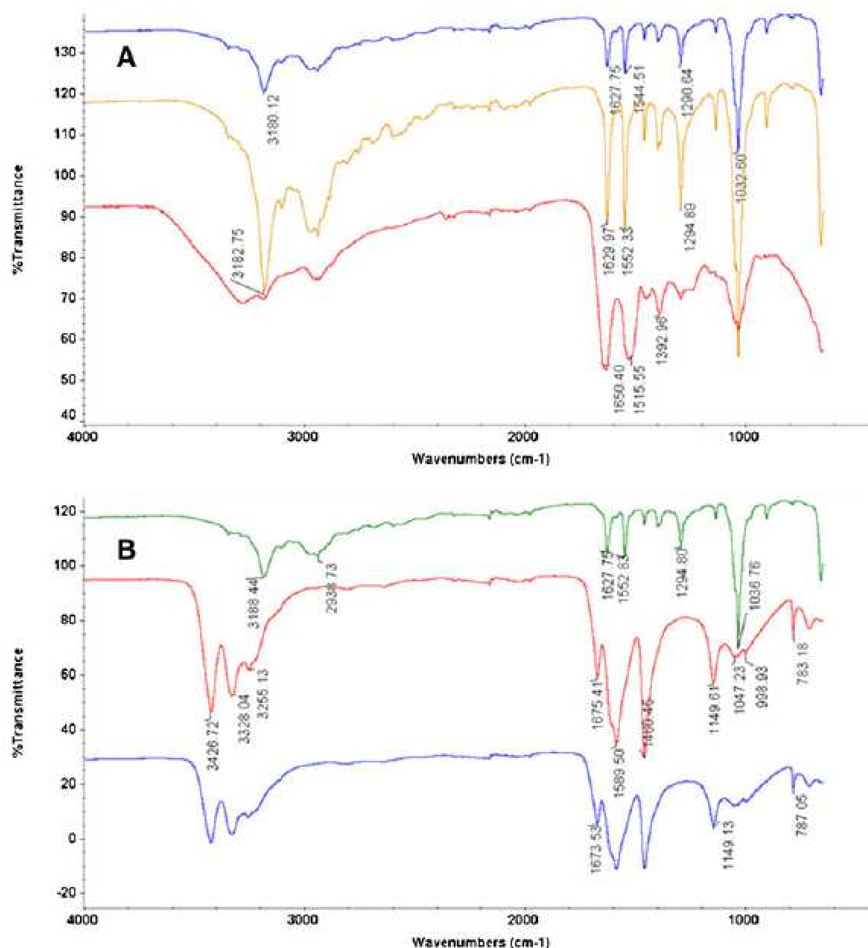
Chemometrical Processing

Chemometrical processing is an additional method to show the similarities and the differences in the investigated berries based on their bioactive compounds. The comparison of the DPPH antiradical activity (μmol TE/g DW) of investigated berries is shown in the Fig. 6a, where the highest values were in Aronia and “Murtilla” non-ripe. In order to exactly compare the quenching ability of the examined berries, the half maximal inhibitory concentration (IC_{50}), which is the concentration of the extract that inhibited DPPH free radical by 50 %, was calculated for a widely used scavenging reaction time of 30 min shown in Fig. 6b. The lower the IC_{50} value, the higher the radical-scavenging activity of the berries.

By comparing the IC_{50} value of the berries water extracts, we found that the highest radical scavenging effect was observed in “Murtilla” non-ripe and Aronia berries with IC_{50} of about 6 mg ml^{-1}. The potency of radical scavenging effect of these two extracts was about ten times greater than in

raspberry extract with the lowest antiradical activity. The scavenging activity of the extracts in decreasing order was: Aronia> “Murtilla” non-ripe > “Murtilla-like” ripe > blueberry (Chile) > “Murtilla-like” non-ripe ≥ blueberry (Poland) and raspberry (Fig. 6b). After the PCA, the dimensionality of data was reduced from 15 measured, calculated, and partially correlated original variables to the new set of uncorrelated variables—principal components, from which first two components accounted for 91.3 % of the total variability.

Fig. 15 Infrared study of FTIR spectra of a 0.0132 μM BSA+30 μM catechin during 1 h at 25 °C (*upper line from the top*), 0.0132 μM BSA+50 μg/ml of water extract of “Murtilla” non-ripe at 0 h time (*second line from the top*), 0.0132 μM BSA (*third line from the top*); **b** 0.0132 μM BSA+30 μM catechin during 1 h at 25 °C (*upper line from the top*), 0.0132 μM BSA+2.4 M urea+50 μg/ml of water extract of “Murtilla” non-ripe during 1 h at 25 °C (*second line from the top*), 0.0132 μM BSA+2.4 M urea during 1 h at 25 °C (*third line from the top*)



These new variables highly correlate with the original antiradical descriptors of absorbance reading and inhibition at 60 min in the first principal component (PC1) and DPPH scavenging activity (μmol TE/g DW) at 60 and 90 min in the second PC. Plot of these PCs (Fig. 6c) shows not very strong clustering tendency among all berry water extracts according to scavenging ability data, but some similarities between fruit groups are evident. Clusters of water extracts of Aronia and “Murtilla” non-ripe fruits, both with the relatively very high antiradical activity are well separated from “Murtilla-like” ripe and rest fruits as well as from raspberries with the lowest antioxidant activity.

A multiparametric approach of canonical discrimination analysis (CDA) was carried out in order to evaluate the influence of all DPPH antiradical parameters in the classification and

differentiation of examined water fruit extracts according to their scavenging ability. Main seven fruit species were totally and correctly separated into relevant clusters. CDA based on the selected antiradical variables indicated that the first two significant canonical discriminant functions with eigenvalues > 1, Wilk's lambda ~ 0, and Chi-square test significance P < 0.000λ explained 98.9 % of cumulative variance (first function 93.8 %).

Taking into account the coefficients of canonical discriminant functions (data not presented here), the most significant contribution to discrimination in the first function was obtained from absorbance readings and inhibition value in reaction time 30 min and in the second function absorbance reading and inhibition in time 60 min. The stepwise discrimination found the DPPHantiradical activity after 1 min of reaction time as the most discriminant variables. Furthermore, the classification matrix gave evidence that the studied water extracts were correct, with 100 % success rate, classified to their fruit classes according to their DPPH scavenging ability.

Mass Spectra Data

The spectrum shows the main m/z peaks found in berries (Fig. 7a–d) in water fraction with relative abundance (RA, %) from 20 to 100 %. The main peak was about 192–193, which mostly belongs to ferulic acid (Gómez-Romero et al. 2011). The RA of the obtained peaks showed the difference in the amount of polyphenol compounds in these samples.

Discussion

It was of great interest to compare "Murtilla-like" with "Murtilla" in order to find out if the "Murtilla-like" bioactivity is on the same level as of original "Murtilla". Therefore, the contents of the bioactive compounds and AA were determined and compared with the widely consumed blueberries, red raspberries, and chokeberries. A number of reviewed articles showed that the main bioactive compounds determining the nutritional quality of berries are polyphenols, anthocyanins, and flavonoids (Battino et al. 2009; Bowen-Forbes et al. 2010; Cuevas-Rodriguez et al. 2010; Dai et al. 2009). As was declared in "Results", the contents of bioactive compounds (polyphenols, flavonoids, anthocyanins, and ascorbic acid) in water extracts was determined and compared, and the significantly highest were in water extract of non-ripe "Murtilla".

Also the antioxidant activity according to ABTS and CUPRAC was significantly higher in water extract of non-ripe "Murtilla". Our results were in agreement with others, showing that water extracts of blackberries contain high amounts of anthocyanins (Dai et al., 2009). The results show promising perspectives for the exploitation of non-traditional tropical fruit species with considerable levels of nutrients and antioxidant capacity. Our data add valuable information to current knowledge of the nutritional properties of tropical fruits, such as the considerable antioxidant capacity found for acerola—Malpighiaemarginata and camu-camu—Myrciariadubia (ABTS, DPPH, and FRAP) and for puçá-preto—Mouriripusa (all methods).

"Murtilla" in comparison with other 18 non-traditional tropical fruits from Brazil has an average value of antioxidants (Rufino *et al*. 2010). For dry matter the order observed was: bacuri> carnauba > yellow mombin> java plum >umbu> cashew apple >mangaba> assai

>murta>gurguri>puçá-coroa-de-frade>uvaia>nance>jaboticaba>jussara>puçá-preto>acerola>camu-camu. When evaluated by the ABTS method, our fruits ranged from 6.3 to 153 μmol TE/g FW and from 16.4 to 1,237 μmol TE/g DW. FRAP values were 11.8–279 and 16.1–2,502 μmol$FeSO_4$/g, respectively. Our data are in agreement with these results. The order of increasing antioxidant capacity, measured by the ABTS method, was: umbu< yellow mombin< carnauba < cashew apple <mangaba<assaí<uvaia< java plum<gurguri<jaboticaba<puçá-coroa-de-frade<murta (Peña-Neira et al. 2007).

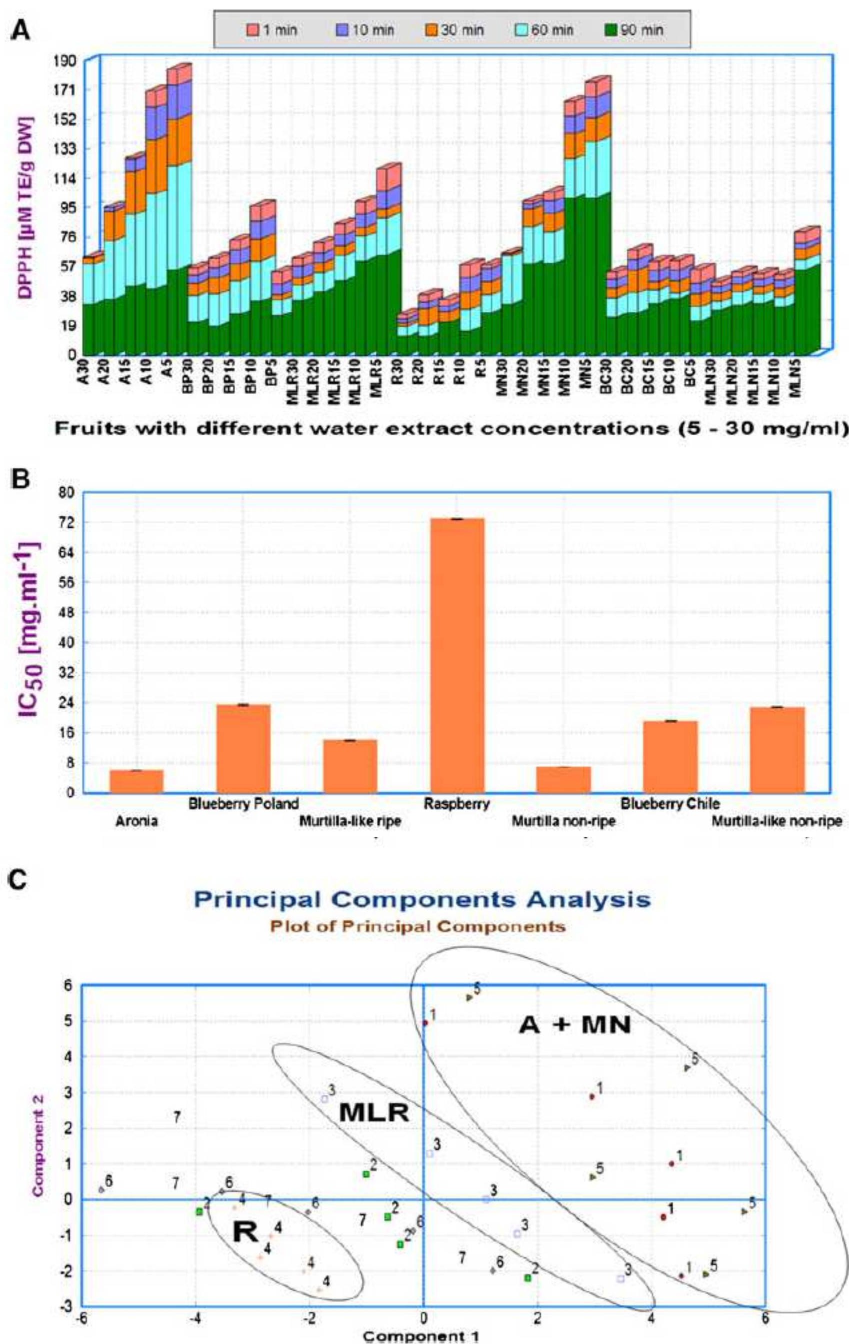


Fig. 16 a Overlap bar chart comparing the water extracts by DPPH antiradical activity (μM TE/g DW) of investigated berries (*A Aronia*, *BP* blueberry Poland, *MLR* "Murtilla-like" ripe, *R* raspberry, *MN* "Murtilla" non-ripe, *BC* blueberry Chile, *MLN* "Murtilla-like" non-ripe) according to reaction time contribution at 1, 10, 30, 60, and 90 min. **b** IC_{50} bar chart of DPPH-radical scavenging activity in the water extract of berries. The lower the IC_{50} values the higher antiradical activity. Data were performed in triplicates ($n=3$) for a reaction time of 30 min, and in the range of extract concentration was from 5 to 30 mg ml^{-1}. **c** Differentiation of the berry water extracts by the principal component analysis. Score plot on the first two components of the DPPH scavenging parameters (*1 Aronia* (*A*), *2* blueberry Poland, *3* "Murtilla-like" ripe (*MLR*), *4* raspberry (*R*), *5* "Murtilla" non-ripe (*MN*), *6* blueberry Chile, *7* "Murtilla-like" non-ripe); extract concentrations: 30, 20, 15, 10, and 5 mg ml^{-1}; reaction times: 1, 10, 30, 60, and 90 min)

Vitamin C of "Murta" was 181 mg/100 g FW (6.98 mg/g DW) which is approximately equal to our results (Fig. 2b). The anthocyanins were about 143 mg/100 g FW (5.52 mg/g DW) and this number is lower than our results (Fig. 2b). The polyphenols in "Murtilla" were 20.55 mg GAE/g DW

and this number is lower than our results (Fig. 2a).

The antioxidant activity (μmol TE/g DW) by ABTS was about 166. A positive and significant correlation was found in this study between vitamin C-extractable polyphenols and ABTS (R^2 0 0.70). Polyphenols and DPPH results expressed as antioxidant concentrations corresponding to 50 % scavenging activity were negatively and significantly correlated (R^2 0 0.72; $P<0.05$); this is due to the fact that the DPPH method yields inversely proportional results. There was also a positive and significant correlation of polyphenols ($P < 0.05$) and ABTS (R^2 0 0.92) assay (Rufino et al. 2010).

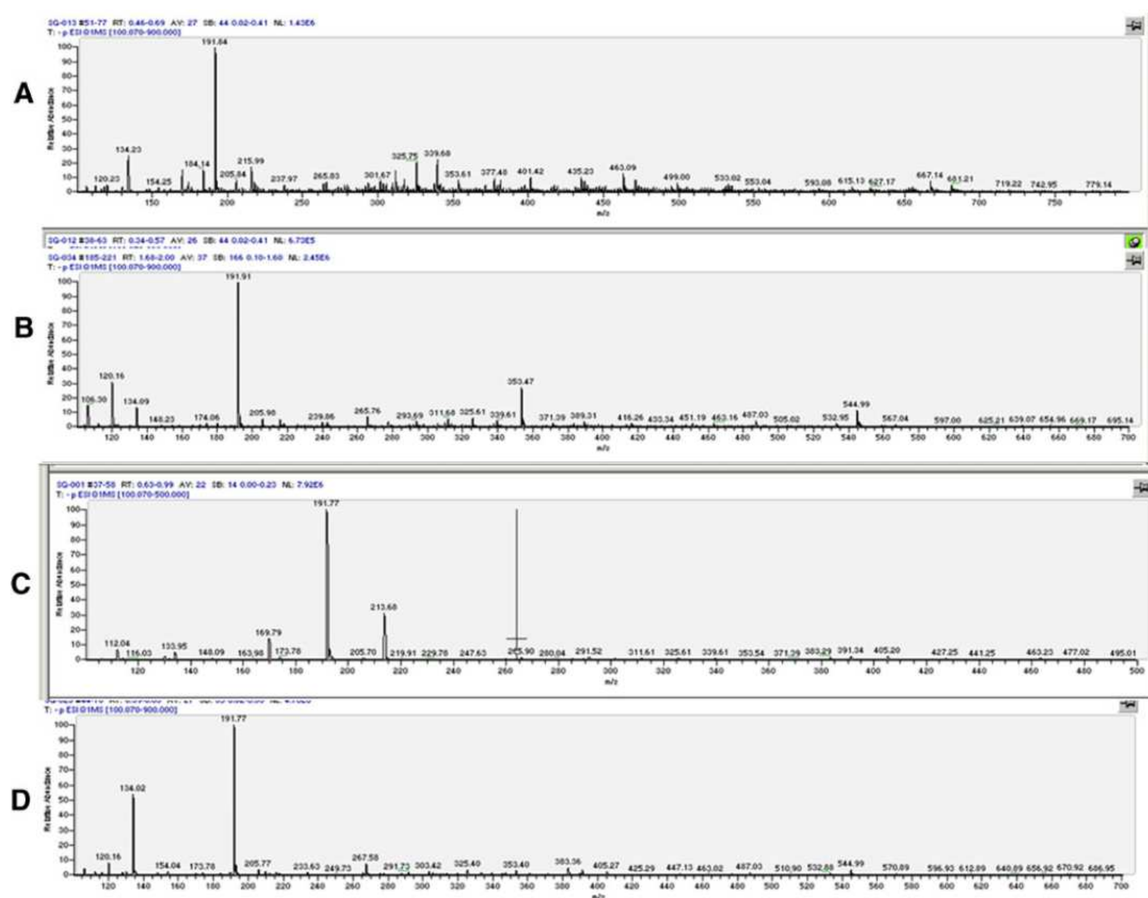


Fig. 17 ESI-MS spectra of water fractions of the following berries: **a**, **b**, **c**, **d** "Murtilla-like" non-ripe, Chilean blueberry, "Murtilla" non-ripe, Polish blueberry, respectively, in negative ion mode

These data are in agreement with our results. Our results correspond also with the research approach of Wu et al. (2006), where concentrations of total anthocyanins varied considerably from 0.7 to 1,480 mg/100 g FW in gooseberry ('Careless' variety) and chokeberry, respectively. Total phenolic content and total anthocyanin content of four berry fruits (strawberry, Saskatoon berry, raspberry, and wild blueberry), chokecherry, and seabuckthorn ranged from 22.83 to 131.88 g/kg and 3.51 to 13.13 g/kg, respectively, which corresponds with our results.

Our data can be comparable with another report (Cuevas-Rodriguez et al., 2010), where the proanthocyanidins (condensed tannins) were present in the blackberry fruits. The average anthocyanin concentration was 49.2 mg/g in the commercial cultivar Tupy, while in the wild genotypes and the breeding line, the range was 361.3 – 494.9 mg/g (cyanidin 3-O-glucoside equivalent). The proanthocyanidin concentration varied widely among wild genotypes (417.5–1,343.6 mg/g CE).

Comparison of different fractions of water extracts from of wild blackberry A. chilensis (Mol)

Stuntz (Elaeocarpaceae), corresponded with our results. Wu et al. (2006) showed that in chokeberry the amount of anthocyanins was 1,480 mg/100 g FW (52.54 mg/g DW), for red raspberry 92.1 mg/100 g FW (6.48 mg/g DW). Also other authors reported similar results. So, Ruiz et al. (2010) found the highest total polyphenol content in maqui, followed by calafate and "Murtilla". Reported high anthocyanin content in calafate berries berries (17.81±0.98 μmol g^{-1}) are comparable with those indices found in maqui (17.88±1.15 μmol g^{-1}).

The AA of "Murtilla-like", blueberries and red raspberries was comparable. Also other reported different AA data in different cultivars harvested in different seasons (Ruiz et al., 2010). According to these authors the means of AA for calafate, maqui, and Murtilla were 74.4 ± 15.9, 88.1 ± 21.5, and 11.7 ± 2.3 μmol TE/g FW, respectively. Seeram (2010) discussed also that phytonutrients ranged from fat-soluble/lipophilic to water-soluble/hydrophilic compounds.

Conclusions in the report of Elberry et al. (2010) are in line with our results about the high antioxidant activity of berries. Our results are in accordance with You et al. (2011), where four Rabbiteye blueberry cultivars (Powderblue, Climax, Tifblue, and Woodward) grown organically and conventionally were compared regarding their chemical profiles and antioxidant capacity in terms of total phenolic content, total anthocyanin content, and antioxidant values by ABTS, DPPH, FRAP, and CUPRAC. Total phenolics, flavonoids, and anthocyanins (mg/g FW) were in blueberry 261–585, 50, and 25–495 and in raspberry 121, 6, and 99; antioxidant activity (μmolTrolox/g FW) for blueberry 14 by ABTS and 25.3 by DPPH assays (Li et al., 2012). The result from this study indicated that blueberries had very high ORAC values, and higher antioxidant capacity than other selected fruits and vegetables (Wulf et al., 2005).

The comparison of the results of different solvents in Dabai fruit parts (methanol, ethanol, ethyl acetate, acetone, and water) and total phenolics, total flavonoids, total anthocyanins, and antioxidant capacity ($ABTS^+$ and FRAP assays) were in accordance with our data (Khoo et al., 2012). The acetone extract had maximum phenol and flavonoid content and showed best DPPH free radical scavenging activity and reducing capacity assessment. Ethyl acetate extract showed best superoxide radical scavenging activity, while aqueous extract showed best hydroxyl radical scavenging activity (Chanda and Kaneria, 2012).

II. KIWI FRUITS

Bioactive compounds and the antioxidant capacity in new kiwi fruit cultivars

Results and discussion

Polyphenols, flavonoids, flavanols and tannins

The combination of determination of bioactive compounds as total phenols, total flavonoids, total flavanols and tannins, determined spectroscopically, and with antioxidant assays, fluorescence and mass spectra can be used in comparison and fingerprinting analysis of new kiwi fruit cultivars. These methods can be used for rapid distinguishing of the cultivars.

The results of the determination of the contents of these bioactive compounds in all seven studied kiwi fruits cultivars are shown in the Table 1. As can be seen, the contents of polyphenols in

ethanol and water extracts were significantly higher than in acetone and hexane extracts (P in all cases < 0.05). The contents of flavonoids in ethanol extract were significantly higher in ‘Haenam’ and ‘Bidan’, in water extracts – in ‘SKK12’ and ‘Hwamei’, in acetone and hexane extracts – in ‘Bidan’ (P in all cases <0.05). The contents of flavanols in ethanol and water extracts were significantly higher in ‘Haenam’, and ‘Bidan’, in acetone and hexane extracts – in ‘Haenam’ (P in all cases < 0.05). The contents of tannins in ethanol extracts were significantly higher in ‘SKK12’, in water and acetone extracts – in ‘Bidan’, and in hexane extracts – in ‘SKK12’ (P in all cases < 0.05).

As can be seen, the contents of the bioactive compounds extracted by different solvents differ significantly: the content of the main bioactive compound – polyphenols was significantly higher in ‘SKK12’, ‘Hwamei’ and ‘Bidan’ (P < 0.05).

Antioxidant capacity

The results of the determination of the level of antioxidant capacity of seven studied kiwi fruit cultivars are shown in the Table 2. As can be seen: (a) according to all assays the significantly highest level of AC in all extracts was in ‘SKK12’, following by ‘Hwamei’ and ‘Bidan’ (P < 0.05). ABTS and CUPRAC are two electron transfer assays and therefore the obtained results are similar. As can be seen, according to all four used assays, the significantly highest level of antioxidant capacity was registered in ‘Bidan’, ‘SKK12’ and ‘Hwamei’ cultivars (P < 0.05). As was shown above, these cultivars have also the highest content of polyphenols among studied cultivars (Table 1).

Fluorometric data

The 3D-FL of kiwi fruit cultivars ethanol extracts differ by the wavelengths of the peaks and their fluorescence intensity (FI), and could be classified according to the fluorescence results to three groups ‘Hayward’(including ‘Daheung’, ‘Haenam’, Hwamei’ and ‘SKK12’), ‘Bidan’ and ‘Hort 16A’. The following common peaks appeared in three groups: at λ ex/em of 290/220, 400/230 and 600/ 210 nm. ‘Hort 16A’ showed one big peak at 400/300 nm, which was not found in any of cultivars. ‘Hwamei’, which is similar to ‘Hayward’ showed one peak at 300/280, characteristic only for this cultivar. At λ ex/em of 700/400 nm the biggest prominent peak was in ‘Bidan’ cultivar, decreasing for ‘Hayward’, ‘Hwamei’ and’ Hort 16A’ (Fig. 1C, B, D and A, respectively).

The binding properties of the kiwi fruit samples in comparison with the pure flavonoids such as catechin are shown in two-dimensional fluorescence spectra (2D-FL). One of the main peaks for HSA was found at λ ex/em of 220/357nm (Fig. 1E). The interaction of HSA and the ethanol extracts of kiwi fruit cultivars (Fig. 1E) showed slight change in the position of the main peak at the wavelength of 357 nm and the decrease in the fluorescence intensity (FI). The following changes appeared when the ethanol extracts of kiwi fruit were added to HSA (initially the main peak at emission 357 nm and FI of 961.00 (Fig. 1E, the upper line is HSA). The reaction with the kiwi fruit extracts and catechin decreased the FI of HSA (Fig. 1E, the lowest line).

The following decrease in the FI (%) occurred during the interaction of ethanol extracts with HSA: HSA + ‘Hayward’ = 3.86; HSA + ‘Haenam’ = 6.71; HSA + ’Hort 16A’ = 7.63; HSA + ‘Bidan’ = 10.18; HSA + ’Bidan’ = 12.03; HSA + ’Hwamei’ = 15.05; HSA + ’SKK 12’=11.65;

HSA+catechin=15.41. The water extracts showed the results of the decrease (%) of HSA intensity (Fig. 1F): HSA + 'Hayward' = 2.03; HSA+'Hort 16A' = 10.79; HSA + 'Bidan' = 15.47; HSA + catechin = 15.89; HSA + 'Hwamei' = 18.76; HSA + 'SKK 12'=21.24.

These data were slightly higher than with ethanol extracts and such strong binding properties of water extracts are proportional to their amount of polyphenols (Table 1). These results were in direct relationship with the antioxidant capacities of the extracts (Table 2). The synergism of bioactive compounds is shown when to the mixture of HSA and extracts of kiwi fruit catechin was added.

Our very recent results showed that the fluorescence is significantly quenched, because of the conformation of proteins, phenolic acids and flavonoids (Namiesnik *et al*., 2013; Cao et al, 2011). This interaction was investigated using tryptophan fluorescence quenching. Our result is in agreement with others that quercetin, as an aglycon, is more hydrophobic and demonstrates strong affinity toward HSA. Other results (Xiao et al., 2011) differ from the reported by us, probably because of the variety of antioxidant abilities of pure flavonoids and different ranges of fluorometry scanning ranges used in a similar study.

The strong binding properties of phenolic show that they may be effective in prevention of atherosclerosis under physiological conditions.Quercetin can suppress HSA. Much of the bioactivities of citrus flavanones significantly appear to impact blood and micro vascular endothelial cells, therefore it was essential to investigate the interaction between kiwi fruit polyphenols and serum albumin. The binding constants ranked in the following order quercetin>rutin>calycosin> calycosin-7-O-(sup)-D-glucoside (formononetin-7-O-(sup)-D-glucoside (Liu *et al*., 2010). 3-D fluorescence can be used as an additional tool for the characterization of the polyphenol extracts of kiwi fruit cultivars and their binding properties.

MS spectra

The ESI-MS in negative ion mode of studied extracts slightly differ between cultivars. As it was shown previously the cultivars were classified according to fluorometric measurements to three groups: 'Hayward' (including 'Daheung', 'Haenam', Hwamei' and 'SKK12'), 'Bidan' and 'Hort 16A'. There were done all the spectra analyses, but only these groups are presented in Fig. 2 and Table 3. In all cultivars the main peak was at m/z 190.97 (100%) corresponded to quinic acid (Table 3, Fig. 2), but small peaks differ from one groupto another (Table 3).

'Hwamei' slightly differ in methanol extracts from the other four cultivars which belong to the 'Hayward' group (Table 3, Fig. 2B). MeOH/water/50/50 showed as well differences in these 3 groups (Table 3, Figs. 2B, F, J). 'Bidan' contained also the main peak with m/z 191(100%) with average peaks different from the first group such as 308.95 and 366.91 (Table 3). MeOH/water/formic acid/50%/49%/1% extracts were different and contained different peaks mostly in 'Hayward' and 'Bidan' groups of m/z 370.97 and 225.02, respectively (Table 3, Figs. G, K).

Acetone fractions of the groups showed one main peak of m/z 191 with a number of small peaks with different masses (Table 3, Figs. 2D, H, L, P). As can be seen all kiwi fruit ethanol extracts characterised by chlorogenic acid of the (M-H) – deprotonated molecule (m/z 353) and the ion corresponding to the deprotonated quinic acid (m/z 191), which was consistent with Sun, Liang, Bin, Li, and Duan (2007). The recorded spectra were in the same scale (in the range between 100 and 600

m/z) for comparison.

We choose negative mode for the MS method because in many publications was described that this mode is the best for analysis of low-molecular phenolic compounds (Gómez-Romero *et al*., 2011; Sun *et al*., 2007). The main peaks were identified and the recorded

Table 11
Bioactive compounds of seven kiwi fruit cultivars in ethanol (Et), water (W), acetone (Ac) and hexane (He) extracts.[1,2,3]

	POL (mg GAE/g)	FLAVON (mg CE/g)	FLAV (μg CE/g)	TAN (mg CE/g)
HaywardEt	4.48 ± 0.44[a]	1.22 ± 0.12[a]	37.84 ± 3.67[de]	2.84 ± 0.26[c]
Daheung Et	4.18 ± 0.40[a]	0.99 ± 0.11[a]	5.82 ± 0.56[a]	1.63 ± 0.16[a]
HaenamEt	6.82 ± 0.55[b]	4.25 ± 0.41[c]	42.96 ± 0.45[e]	2.85 ± 0.21[c]
BidanEt	11.45 ± 1.12[c]	4.32 ± 0.38[c]	15.80 ± 1.51[c]	2.48 ± 0.23[b]
Hort16AEt	10.23 ± 1.07[c]	1.23 ± 0.09[a]	31.88 ± 3.21[d]	2.88 ± 0.28[c]
SKK12Et	14.48 ± 1.46[d]	2.39 ± 0.21[b]	10.53 ± 1.07[b]	3.01 ± 0.28[c]
HwameiEt	13.11 ± 1.29[cd]	2.23 ± 0.21[b]	9.46 ± 0.98[b]	2.81 ± 0.27[c]
HaywardW	5.30 ± 0.45[a]	0.57 ± 0.12[a]	16.35 ± 1.65[b]	1.17 ± 0.14[a]
DaheungW	5.50 ± 0.54[a]	0.55 ± 0.06[a]	7.90 ± 0.78[a]	1.57 ± 0.14[b]
HaenamW	7.69 ± 0.69[b]	0.70 ± 0.09[b]	8.87 ± 0.88[a]	1.17 ± 0.11[a]
BidanW	13.97 ± 1.32[d]	1.00 ± 0.11[b]	39.92 ± 3.83[d]	3.04 ± 033[d]
Hort16AW	11.08 ± 1.14[c]	1.37 ± 0.13[c]	8.59 ± 0.81[a]	2.37 ± 2.24[c]
SKK12 W	16.34 ± 1.11[e]	1.75 ± 0.07[d]	19.68 ± 1.94[c]	1.60 ± 0.03[b]
HwameiW	14.23 ± 1.39[d]	1.62 ± 0.11[d]	14.47 ± 1.44[ab]	2.50 ± 0.15[c]
HaywardAc	1.15 ± 0.05[a]	0.61 ± 0.07[b]	18.91 ± 1.87[e]	1.42 ± 0.18[c]
DaheungAc	0.84 ± 0.07[a]	0.48 ± 0.06[a]	2.98 ± 0.27[a]	0.82 ± 0.09[a]
HaenamAc	1.82 ± 0.04[b]	2.11 ± 0.24[d]	21.43 ± 2.32[f]	1.43 ± 0.16[c]
BidanAc	3.39 ± 0.33[d]	2.17 ± 0.22[d]	7.84 ± 0.78[c]	1.25 ± 0.13[b]
Hort16AAc	2.74 ± 0.21[c]	0.62 ± 0.08[b]	15.91 ± 1.58[d]	1.44 ± 0.15[c]
SKK12Ac	5.11 ± 0.52[e]	1.21 ± 0.23[c]	5.24 ± 0.51[b]	1.51 ± 0.16[c]
HwameiAc	4.85 ± 0.48[e]	1.12 ± 0.12[c]	4.71 ± 0.47[b]	1.45 ± 0.14[c]
HaywardHe	0.49 ± 0.03[a]	0.42 ± 0.07[a]	12.63 ± 1.32[d]	0.95 ± 0.9[b]
DaheungHe	0.31 ± 0.04[a]	0.32 ± 0.03[a]	1.97 ± 0.19[a]	0.55 ± 1.2[a]
HaenamHe	1.15 ± 0.13[b]	1.43 ± 0.16[c]	14.31 ± 1.34[e]	0.95 ± 0.7[b]
BidanHe	2.07 ± 0.25[c]	1.45 ± 0.14[c]	5.26 ± 0.52[c]	0.83 ± 0.6[b]
Hort16AHe	1.67 ± 0.14[bc]	0.41 ± 0.04[a]	10.63 ± 1.13[cd]	0.96 ± 0.5[b]
SKK12He	3.42 ± 0.33[d]	0.81 ± 0.08[b]	3.49 ± 0.32[b]	1.03 ± 0.09[b]
HwameiHe	3.04 ± 0.33[d]	0.75 ± 0.07[b]	3.14 ± 0.31[b]	0.97 ± 0.09[b]

POL, polyphenols; FLAVON, flavonoids; FLAV, flavanols; TAN, tannins; CE, catechin equivalent; GAE, gallic acid equivalent; HaywardEt, DaheungEt, HaenamEt, HwameiEt, Hort16AEt, SKK12Et and BidanEt, kiwi fruit cultivars extracted with 100% ethanol; HaywardW, DaheungW, HaenamW, HwameiW, Hort16AW, SKK12W and BidanW, kiwi fruit cultivars extracted with water; HaywardAc, DaheungAc, HaenamAc, HwameiAc, Hort16AAc, SKK12Ac and BidanAs, kiwi fruit cultivars extracted with acetone; HaywardHe, DaheungHe, HaenamHe, HwameiHe, Hort16He, SKK12He and BidanHe, kiwi fruit cultivars extracted with hexane.
[1] Values are means ± SD of 5 measurements.
[2] Values in columns for every bioactive compound with the same solvent bearing different superscript letters are significantly different ($P < 0.05$).
[3] Per g dry weight.

Table 12
The antioxidant capacities of seven kiwi fruit cultivars (μmolTE/g DW) in ethanol[A], water[B], acetone[C], and hexane[D] extracts.[1,2,3]

	Hayward	Daheung	Haenam	Bidan	Hort 16A	SKK12	Hwamei
ABTS[A]	18.21 ± 1.65[a]	17.42 ± 1.65[a]	22.43 ± 2.18[a]	34.25 ± 3.23[c]	31.15 ± 3.11[b]	37.18 ± 3.65[c]	33.25 ± 3.31[b]
ABTS[B]	20.41 ± 2.11[a]	22.40 ± 2.23[a]	26.18 ± 2.43[a]	39.16 ± 3.87[c]	34.12 ± 3.41[b]	42.14 ± 4.32[d]	39.35 ± 3.87[c]
ABTS[C]	4.82 ± 0.45[a]	4.05 ± 0.42[a]	5.42 ± 0.52[a]	12.41 ± 1.24[ab]	11.12 ± 1.11[ab]	14.15 ± 1.43[b]	13.16 ± 1.31[b]
ABTS[D]	1.61 ± 0.15[a]	1.42 ± 0.14[a]	1.83 ± 0.18[a]	4.23 ± 0.41[b]	4.11 ± 0.41[b]	4.83 ± 0.48[b]	4.52 ± 0.45[b]
CUPRAC[A]	20.18 ± 2.04[a]	19.44 ± 1.87[a]	24.12 ± 2.32[ab]	35.42 ± 3.23[bc]	32.14 ± 2.16[b]	38.15 ± 3.87[c]	34.18 ± 3.21[bc]
CUPRAC[B]	21.14 ± 2.11[a]	23.40 ± 1.87[a]	27.41 ± 2.12[b]	40.18 ± 3.23[d]	35.61 ± 2.76[c]	43.27 ± 3.23[d]	40.91 ± 3.45[d]
CUPRAC[C]	4.01 ± 0.32[a]	4.94 ± 0.27[a]	6.12 ± 0.54[ab]	13.13 ± 1.21[c]	12.43 ± 0.85[b]	15.25 ± 1.32[d]	14.21 ± 1.34[c]
CUPRAC[D]	1.51 ± 0.13[a]	1.38 ± 0.11[a]	1.73 ± 0.14[ab]	4.11 ± 0.41[bc]	3.85 ± 0.34[b]	4.63 ± 0.43[c]	4.41 ± 0.27[bc]
FRAP[A]	6.12 ± 0.56[a]	5.42 ± 0.54[a]	10.21 ± 1.01[ab]	18.44 ± 1.76[c]	11.25 ± 1.12[b]	21.15 ± 1.71[c]	20.14 ± 1.98[c]
FRAP[B]	7.12 ± 0.65[a]	7.88 ± 0.67[a]	11.33 ± 1.08[ab]	21.32 ± 1.78[c]	13.12 ± 1.31[b]	24.55 ± 2.18[c]	23.11 ± 2.11[c]
FRAP[C]	1.58 ± 0.15[a]	1.15 ± 0.09[a]	2.43 ± 0.18[ab]	4.75 ± 0.28[c]	3.81 ± 0.32[b]	5.36 ± 0.43[c]	5.05 ± 0.41[c]
FRAP[D]	0.53 ± 0.04[a]	0.48 ± 0.03[a]	0.81 ± 0.07[ac]	1.65 ± 0.09[c]	1.31 ± 0.12[b]	1.98 ± 0.11[d]	1.79 ± 0.14[d]
DPPH[A]	6.95 ± 0.54[a]	5.80 ± 0.45[a]	7.65 ± 0.45[ab]	14.41 ± 1.34[c]	11.18 ± 1.13[b]	17.23 ± 1.43[d]	15.42 ± 1.28[c]
DPPH[B]	6.08 ± 0.56[a]	6.90 ± 0.43[a]	9.14 ± 0.41[ab]	17.15 ± 1.54[c]	13.24 ± 1.43[b]	18.42 ± 1.67[d]	17.85 ± 1.87[c]
DPPH[C]	1.75 ± 0.17[a]	1.41 ± 0.12[a]	2.18 ± 0.15[ab]	4.15 ± 0.32[c]	3.18 ± 0.23[b]	4.87 ± 0.28[c]	4.37 ± 0.32[c]
DPPH[D]	0.65 ± 0.07[a]	0.52 ± 0.05[a]	0.74 ± 0.08[ab]	1.48 ± 0.12[c]	1.21 ± 0.09[b]	2.03 ± 0.04[d]	1.74 ± 0.06[c]

[1]Values are means ± SD of 5 measurements; [2]Values in columns for kiwi fruits with the same solvent bearing different superscript letters are significantly different ($P < 0.05$); [3]per g dry weight. Cupric reducing antioxidant capacity (CUPRAC), 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Ferric-reducing/antioxidant power (FRAP).
[A,B] Extracted at room temperature in concentration of 25 mg lyophilized sample in 1 mL ethanol, 1 mL water, respectively.
[C] Extracted at room temperature in concentration of 40 mg lyophilized sample in 1 ml acetone.
[D] Hexane.
[1] Values are means ± SD of 5 measurements.
[2] Values in rows with different superscript letters are significantly different ($P < 0.05$).
[3] Per g dry weight.

MS spectra can be used asa fingerprint for characterisation of different kiwi fruit cultivars, based on the percentage of the main peaks. The most abundant is chlorogenic acid. This is in agreement with Mittelstadt et al., (2013), who showed that one of the novel aspects of kiwi fruit is the presence of a high level of quinicacid which contributes to the flavour of the fruit. Quinic acid

metabolism intersects with the shikimate pathway, which is responsible for the de novo biosynthesis of primary and secondary aromatic metabolites. Our results are in accordance with Clifford (2000),

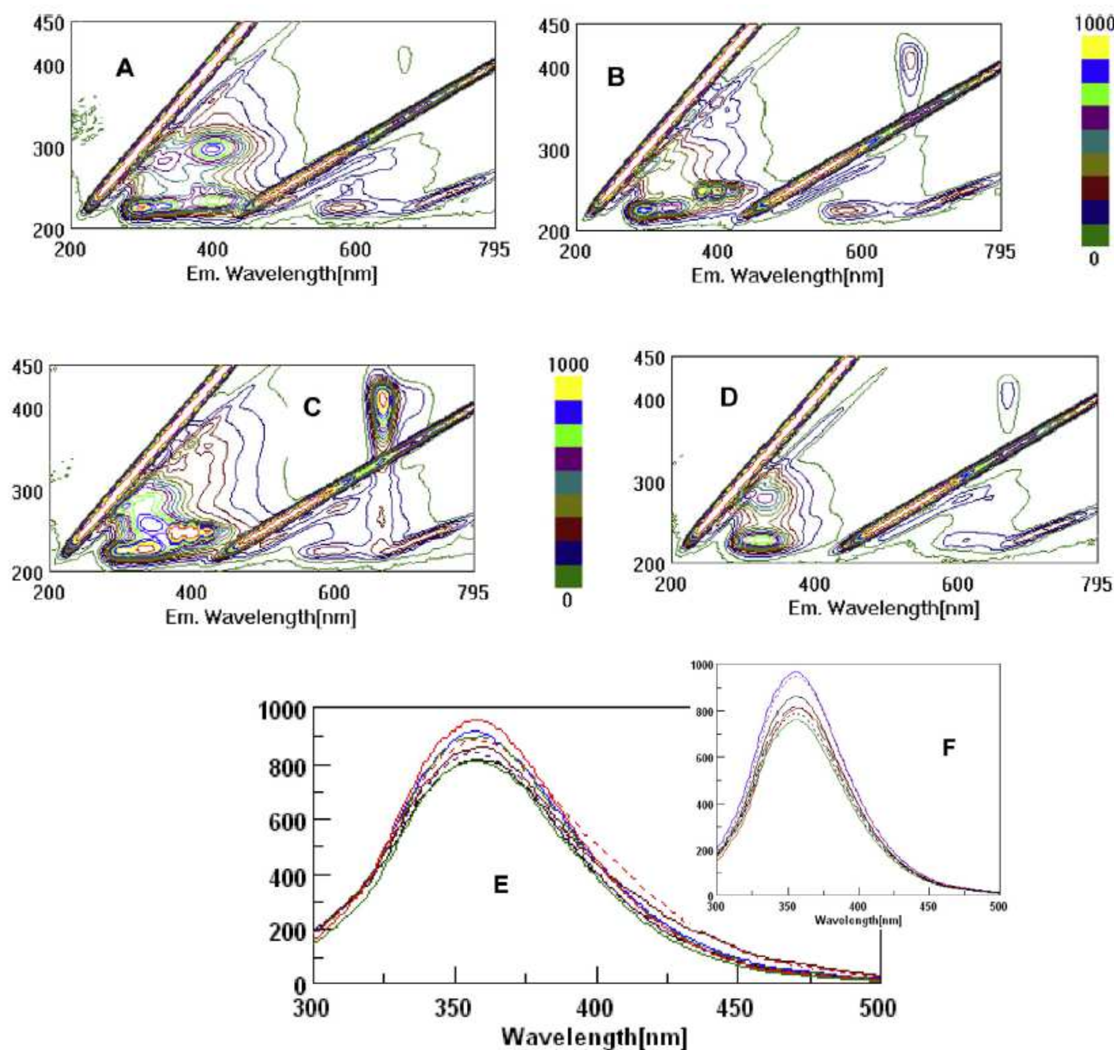


Fig. 18 Contour maps of three dimensional fluorescence (3D-FL) spectra of ethanol extracts of A, B, C, D, 'Hort16A' 'Hayward'; 'Bidan', D, and 'Hwamei'. 2D-FL spectrum illustrate the interaction between humam serum albumin (HSA), catechin, ethanol (E) and water (insert F) extracts of kiwi fruit cultivars. The change in the fluorescence intensity as a result of binding affinity with kiwi fruit extracts: E, HSA [first line from the top with fluorescence intensity (FI) of 961.00]; HSA + 'Hayward' (second line from the top with FI = 923.94), HSA + 'Haenam' (third line, FI = 896.54), HSA + 'Hort16A' (fourth line, FI = 887.66), HSA + 'Bidan' (fifth line, FI = 863.18), HSA + 'Hwamei' (sixth line, FI = 845.40), HSA + 'SKK12' (seventh line, FI = 816.41), HSA + catechin (eighth line, FI = 812.90). Insert F, HSA [first line from the top with fluorescence intensity (FI) of 967.64]; HSA + 'Hayward' (second line from the top with FI = 948.00), HSA + 'Hort16A' (third line, FI = 863.23), HSA + 'Bidan' (fourth line, FI = 817.90), HSA + catechin (fifth line, FI = 813.85), HSA + 'Hwamei' (sixth line, FI = 786.39), HSA + 'SKK12' (seventh line, FI = 762.12). In all reactions were used the following conditions: HSA (2.0×10^{-6} mol/L); catechin (1.7×10^{-6} mol/L); ethanol extracts in concentration of 50 μg/mL. The binding was during 1 h at 25 °C. Fluorescence intensities are on *y*-axis and emission wavelengths – on *x*-axis.

Fiorentino et al. (2009) and Sârbu et al. (2012), where fingerprinting of kiwi fruit was suggested.Palafox-Carlos *et al*. (2012) showed the interactions of four major phenolic compounds (chlorogenic, gallic, protocatechuic and vanillic acid) found in 'Ataulfo' mango pulp. Significant synergism was found in the majority of the all combinations, as well as the combination of the four phenolics. Cultivars of fruits and vegetables even grown in the same geographic and climatic conditions could different significantly and therefore, it must be taken into consideration (Koh et al., 2009; Toledo et al., 2008).

Manolopoulou and Papadopoulou (1998), described such differences in kiwi fruit cultivars. However, in their study were investigated mainly respiratory and physicochemical changes of four

kiwi fruit cultivars during cool-storage. Manolopoulou and Papadopoulou (1998) investigated only four cultivars: Allison, Bruno, Hayward and Monty harvested at the proper stage of maturity. They investigated respiration rates, production of ethylene, shelf-life. Among bioactive compounds only ascorbic acid content was measured. No changes in antioxidant activity were described. Therefore, it was decided to study seven well known kiwi fruit cultivars, determine and compare contents of main bioactive compounds and the level of the antioxidant capacity in order to find the best for human consumption. It must be underlined once again that these fruits were at the same stage of ripening and grown in the same geographic and climatic conditions. Therefore, no doubt, the determined data must be reliable.

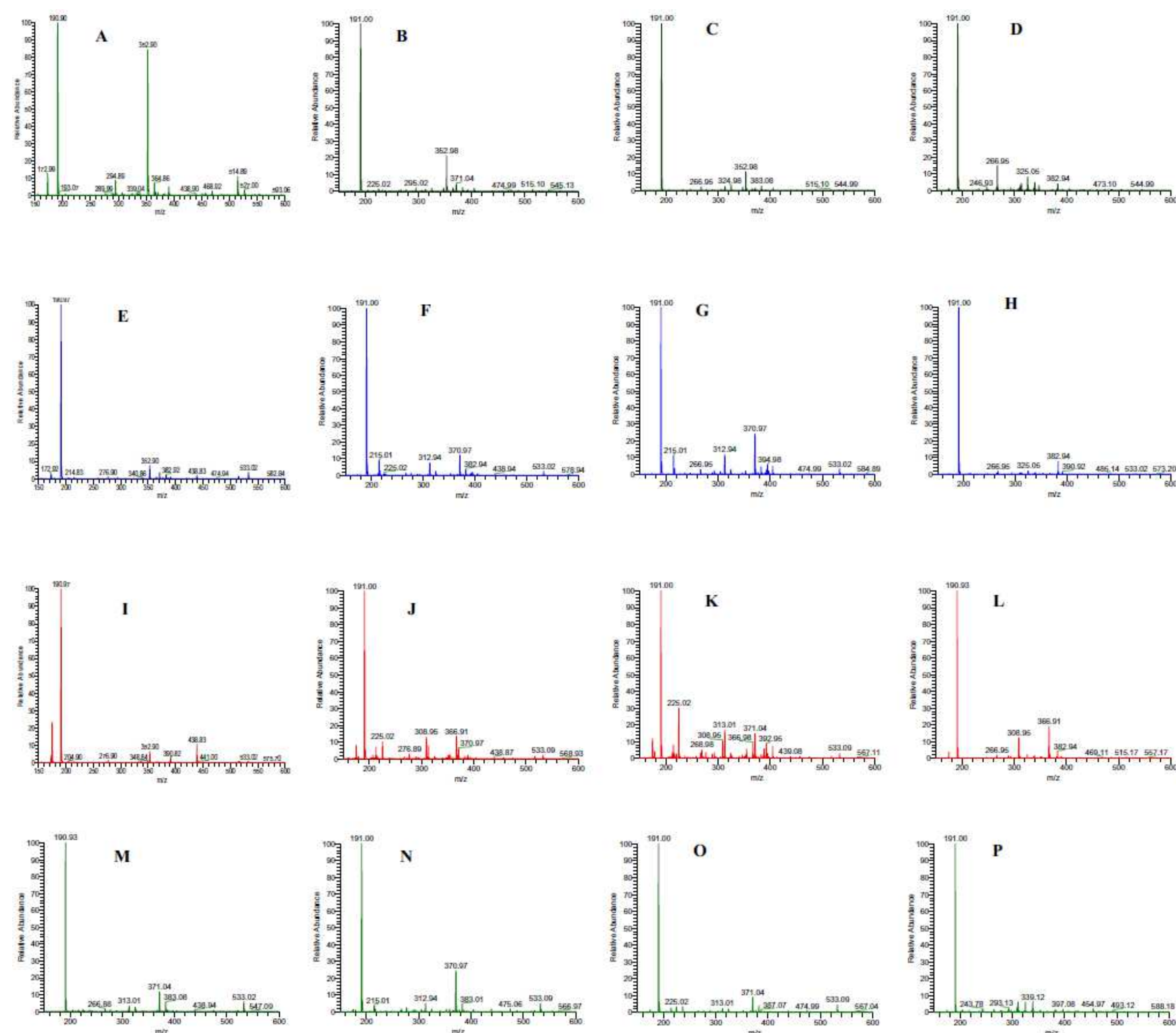


Fig. 19 ESI-MS spectra in negative ion mode of kiwi fruit cultivar groups extracts. A, B, C, D, EtOH, MeOH/water, MeOH/water/acid, acetone of 'Hort 16A'; E, F, G, H, EtOH, MeOH/water, MeOH/water/acid, acetone of 'Hayward'; I, J, K, L, EtOH, MeOH/water, MeOH/water/acid, acetone of 'Bidan'; M, N, O, P, EtOH, MeOH/water, MeOH/water/acid, acetone of 'Hwamei'.

The results of present investigation show that all kiwi fruit cultivars contain high quantities of bioactive compounds. Also our previous data (Park et al., 2008) and of others (Amodio et al., 2007; Jeong et al., 2007; Tavarini et al., 2008) are in agreement with our present results. However, the results are different for different cultivars (Castaldo et al., 1992; Du et al., 2009; Samadi-Maybodi, &Shariat, 2003). So, the contents of the main bioactive compound – polyphenols was significantly higher in 'SKK12', 'Bidan' and 'Hwamei' ($P < 0.05$). The obtained results depend on the year of

collection and the extraction procedure, therefore our recent published results differ from the presently reported (Park et al., 2011). Also the significant highest level of antioxidant capacity and binding abilities were registered in the same cultivars: ‘SKK12’, ‘Bidan’ and ‘Hwamei’ (P < 0.05).

III MEDICINAL PLANTS

Chemical Composition, Antioxidant and Anticancer Effects of the Seeds and Leaves of Indigo (Polygonum tinctoriumAit.) Plant

Results

Bioactive Compounds

The results were summarized in the Table 1. As can be seen, the significantly highest content of polyphenols and flavonoids was in prolipid and mature leaves, flavanols— in seeds, and tannins— in prolipid, immature, and mature leaves (P<0.05 in all cases).

Mass Spectra

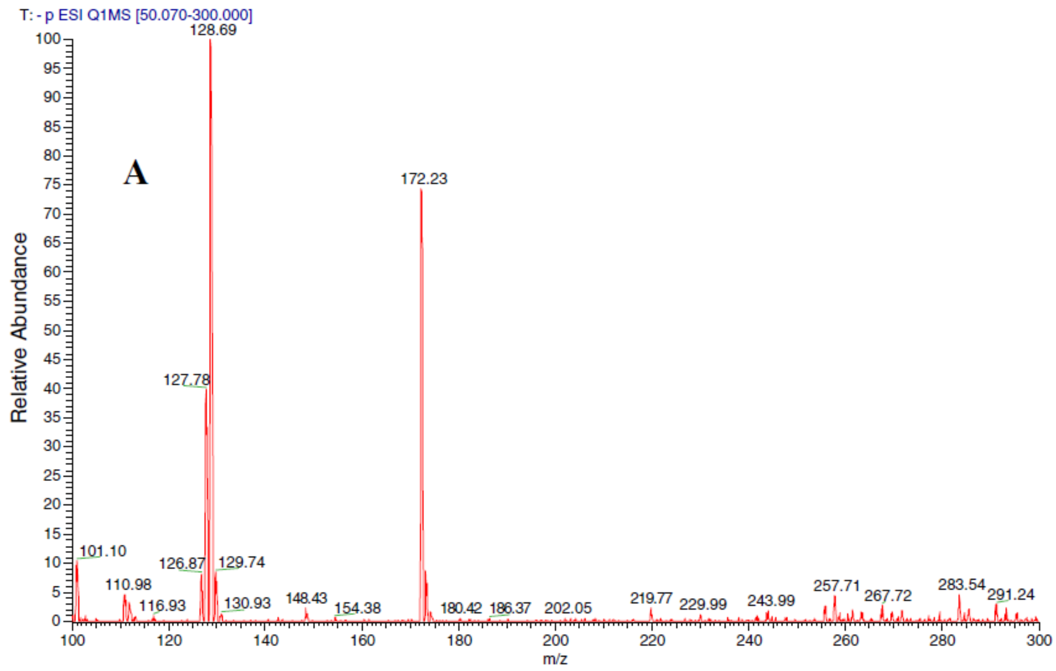
Gallic acid (Fig. 1a) and quercetin (Fig. 1b) were used as standards. The spectrum shows the main m/z peaks found in seeds (Fig. 2a) in methanol fraction: at 106, relative abundance (RA)=58 %; benzoic acid at 120 has RA=100 %; and methyl vanillate at 180 has RA=18 %, at 214 RA=40 %. The peaks in seeds were not found in immature leaves at the same location (Fig. 2b): at 104, RA=20 %; in comparison with the one at 106, RA was higher as twice as in leaves; instead of peak at 120, the peak appeared at 134 (RA=75) of *p*-hydroxybenzoic acid; at 192 (RA=100 %) of scopoletin; at 356 (RA=40 %) and at 365 (RA=18 %). The same fraction for mature leaves showed the following peaks (Fig. 2c): one of the main peaks was located at 134 for p-hydroxybenzoic acid with RA=95 %, slightly higher than for immature ones; at 192 (RA=100 %) of scopoletin, than the other ones at 355 (RA=100 %), 365 (RA=40 %), and 611 (RA=20 %).

Table 13 Bioactive compounds in methanol extracts of the studied samples per dry weight (DW)

Sample	Polyphenols mg GAE	Flavonoids mg CE	Flavanols μg CE	Tannins mg CE
Seeds	5.14±0.3 a	3.842±0.2 a	1,568.95±79.1 b	1.14±0.05 a
Immature leaves	11.55±0.5 b	5.175±0.2 a	432±4.4 c	2.56±0.1 b
Mature leaves	14.22±0.7 c	6.079±0.3b	213±2.1 d	2.95±0.2b
Prolipid	16.64±0.7 c	6.566±0.3 b	1,109.65±54.2 a	3.18±0.3 b

Values are means ± SD of five measurements. Values in columns for every bioactive compound with the same solvent bearing different letters are significantly different ($P<0.05$).

CE catechin equivalent, *GAE* gallic acid equivalent



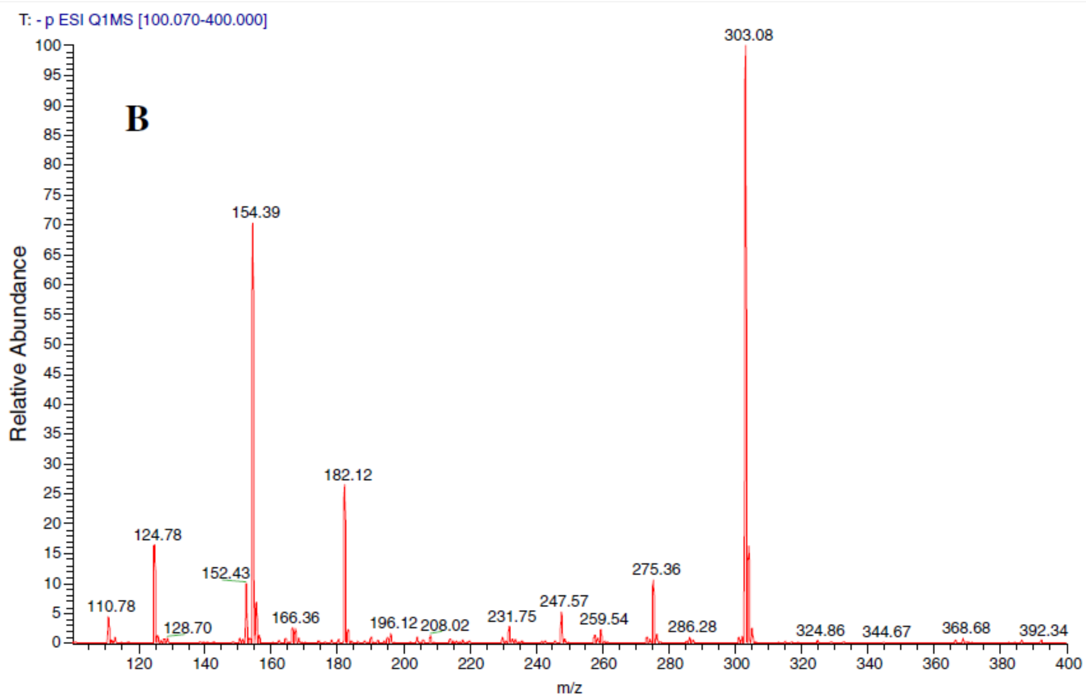


Fig. 20 ESI-MS spectra of **a** gallic acid; **b** quercetin

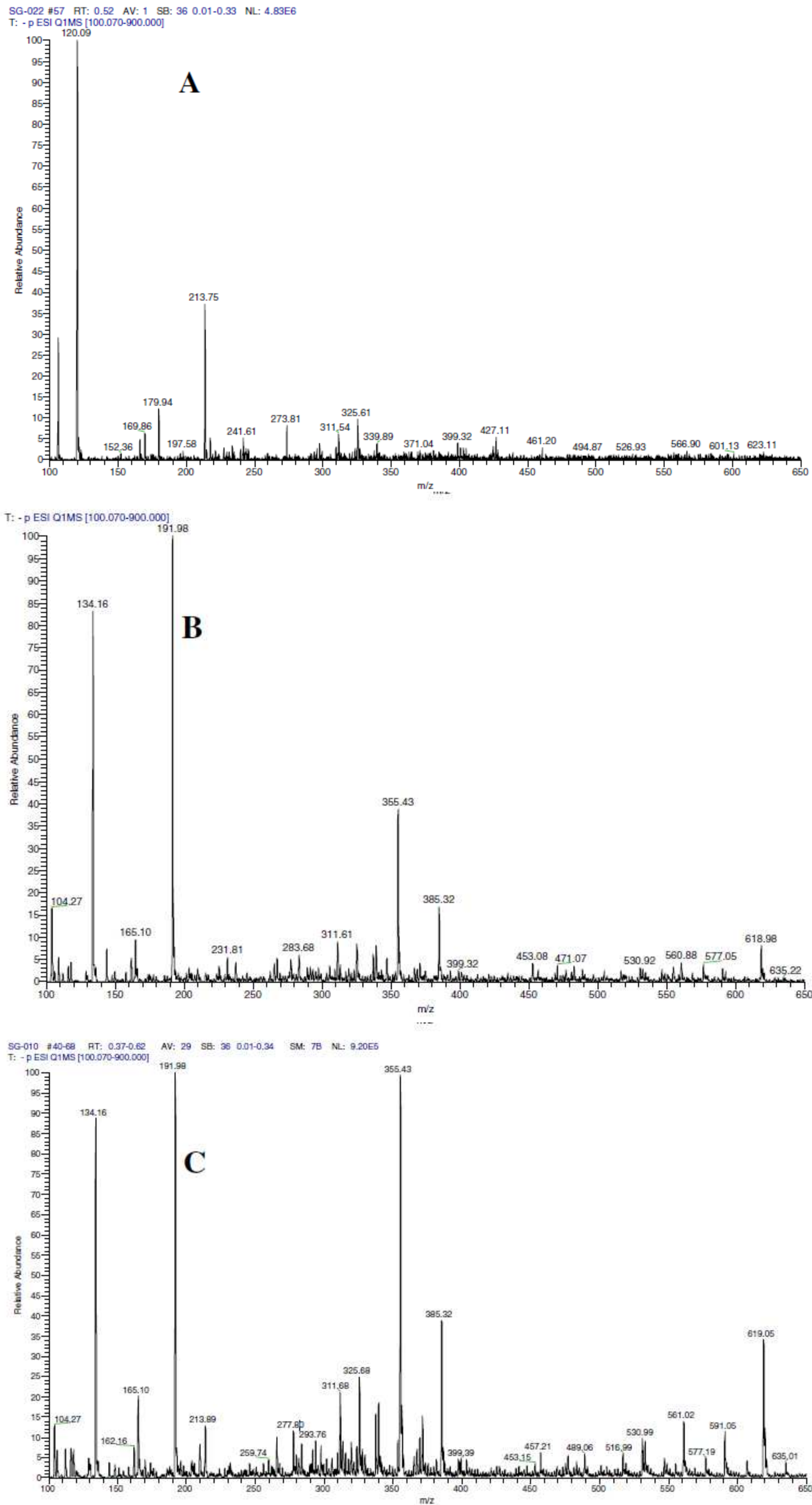


Fig. 21 ESI-MS spectra of methanol fractions of seeds (**a**), immature leaves (**b**), and mature leaves (**c**) of indigo plant in negative ion mode

The spectrum shows the main m/z peaks found in seeds (Fig. 2a) in ethyl acetate fraction: at 174 (RA=20 %) for coniferaldehyde, at 188 (RA=100 %), at 312 (RA=45 %), at 330 (RA=65 %), and at 340 (RA=40 %). Only two peaks were in immature leaves (Fig. 3b) with slightly different abundance such as at 314 with RA of 50 % and at 340 with RA of 75 %. Other peaks were for p-hydroxybenzoic acid at 138 (RA=20 %); vanillic acid at 166 (RA=100 %); at 266 (RA=40 %) for apigenin; at 294 (RA=100 %) for catechin, at 326 with RA=70 % and 619 with RA of 15 %.

The peaks appeared for mature leaves in the same fraction were the following (Fig. 2c): at 134 (RA=55 %) for p-hydroxybenzoic acid, the same peak was in immature leaves with slight shift. Another peaks appeared at 165 (RA=100 %) for vanillic acid for both leaves; at 192 (RA=50 %) for scopoletin only in immature leaves; at 215 (RA=50 %); at 286 (RA=50 %); at 294 (RA=85 %) forcatechin for both leaves; and at 330 (RA=60 %) and at 618 (RA= 50 %) which were shown in both leaves. The obtained results showed the same location of the peaks in both leave samples, only with higher amounts of the compounds showing different relative abundances.

The Antioxidant Activity

The results of the determination of the antioxidant activity in the studied samples are summarized in the Table 2. As can be seen, according to ABTS test, the significantly higher antioxidant activity was in mature leaves, according to CUPRAC—in prolipid and mature leaves, and according to FRAP—in prolipid, mature, and immature leaves ($P<0.05$ in all cases).

Fluorimetry

3D-FL (Fig. 4A, B) spectra illustrated the elliptical shape of the contour maps (Aa, Ba) and cross maps (Fig. 4Ab, Bb) of the main peaks for indigo methanol and ethyl acetate extracts of mature leaves. The main peaks for methanol extracts appeared at λ ex/em of 260/310 with fluorescence intensity (FI) of 889.58 and another one at λ ex/ em of 260/360 nm with FI of 776.07 (Fig. 4Aa, C, Ab). The ethyl acetate fraction had slightly different peaks: at λ ex/em of 260/320 with FI of 169.59 and at λ ex/em of 260/360 nm with FI of 165.94 (Fig. 4Ba, D, Bb). One of the main peaks for 2×10^{-5} M/L BSA was found at λ ex/em of 225–230/335 nm with FI of 877.60 (Fig. 4E, upper curve).

The interaction of BSA and ethyl acetate extract of indigo mature leaves (Fig. 4E, middle curve, with FI=715.61) and BSA and indigo (Fig. 4E, lower curve, with FI=650.81) showed the peak of 335 nm and decrease in the fluorescence intensity (FI). These results are in correspondence with the amount of polyphenols, antioxidant activity, and MS bioactivity data that the methanol extract is more bioactive than the ethyl acetate.

The decrease in fluorescence intensity of BSA was about 18.5 % for the ethyl acetate fraction and 25.8 % for methanol fraction, showing higher quenching activity of methanol extracts of polyphenols. The interaction between methanol and ethyl acetate polyphenol extracts of indigo and BSA showed that indigo has a strong ability as other studied medicinal plants to quench the intrinsic fluorescence of BSA by forming complexes.

FTIR Spectra

The FTIR spectra of methanol extract of prolipid (Fig. 5A, upper curve) was compared with ethyl acetate extract of indigo mature leaves (Fig. 5B, middle curve) and with methanol extract of indigo mature leaves (Fig. 5C, lower curve). Noticeably, the presence of wavelengths of FTIR spectra of gallic acid at 860, 1,025, 1,100, and 1,654 cm^{-1}, tannic acid at 1,172, 1,511, and 1,627, and p-coumaric acid at 1,124, 1,171, 1,508, and 1,638 cm^{-1} were observed in samples analyzed.

The wavelength of FTIR spectra corresponding for vanillin was 1,498, 1,534, 1,617, 1,654, and 3,392 cm^{-1} (Kannan et al., 2011; Nirmaladevi et al., 2010). The main bands presenting in the samples are the following: the band of 1,029 cm^{-1} (–C-O alcohols) is exactly found in ethyl acetate extract of mature leaves (Fig. 5, line b) with a small shift at 1,017 $cm^{-1}$for the dry substance (Fig. 5, line a) and for methanol extract of 1,033 cm^{-1} (Fig. 5, line c).

The band of 1,280 cm^{-1} (–OH aromatic) appeared in slightly different location of 1,201 cm^{-1}. Other peaks appeared at 1,319–1,397 cm^{-1}. The peak of 1,422 cm^{-1} (–C-O alcohols) appeared only in prolipid. The peak of 1618 cm^{-1} (C0C aromatic and C0C alkenes) appeared in all the samples with a shift at 1,650 and 1,597 cm^{-1} for carbonyl substituents. The broad band of 3,309, 2,925 and 2,917 cm^{-1} belong to glycosidic groups O-H. FTIR of quercetin as a standard showed broad phenolic OH band centered around 3,404 cm^{-1}, characteristic -CO stretching at 1,663 cm^{-1} aromatic bending and stretching around 1,091 and 1,663 cm^{-1}, and -OH phenolic bending around 1,197 and 1,374 cm^{-1} (Kim et al., 2012). FTIR spectra of water extracts of mature indigo leaves (Kim et al., 2012) showed a peak characteristic -CO stretching at 1,634 cm^{-1} aromatic bending and the peaks at 2,925 and 2,852 cm^{-1} are related to the C–H bond of saturated carbons, which are different from our results of methanol and ethyl acetate extracts.

Matching between the peaks in the range from 4,000 to 400 cm^{-1} of (prolipid methanol extract)/(indigo ethyl acetate extract)065.08 %, (prolipid methanol extract) /(indigo methanol extract) 076.52 %, and (indigo ethyl acetate extract)/(indigo methanol extract)069.41 % (Fig. 5). Matching between the peaks of the water extracts of indigo mature leaves and the same substances in the same range of the peaks in prolipid was slightly higher of about 78.38 % (Kim et al., 2012; Lanslay & Newman, 2007).

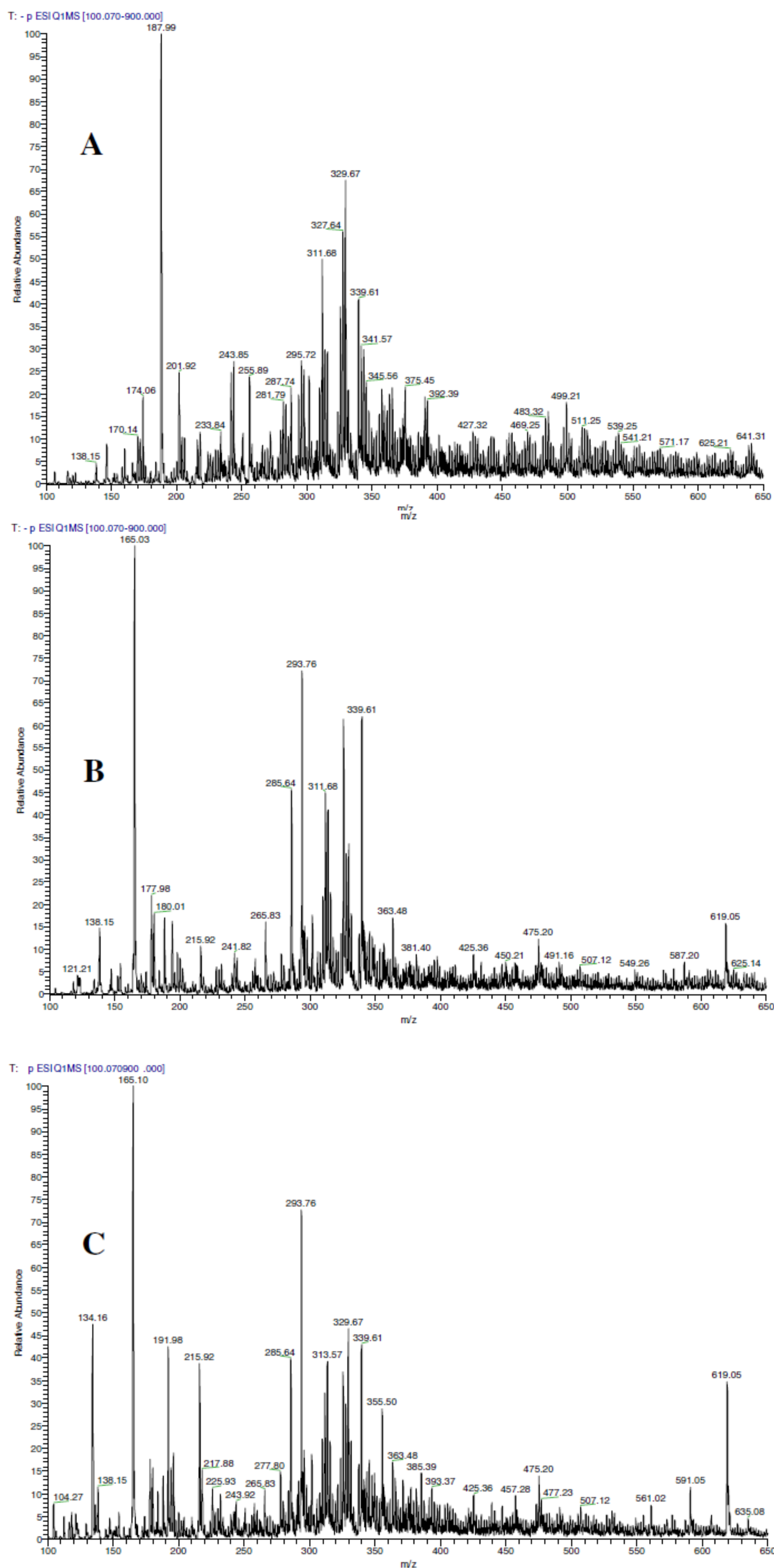


Fig. 22 ESI-MS spectra of ethyl acetate fractions of seeds (a), immature leaves (b), and mature leaves (c) of indigo plant in negative ion mode

Anticancer Activity

It was observed that the percentage of proliferativity of the methanol and ethyl acetate extracts of mature leaves and prolipid samples on two cell lines (Fig. 6a, Calu-6 for human pulmonary carcinoma and Fig. 6b, SNU-601 for human gastric carcinoma) was different. The proliferativity (in percent) for concentrations of 800 μg/mL for methanol and ethyl acetate extracts of prolipid on Calu-6 were 75.49 and 79.24 %, respectively, and on SNU601 were 77.42 and 80.45 %, showing the highest antiproliferative activity in comparisonwith mature leave sample for Calu-6 (76.12 and 80.22 %) and SNU-601 (79.43 and 82.26 %). Our investigation shows that antioxidant activity of the studied samples was highly correlated with their antiproliferative activity.

Table 14 Antioxidant activities (in micromole Trolox equivalents) in methanol extracts of the studied samples per dry weight (DW)

Sample	ABTS	CUPRAC	FRAP
Seeds	68.326±3.4 b	29.27±1.3 a	12.21±0.6 a
Immature leaves	134.438±6.6 c	30.62±1.4 a	19.79±0.9 b
Mature leaves	185.464±9.1 d	59.46±2.8 b	20.91±1.1 b
Prolipid	206.24±20.2a	64.65±3.1 b	22.68±1.2 b

Values are means ± SD of five measurements. Values in columns for every bioactive compound with the same solvent bearing different letters are significantly different ($P<0.05$).

ABTS 2,2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt, *CUPRAC* cupric reducing antioxidant capacity, *FRAP* ferric-reducing/antioxidant power

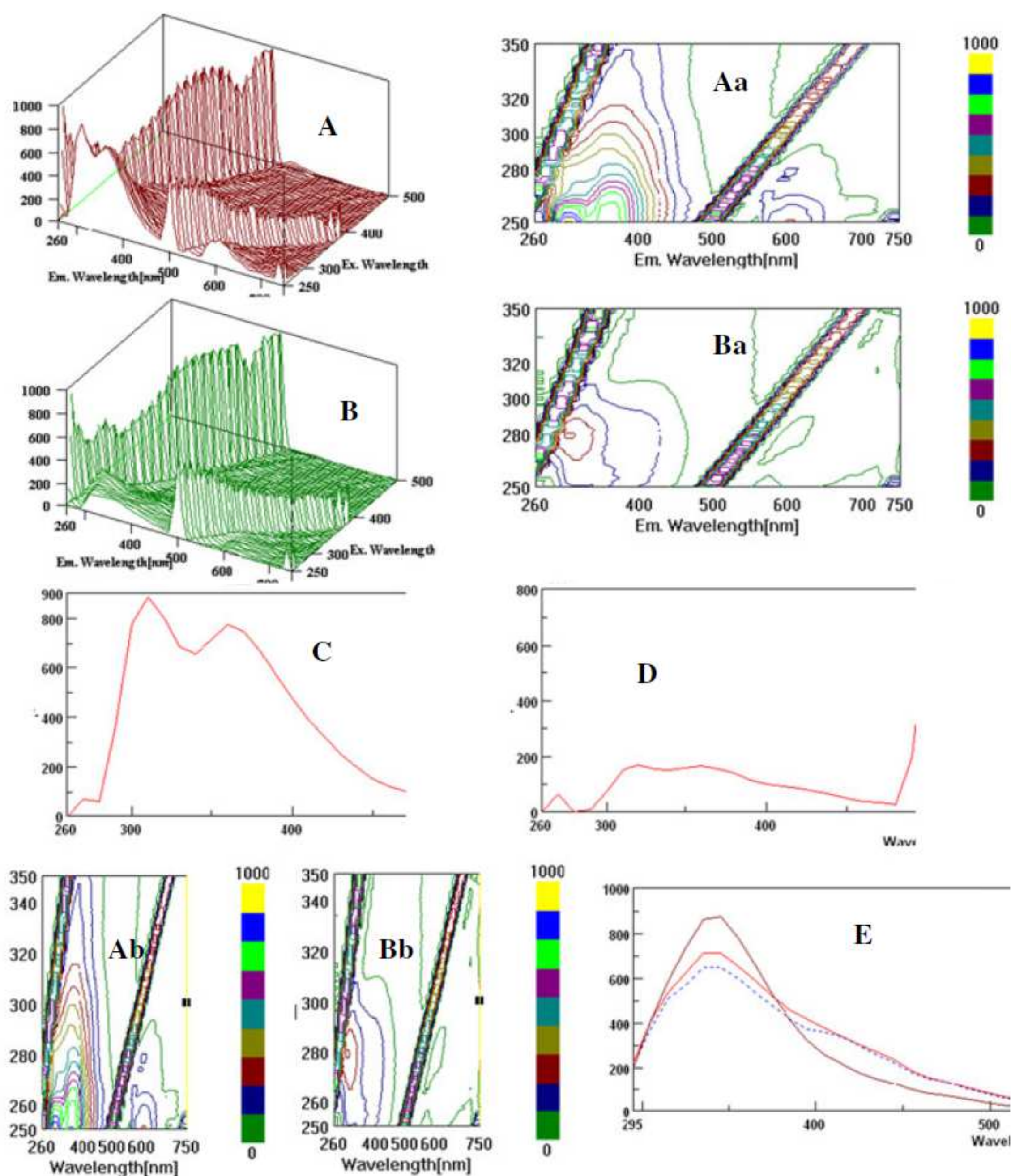


Fig. 23 *A* three-dimensional fluorescence (3D-FL) spectrum of methanol extract (0.001 mg/mL) of indigo mature leaves, *Aa* elliptical shapes of the contours of methanol extracts of indigo mature leaves, *B* 3D-FL spectrum of ethyl acetate extract (0.001 mg/mL) of indigo mature leaves, *Ba* elliptical shapes of the contour map of ethyl acetate extracts of indigo mature leaves, *C* 2D-FL of methanol extract of indigo mature leaves, *D* 2D-FL of ethyl acetate extract of indigo mature leaves, *Ab* cross maps of methanol extracts of indigo mature leaves, *Bb* cross maps of ethyl acetate extracts of indigo mature leaves, *E* change in the fluorescence intensity (ID) as a result of binding affinity of: 2.0×10^{-4} mol/L of BSA (*upper line*); BSA and 40 µg/mL of indigo leave methanol extract (*middle line*); BSA and 40 µg/mL of indigo leave ethyl acetate extract (*lower line*); the 3D-FL were run emission mode and fluorescence intensity up to 1,000, emission wavelengths from 260 to 750 nm and excitation wavelengths from 250 to 500 nm; scanning speed was 1,000 nm/min, For *Aa*, *Ba*, *C*, *D*, *Ab*, *Bb*, and *E*, emission wavelength on x-axis and fluorescence intensity on y-axis for *C*, *D* and *E*; for *Aa*, *Ba*, *Ab* and *Bb*, excitation wavelength on y-axis

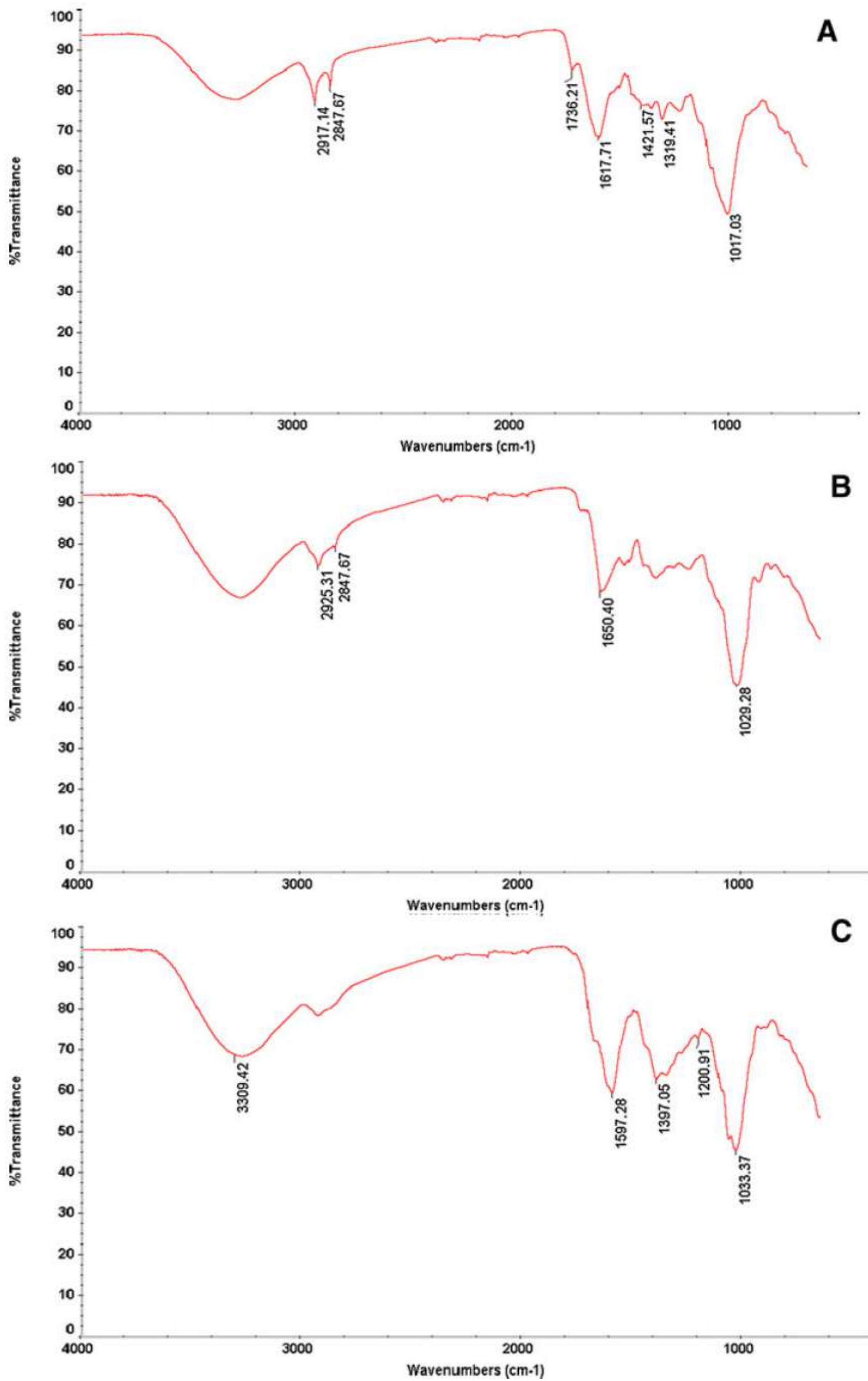


Fig. 24 Infrared study of FTIR spectra of **a** methanol extract of prolipid; **b** ethyl acetate extract of indigo mature leaves; and **c** methanol extract of indigo mature leaves

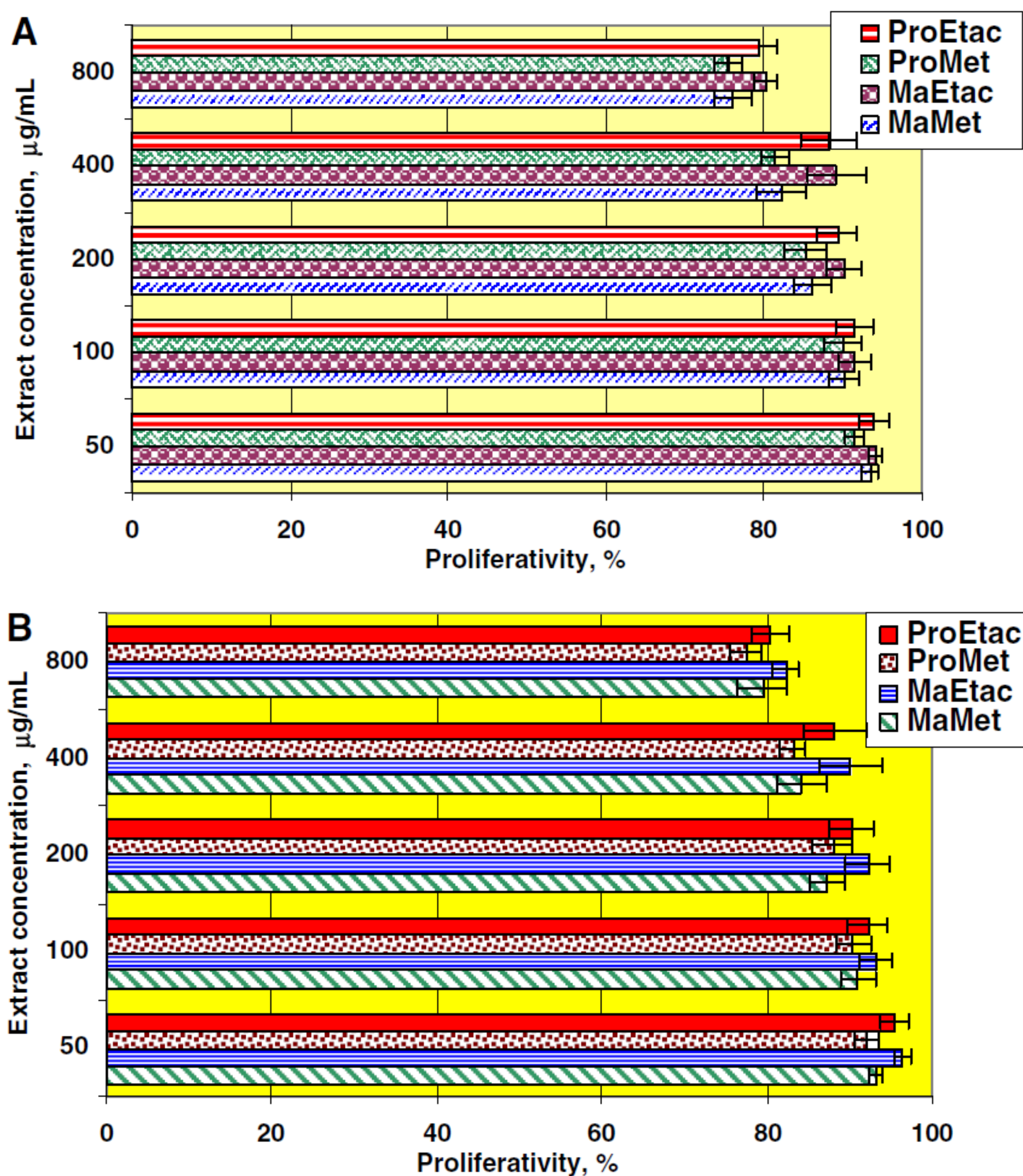


Fig. 25 The proliferativity (in percent) of human cancer cells of the **a** CALU-6 and **b** SNU-601 lines, in the presence of methanol and ethyl acetate indigo mature leaves and prolipid. Each point represents the mean ± SD (n=6). Abbreviations: *Proetac* prolipid ethyl acetate extract, *ProMe* prolipid methanol extract, *MaEtac* mature leaves ethyl acetate extract, *MaMet* mature leaves methanol extract

Discussion

The obtained results of our research can be connected with the recently performed experiments in order to find the scientific basis for the health properties of this plant (Ho and Chang, 2002), where the antinociceptive, anti-inflammatory, and antipyretic effects of indigo plant root methanolic extract were evaluated. The indigo plant root extracts significantly and dose-dependently inhibited the writhing responses of mice and decreased the licking time in both the early and late phases of the formalin test. However, as was stated, the research on the content of bioactive

compounds and the antioxidant and anticancer activities of indigo was limited. Therefore, some other plants were reviewed in order to compare the obtained results. So, Dall'Acqua et al. (Dall'Acqua et al., 2008; Ahmad et al., 2012) evaluated *in vitro* antioxidant properties of some traditional medicinal plants: investigation of the high antioxidant capacity of *Rubus ulmifolius* used in Sardinia as tea beverages or as decoction for medicinal purposes.

Among the various species, R. *ulmifolius* resulted as the more bioactive with all the used methods. Phytochemical investigation revealed several phenolic compounds as caffeic acid, ferulic acid, quercetin, kaempferol-3-Oglucuronide, kaempferol-3-O-(6''-p-coumaroyl)-β-D-glucopyranoside, kaempferol-3-O(6''-caffeoyl)-β-D-glucopyranoside, and many others which are in accordance with our MS data, which are responsible for the antioxidant properties. Our results can be compared with Generalic et al. (Generalic et al., 2011), who studied the phenolic profile and antioxidant properties of Dalmatian sage.

The results strongly indicate that Dalmatian sage leaves are rich source of valuable phenolics, mainly phenolic acids, with extremely good antioxidant properties. The presence of resveratrol or its derivates was confirmed in all extracts. The authors found that the best results for total phenols and flavonoids, as well as the best antioxidant properties were obtained for May sage.

Our results can be compared with the phytochemical composition and antioxidant activity of wild medicinal plants, based on chemical, biochemical, and electrochemical methods. So, F. *ulmaria* was found to be the richest in antioxidant phytochemicals, such as phenolics (228 mg GAE/g DW) and flavonoids (62 mg CE/g DW). The antioxidant activity was found to vary in the order: F. ulmaria>S. nigra>C. multiflorus, irrespective of the analysis method (Borros et al., 2011). Seven compounds related to flavonoids and a mixture of two caffeic acid esters were isolated from L. erythrorhizonSiebet. Zucc. and identified by spectroscopic methods with good radical scavenging activities toward ABTS but showed moderate inhibition of DPPH (Han et al., 2008) .

The presented MS data (Figs. 1, 2, and 3) were in accordance with others (Selvius and Armitage, 2011), where for direct identification of the organic dye compounds quercetin, indigotin, and alizarin in reference materials, in solution, by use of direct analysis in real time ionization and high-resolution time-of-flight mass spectrometry was done. These data are in accordance with Mantzouris *et al*. (Mantzouris et al., 2011) that the treatment by the standard HCl dyestuff extraction method revealed different flavonoids and phenolic acids, where some of them are listed: apigenin, ellagic acid, fisetin, indigotin, indirubin, kaempferol, naringenin, quercetin, and others. Our results exactly in accordance with others (Manhita et al., 2011), where the composition of the natural dyes was determined after different extraction procedures.

The efficiency of eight different procedures used for the extraction of natural dyes was evaluated using contemporary wool samples dyed with cochineal, madder, woad, weld, brazilwood, and logwood. Comparison was made based on the LC-DAD peak areas of the natural dye's main components which had been extracted from the wool samples. Among the tested methods, an extraction procedure with Na_2EDTA in water/DMF (1:1, v/v) proved to be the most suitable for the extraction of the studied dyes, which presented a wide range of chemical structures (Manhita et al., 2011).

The present results can be compared with our recent ones (Kim et al., 2012), where the water extract of indigo plant was analyzed. In water extract, the polyphenols and flavonoids were significantly higher in prolipid, flavanols—in indigo seeds. Our results are in accordance with Fialova

et al. (Fialova et al., 2009), where in leaves of Isatistinctoria L. the following indices were determined: total polyphenols (3.03 %), tannins (1.05 %), and total flavonoids (expressed as isoquercitrin 0.3 %). The phenolic compounds showed higher radical scavenging activity that vitamin C (Nadour et al., 2012). The antioxidant activity was the highest in prolipid, followed by indigo mature leaves.

Exactly the same relationship was obtained in methanol and ethyl acetate extracts, but the highest value was in methanol fraction (Kim et al., 2012). The composition of the indigo plant depends on the extraction procedure. Results of the study of five plants, of which four are endemic to Turkish flora (Tepe et al., 2006) showed that the plants were screened for their possible in vitro antioxidant activities by two complementary test systems (DPPH and βcarotene/linoleic acid). In the first case, Pelargonium endlicherianum extract exerted greater antioxidant activity with an IC_{50} value of 7.43±0.47 μg/mL, followed by Hieraciumcappadocicum of 30.0 ± 0.14 μg/mL.

When compared to the synthetic antioxidant BHT (18.0±0.40 μg/mL), the methanolic extract of *P. endlicherianum* exhibited more than twofold greater antioxidant activity. In the β-carotene/linoleic acid test system, the most active plant was *P. endlicherianum* with 72.6 %±2.96 inhibition rate, followed by *H. cappadocicum* (55.1 %±2.33) and Verbascumwiedemannianum (52.5 % ± 3.11). The results of antioxidant activities of indigo plant (Table 2) are in agreement with the above-cited data. A strong correlation between TEAC values and those obtained from CUPRAC assay implied that antioxidants in these plants were capable of scavenging free radicals and reducing oxidants. A high correlation between antioxidant capacities and their total phenolic contents indicated that phenolic compounds were a major contributor of antioxidant activity of these plants.

Our results on cytotoxicity are in accordance with others (Costa et al., 2005; Itharat et al., 2004; Sandoval et al., 2002). The antioxidant activity of maca (*Lepidium meyenii*) was assessed by the inhibition of peroxynitrite. Maca (mg/mL) protected RAW 264.7 cells against peroxynitriteinduced apoptosis and increased ATP production in cells treated with H_2O_2 (1 mM). The concentration of catechins in maca was lower than in green tea (2.5 vs. 145 mg/g). Maca has the capacity to scavenge free radicals and protect cells against oxidative stress.

Our results can be compared with the recent work of Lin et al. (2009). The extract of indigo naturalis (QD) and its main components indirubin, indigo, and tryptanthrin in human neutrophils were investigated for their anti-inflammatory effects. QD showed the significant inhibition of superoxide anion, attenuated the formyl-lmethionyl-l-leucyl-l-phenylalanine (FMLP)-induced phosphorylation of extracellular regulated kinase; QD inhibited calcium mobilization caused by FMLP. On the other hand, neither indirubin, indigo, nor tryptanthrin produced similar changes in human neutrophils.

The plant extracts were tested for cytotoxicity by the brine shrimp lethality assay, sea urchin eggs assay, hemolysis assay, and MTT assay, using tumor cell lines (Costa et al., 2005). The extract of *Oroxylum indicum* showed the highest toxicity on all tumor cell lines tested, with an IC50 of 19.6 μg/mL for CEM, 14.2 μg/mL for HL-60, 17.2 μg/mL for B-16 and 32.5 μg/mL for HCT-8. On the sea urchin eggs, it inhibited the progression of cell cycle since the first cleavage (IC50=13.5 μg/mL). As was recently shown by Heo et al., (2007) that the Korean medicinal plants, which were used for a long time as traditional seasoned salads, possess anticancer activity.

Our studies on cytotoxicity are in correspondence with Iwaki and Kurimoto (2002), where it was shown that tryptanthrin and indirubin, both compounds originating from indican in the leaves of

P. *tinctorium*, are responsible for many of the biological activities of this plant. Tryptanthrin has a potent anti-inflammatory activity and shows growth inhibitory activity against cancer cell lines *in vitro*. The effect of this substance on azoxymethane-induced intestinal tumorigenesis in rats with carcinogenesis in the intestines is closely associated with inflammation. Tryptanthrin inhibited the incidence of intestinal tumors. Indirubin has been reported to possess an anti-leukemic activity and P. *tinctorium* also contains various anti-oxidative substances, such as gallic acid and caffeic acid, with potential anti-tumor activity.

We consider it likely that P. *tinctorium* shows cancer preventive activity as a consequence of the integral effects of these substances. Our results are in accordance with others (Xie et al., 2012), where diploid leaf extracts of *Gynostemma pentaphyllum* Makino, which is used in tea and food, had strongest inhibition on inflammation and HT-29 proliferation, but these extracts had different order of antiproliferative properties in the LNCaP cells. The interaction between water polyphenol extracts of indigo mature leaves and BSA showed that indigo has a strong ability, as other medicinal plants, to quench the intrinsic fluorescence of BSA by forming complexes (Kim et al., 2012). Better ability is shown by methanol extract. The application of IR spectroscopy and fluorescence in herbal analysis is still limited when compared to other areas. The representative IR spectra from the mid-IR region (4,000–800 cm^{-1}) for ethyl acetate and methanolic extracts were observed. The three extracts in the region of polyphenols showed slight variation in bands than the standards.

Bioinformatics

According to the developmental biology and embryology dictionary, bioinformatics have been define as; *computational biology; the application of the power of computational biology to solutions of complex biological data analysis; the building and manipulation of biological databases; the application of information technology, statistics, and mathematics to biological problems involving large volumes of data with complex interrelationships; it provides the foundation for much modern biomedicine and biotechnology* (Frank, 2012)

Because they are building around developing software which are useful for biological knowledge, One might think of bioinformatics as a concept of ‘using computer to solve biological and biomedical question’, like searching biological databases, compare sequences and find out protein structure. They are method for storing, retrieving, organizing and analyzing biological data.

Benefit of using Bioinformatics

When compare bioinformatics with traditional ways of performing biological experiments, as *in vivo* and *in vitro*. The Bioinformatics application is much faster and more effective in many ways.

One might look back on sequence analysis, at one of the old method called *pattern matching*, the sequences were assembled, analyzed and compared by writing them manually on pieces of paper, then taping them side by side on a board or a wall in laboratory. Before computer and bioinformatics were available, no one would have guess that anyone can manually put the algorithms into memory banks for analysis on molecular sequences as digital numbers.

Searching up to date high quality databases is one of its strong point, and there are much more tools that bioinformatics can provide. It let as retrieving protein sequences, simulating protein 3-D structure, compare sequence, making multiple protein sequence alignment, and even helps us transferring knowledge from model plants to non-model plants.

One might compare the usage of bioinformatics to an entire lab with some expensive equipments and knowledgeable staffs, who can work fast, effective and tirelessly. Of cause, like how effective it is, bioinformatics are also a cost effective method. Some tools that available online even cost us nothing to use.

Computational perspective

Algorithm

Since "No human being can write fast enough, or long enough, or small enough" (Boolos and George, 1974; Jeffrey and Richard, 1999), even if one can carry out a simple calculation. It is impossible to do it effective enough on complex equation, because on some equations, they might need more than a million operations to reach their conclusions. It takes times need on progress to reach the definite answer.

That is why computer for calculation is needed. The researcher has role of his own, a better way to solve a problem, by "finding a set of rules that precisely defines a sequence of operations", which was called Algorithm.

Efficiency

While calculating might done by hand, to measure how effective algorithm is. Counting the operations (addition, multiplication, comparison, etc) that had been performed is done, because it is factor of the time computer would use for running.

To sum it up, measuring how effective algorithm is done by time, so the common way to evaluate the efficiency of method is by considering the number of operations required. The example is as follows.

If an algorithm requires $10n^2$ operations on an input of length n, then one knows how many operations will be needed for any input, and how fast per second the computer can perform, one can calculate the running time on the machine easily.

O notation

If the algorithm requires $10n^2+15n+5$ operations on an n-long input. It means that as n grows larger. The main factor which determines volume of time the algorithm requires will be on $10n^2$. Since the contribution of the lower-order terms $15n+5$ will become tiny compare to $10n^2$. And the constant 10 of $10n^2$ is not much important, when it comes to the rate of growth on the number of operations.

By example above, the effectiveness of algorithm should be focus on the main tread, so if algorithm that takes $10n^2+15n+5$ operations require "$O(n^2)$" time (oh of n squared), or is "an $O(n^2)$ algorithm". This means that the algorithm's running time increases quadratically with the input length.

NP-completeness

The scientists use computer in the development of an optimal algorithm for a specific problem, mainly polynomial algorithm. Several problems have said algorithm, so making the exponent c in $O(n^c)$ as small as possible will developed. Anyhow, polynomial algorithm is still on proving mathematically.

A dozen of problems have been identified but they are not recognized as polynomial. Only exponential algorithms are recently developed from years of research. Until now, they were unable to mathematically prove the existence of polynomial algorithm. However, every problems will have the polynomial algorithm if one of thousands problems is proved to have said algorithm.

Those problems were known as *NP-complete.* When the problem is indicated as NP-Complete, it means that the problem is highly improbable to have an algorithm that can solve it precisely for all possible input in polynomial time.

Difficult problems

Once the problem is identified as NP-complete, it is known that no algorithm can be used for every problem's instance in a specific polynomial time. Approximation algorithms are the possible way to provides almost optimal solutions.

Another solution is *probabilistic algorithms,* as the problem can be solved in polynomial average time, which its run time can be exponential in the worst-case. *Heuristics* is the fast algorithms but it is not guaranteed to have an optimal solution because it is developed and evaluated on the basis of their performance on problems in the real life without proven guarantee of their quality.

Finally, *exhaustive algorithm* can be developed to try every possible solution with time-saving feature from computational shortcuts but exponential time is required for this method. Thus, it is only useful for moderate size of inputs (Paveland Ron, 2011).

Application

Protein sequences

Proteins are molecules that assembled from approximately 100-500 amino acids. Amino acids themselves are molecules consist of carbon, hydrogen, oxygen, nitrogen and sulfur atoms. The table of 20 codes below has been designed by International Union of Pure and Applied Chemistry committee (IUPAC).

Many methods had to be created to analyze molecular sequences in the form of texts when computational biologists began to input the data into the memory banks. After that, bioinformatics started as the protein sequences analysis application has been programmed on the computer.

Reading protein sequence

In proteins, their amino-acid molecules might have their own unique characteristic, but all of them have an identical pair of hooks like NH2 and COOH. Between the successive residues in the protein sequence, the *peptidic bonds* were formed by atoms.

In 1958, Drs. Kendrew and Perutz determined the first protein structure by using X-ray crystallography technique (1962 Nobel Prize) and they found that protein has distinctive shapes, which encoded in amino acids sequence.

From their speculation, if the protein has similar sequences, they would have the same shapes. Likewise, the same sequence of amino acids would encode the same sequence of protein. Its shape would dictate the protein's function as the below linkage that was logically established as:

SEQUENCE => STRUCTURE => FUNCTION

After specialized area called s*tructural bioinformatics* was developed. It is easier to generate computer 3D image, which is helpful for the navigation between sequences and 3D display.

The Genetic Code: Analyzing Protein sequences by DNA Sequences

Genetic code, the relationship between the codons of messenger RNA and the amino acids of the corresponding protein, is easy to sequencing the DNA directly when compared to determining the protein sequence. However biologist can read protein sequence directly in the DNA sequence because not every protein in a given organism can be synthesized is encoded in the DNA sequence of its genome.

To find the correspondent between 4-nucleotide sequence of A, T, G and C and a set of 20 amino acids, one can translate DNA sequence to analogous protein sequence with Universal Genetic Code provided below.

Table 15: Universal Genetic Code

	T	C	A	G
T	TTT Phe (F) TTC Phe (F) TTA Leu (L) TTG Leu (L)	TCT Ser (S) TCC Ser (S) TCA Ser (S) TCG Ser (S)	TAT Tyr (Y) TAC Tyr (Y) TAA Stop TAG Stop	TGT Cys (C) TGC Cys (C) TGA Stop TGG Trp (W)
C	CTT Leu (L) CTC Leu (L) CTA Leu (L) CTG Leu (L)	CCT Pro (P) CCC Pro (P) CCA Pro (P) CCG Pro (P)	CAT His (H) CAC His (H) CAA Gln (Q) CAG Gln (Q)	CGT Arg (R) CGC Arg (R) CGA Arg (R) CGG Arg (R)
A	ATT Ile (I) ATC Ile (I) ATA Ile (I) ATG Met (M)	ACT Thr (T) ACC Thr (T) ACA Thr (T) ACG Thr (T)	ATT Asn (N) AAC Asn (N) AAA Kys (K) AAG Lys (K)	AGT Ser (S) AGC Ser (S) AGA Arg (R) AGG Arg (R)
G	GTT Val (V) GTC Val (V) GTA Val (V) GTG Val (V)	GCT Ala (A) GCC Ala (A) GCA Ala (A) GCG Ala (A)	GAT Asp (D) GAC Asp (D) GAA Glu (E) GAG Glu (E)	GGT Gly (G) GGC Gly (G) GGA Gly (G) GGG Gly (G)

To use the table above, reading the sequence from a starting point in DNA sequence for 3 nucleotides at a time. After that, read Universal Genetic Code table for the triplet (or *codons*) which correspond to the amino acid.

Example of how DNA decoded:

1. Read the DNA sequence:
GACGAGTGCNNNGACGAG

2. Decompose it into successive triplets:
GAC GAG TGC NNN GAC GAG

3. Translate each triplet into the corresponding amino acid:
DECXDE

Example above is the basic knowledge of how bioinformatics change sequence from protein to DNA sequence. By just knowing where our protein coding region starts in a DNA sequence. It is not hard for bioinformatics tools to imitate call and generate the corresponding amino acid sequence.

There are many online tools which can translate Protein, DNA or RNA sequence (ex: from ExPAsy server), and some can even translate in real time, so user can read any sequence as virtual of other sequences.

Bioinformatics tools on DNA/RNA

There is much more bioinformatics tools, which can use in DNA/RNA purpose such as:

- Retrieving DNA sequences from databases
- Computing nucleotide compositions
- Identifying restriction sites
- Designing polymerase chain-reaction (PCR) primers
- Identifying open reading frames (ORFs)
- Predicting elements of DNA/RNA secondary structure
- Finding repeats in sequences
- Computing the optimal alignment between two or more DNA sequences
- Finding polymorphic sites in genes (single nucleotide polymorphisms, SNPs)
- Assembling sequence fragments

Bioinformatics: Genome

Since Genome is “the complete DNA sequence of an organism”, the sequence which are being deal with are much longer compare to Protein/DNA/RNA sequence, almost in length of million-bp (base pairs) for microbes and several billion-bp for organism.

Bioinformatics have to deal with physical mapping, genetic mapping, sequence of entire genomes, and have to design tools and databases that capable to store, query, analyze, and display them in most user friendly interface.

Most genome bioinformatics tools tend to concentrate on mining individual genes for information, since biologists often use it for specific genes that they were interested in. Those bioinformatics tools include:

- Basic sequence-alignment programs
- Phylogenetic/classification methods
- Display tools which adapted to relatively small-sequence objects (example: in thousand characters length)

When compare with “gene by gene” approach from early era, today it is not strange to obtain new DNA sequences without knowledge what was before the result. Since sequencing now have developed better method, the genes can be both sequencing and discover at the same times.

Protein Data Bank

Some bioinformatics tools can be used in helping to predict the potential effect of a mutation, for example: help choosing the right protein fragment to make antibodies. But those usefulness are far less useful, since they fall short of being the real thing, the detailed spatial representation of molecule.

The good point for biologists is, there is plenty of information about experimental 3D structure available online. Since there are many databank like Genbank, EMBL (European Molecular Biology Laboratory), DDJ (DNA Databank of Japan), and for 3D structure from structural biologist is: the Protein Data Bank (PDB).

Figure 26*:* Example of feature from Protein Data Bank Website

Bioinformatics tools

Those previous subjects were some use of bioinformatics tools, but it might not even cover all the basic. There are so many tools for many purposes, example: PCR primers, Analyzing DNA composition, Counting words in DNA sequences, Finding internal repeats in sequence, Finding Protein Coding Regions, ORFing the DNA sequence, Finding internal exons in vertebrate genomic sequences, Predicting the main physico-chemical properties of a protein, Extinction coefficients, Digesting a protein in a computer, etc.

Even so, when compare to other subjects in science field. Bioinformatics are quite small, new, haven't got much information, and lack a developer. It takes sometimes to learn specific bioinformatics tools, and since they require the profession in various fields. It even takes more time if one wants to develop them, but the results should be worthwhile. If one can applies many different bioinformatics tools together, and use them on real research.

The Development of Bioinformatics and Goal

Only biologist and chemist might be sufficed to use all bioinformatics tools around the world, but for the future, the development. It is quite challenging for bioinformatics when they need profession in multiple disciplines to work together, and even when finally gathering them, since computer engineer, mathematician, biologist, etc. does not seem to talk in same language when it come to their difference in profession. They hardly understand what was other disciplines were doing, and conversely.

All around goal of bioinformatics include:

- The development of computer programs in field of molecular biology, to increase the understanding, obtain information more effectively, solve complicate problem, and make tools that is easy to access and manage.
- To develop new or find more effective algorithm, and data management, for example like comparative between sequence data for locate the same genes that they share, or to predict sequence/protein structure and function.

The example of bioinformatics develop include data mining, learning algorithms for machine, pattern recognition, and visualization. Other major research include sequence alignment, protein structure alignment/structure prediction, prediction of gene expression and protein–protein interactions, drug design/discovery, genome assembly, gene finding, genome association studies, and the modeling of evolution.

Case studies

Bioinformatic Analysis for Anticancer Effects of Flovonoids in Vegetables and Fruits

The risk of cancers can be circumvented by the intake of fruits and vegetables. Their chemopreventive characteristic is concerned with phytochemicals like flavonoids. With the usage of several bioinformatics computer programs, the molecule's mechanisms of action and properties can be examined by their chemical structures.

The PASS (Prediction of Activity Spectra for Substances) software has been used as the tool for bioinformatics for the prediction of over 300 pharmacological activities and biochemical mechanism on the basis of structural formula of a substance. It can also identify the new target to find mechanisms in some ligands.

The PASS software also used to evaluate anticancer properties in the three groups of flavonoids which consist of Isoflavones (*Biochanin A, Glycitein*), Flavonols (*Kaempferol, Morin, Quercetin*) and Flavone (*Luteolin*). From the evaluation, every agents showed high Protein Tyrosin Kinase Inhibitory effects (≥0.6). The further study suggested that Isoflavones (the derivation of 3-phenylchromen-4-one or 3-phenyl-1,4-benzopyrone) showed higher PTK Inhibitory effects when compared with Flavonols and Flavone which implied that *Biochanin A* with Pass activity of 0.758 is the most potent agent. However, the lower PTK inhibitory effects in the Flavonols and Flavone, Drug likeness score are higher than Isoflavones.

Pa and Pi values can be applied on PASS prediction as the measures of the activity and inactivity of compounds in which Pa is the probabilities to become active (close to 1.000) and Pi is the probabilities to become inactive (close to 0.000).

Table 16: PASS prediction of flavonoids

	Molecules	*Pa*	*Pi*	*Drug likeness*
1	Biochanin A	0.758	0.003	0.887
2	Glycitein	0.716	0.003	0.889
3	Kaempferol	0.608	0.006	0.962
4	Luteolin	0.635	0.005	0.959
5	Morin	0.608	0.006	0.965
6	Quercetin	0.607	0.006	0.937

Drug likeness can also be estimated by the PASS software.(W.P. Walters et al., 1998) Drug likeness is the definite score came from the estimation of molecular structure followed by the indication of the particular molecule that have biologically active proportional properties or therapeutic potential. From the result, all of the agents have drug likeness more than 0.8, thus, they can be used as drug. Among these agents, Morin is the most efficient drug because of its highest drug likeness score of 0.965.

An integrated bioinformatics approach to improve two-color microarray quality-control

For the measurements of gene expression in the large-scale, Omics technology is involved and constantly improving which specify the necessity of an extensive analyses in bioinformatics for array quality assessment before and after analysis of pathway and clustering of gene expression. The impact of quality control and normalization steps on the biological conclusions were tested in the research that emphasized on the effect of polyphenols in the red wine on rat colon mucosa. The adapted normalization has solved various artifact issues found in the pathway analysis, clustering and data visualization integration. In the analysis of microarray data, point to point standard analysis procedure that is based on a usage of data visualization and clustering has been applied.

Then, the analysis of clustering highlighted about 700 genes exhibiting dissimilar patterns in 3 rats out of 10 after the completion of a standard analysis. According to the analysis, while Fischer 344 rats (inbred) genetically very much resembling each others, the short-term dietary intervention without pharmacological or chemical treatment and/or to a variability of inter-individual cannot be assigned due to their difference expression profiles. The functional analysis on these data by GenMapp/MAPPFinder has revealed the oxidative stress and cell adhesion are related to the up regulation of pathways which were contrasted with the previous studies performed in the lab that expressed a potential antoxidant effect of polyphenolic treatments on rat colon mucosa (Giovannelli L. et al., 2000; Dolara P. et al., 2005)

The analytical approaches to the biological experiment in this document had showed the quality control and improvement in the general workflow which consists of different process that begins from flagged features removal, background subtraction and a global normalization.

Hierarchical clusterization is the next step that visualizes the experimental groups' expression profiles which indicate the appearance of biological differences among groups (rats). If the biological results are not in contrast with these differences, the identification of technical artifacts presence by microarray data visualization will be executed. The three possibilities presented are (1) No possible further analysis because the complete array quality is bad; (2) The quality of the array is bad but improvement is possible; (3) The quality of the array is satisfactory and the further functional analysis can be done.

In another case, new local normalization is the possible step in the workflow and functional analysis will be made after the quality of the array reaches an acceptable level followed by the re-plot

of the genes involved in modulated pathway back to the microarray's original matrix which can reveal or exclude any "local effects".

The proposed workflow contributes to the integration of the observation on an extensive physical data, pathway analysis, clustering and dedicated normalization procedures. All of these are considered as the improvement of microarray analysis.

Whole-genome sequencing to Control antimicrobial resistance

One of the latest improvements in sequencing technologies like whole-genome sequencing (WGS) has become a crucial tool that deals with a major threat in modern healthcare namely antibiotic resistance.

WGS has been widely applied in many activities including antibiotic steward ship of currently available drugs from the surveillance and elucidation of the factors related with the emergence and persistence of resistance. The improvement of novel antibiotics and diagnostics test is also the notable contribution of WGS.

With the help of various proof-of-principle researches, WGS has become the valuable tool for day-to-day infection control and diagnostic tool to detect antibiotic resistance in some pathogens. However, routine WGS cannot be introduced on a large scale until the appropriate data analysis platforms are properly developed.

CONCLUSIONS

1. The bioactivity of blueberries is significantly higher than the bioactivity of other studied samples; however, this index in the gooseberries is comparable with blueberries and cranberries. The bioactivity of Chilean “Murtilla” berries is significantly higher than the bioactivity of other studied samples; however, this index in the “Murtilla-like” berries is comparable with blueberries and raspberries. The antiproliferative properties of the investigated samples are in correlation with the antioxidant activity. There are many reports on the antioxidant properties of berries; however, there is little information about the binding properties of blueberries and cranberries and even less information about gooseberries. The gooseberry, in comparison with cranberries and blueberries, showed a lower amount of bioactive compounds. Therefore, some of the methods used in this work such as fluorescence were done for the first time. The binding properties of the samples are in correlation with the antioxidant activity. 3-D fluorescence and FTIR spectroscopy was used as an additional tool for the characterization of the polyphenol extracts during different stages of ripening and in different berries cultivars. It is a necessity of discovering new plant breeding and genetic studies of berries with the expression of compounds for human health. The advanced analytical methods such as 3D-FL and FTIR spectroscopy used in this study can be applied for any of the food analysis.

2. Seven relatively new kiwi cultivars were divided to three groups mostly based on fluorometric measurements and supported by MS-spectra. The contents of bioactive compounds, antioxidant activity and binding properties are significantly higher in SKK12’, ‘Bidan’ and ‘Hwamei’ cultivars. The SKK12’, ‘Bidan’ and ‘Hwamei’ and to less degree other four studied cultivars could be a valuable addition to known disease preventing diets.

3. It was found that polyphenols, flavonoids, and flavanols were significantly higher in prolipid, following by indigo mature leaves, immature leaves, and seeds. The ability of indigo to quench the intrinsic fluorescence of BSA, relatively high content of polyphenols compounds and anticancer properties can be used as a new source of antioxidants.

4. Some of the active compounds may have synergistic interactions with other compounds as was shown when quercetin was added to the reaction of HSA. This work demonstrated relatively high antioxidant and binding properties of the investigated berries, fruits and medicinal plants in different extracts, especially in water extracts, which can be widely used as tea drink. The possibility of benefits of the consumption of these plants for everyday human health and pharmaceutical use can be suggested.

ABSTRACT

The objective of this study was to evaluate the antioxidant and binding effects of some less studied berries (gooseberry (*Physalis peruviana*), in comparison with blueberry (*Vaccinium corymbosum*), and cranberry (*Vaccinium macrocarpon*), Murtilla-like" (*Myrteola nummularia* (Poiret) Berg.)) seven kiwi fruit cultivars, seeds and leaves of indigo (*Polygonum tinctorium Ait.)* plant in the model of interaction with human serum albumin (HSA). The relationship between the scavenging properties of dietary polyphenols of the investigated plants, their antioxidant, anticancer effects and their affinities for HSA were investigated by Fourier transform infrared, radical scavenging assays, three-dimensional fluorescence spectroscopy, and electrospray ionization-MS in negative mode. In order to perform the extraction and identification of the antioxidants present in the samples, different types of extraction solvents were used, such as water, methanol, ethyl acetate, and diethyl ether. Polyphenols, tannins, anthocyanins, ascorbic acid were studied by spectroscopic methods. Total antioxidant capacities (TACs) were determined by ABTS, FRAP, 2,2- diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH), Cupric reducing antioxidant capacity (CUPRAC), and β-carotene linoleate assay (β-carotene). It was found that the contents of the bioactive compounds and the level of antioxidant capacities in different extracts differ significantly ($P < 0.05$). The total phenolic content was the highest ($P<0.05$) in water extract of blueberries (46.6±4.2 mg GAE/g DW). The highest antioxidant activities by DPPH and CUPRAC were in water extracts of blueberries, showing 108.1±7.2 and 131.1±9.6 µMTE/g DW with correlation coefficients of 0.9918 and 0.9925, and by β-carotene at 80.1±6.6 % with correlation coefficient of 0.9909, respectively. Gooseberry water extracts contained: polyphenols (mg GAE/g DW)—5.37 ± 0.6, tannins (mg CE/g DW)—0.71 ± 0.2, anthocyanins (mg CGE/g DW)—12.0 ± 1.2, ascorbic acid (mg AA/g DW)— 5.15 ± 0.5, and TACs (µMTE/g DW) by ABTS and FRAP assays were 15.53 ± 1.6 and 6.51 ± 0.7, respectively. "Murtilla-like" water extracts contained polyphenols (mg GAE/g)—19.13±0.9, flavonoids, (mg CE/g)—3.12±0.1, anthocyanins (mg CGE/g)—120.23±5.4, and ascorbic acid (mg/g)—2.20±0.1; and antioxidant activities (µmolTE/g) by ABTS and CUPRAC assays were 200.55±8.7 and 116.76±5.7, respectively. Polyphenol content highly correlated with antioxidant activity (R^2 from 0.94 to 0.81).

Chemometrical processing was done on the basis of kinetic data of two variables (concentration and reaction time) by DPPH scavenging reaction. Bioactive compounds and the antioxidant capacities were significantly higher in 'Bidan' and 'SKK12' kiwi fruit cultivars than in other studied samples. The extracts of the investigated plants exhibited high binding properties with human serum albumin (HSA) in comparison with catechin and quercetin. 3-D fluorescence and FTIR spectroscopy can be applied as additional analytical tools for rapid estimation of the quality of food products In conclusion, based on fluorescence profiles the seven new kiwi fruit cultivars can be classified for three groups: 'Hayward' (including 'Daheung', 'Haenam', Hwamei' and 'SKK12'), 'Bidan' and 'Hort 16A'. In MS – profiles some differences in the peaks were found between the cultivar groups and different berries. All studied fruits could be a valuable food supplementation to known disease preventing diets.

Keywords: Berries; Extracts; Bioactive compounds; Antioxidant and anticancer activities; Binding properties; Food consumption; Medicinal plants; analytical methods; Kiwi fruits cultivars

ABSTRACT (POLISH)

Streszczenie

Celem badań była ocena aktywności antyoksydacyjnej i wiązania z białkami wyciągów z mało dotychczas zbadanych owoców jagodowych [miechunka peruwiańska (*Physalis peruviana*) w porównaniu z borówką wysoką (*Vaccinium corymbosum*), żurawiną wielkoowocową (*Vaccinium macrocarpon*) i mirteolą „Murtilla-like" (*Myrteola nummularia* (Poiret) Berg)], siedmioma odmianami uprawowymi owoców kiwi oraz nasionami i liśćmi rdestu balwierskiego (*Polygonum tinctorium Ait.*) w modelowym układzie oddziaływania z albuminą surowicy ludzkiej (HSA). Zależność między zdolnością wychwytu wolnych rodników przez polifenole zawarte w wyciągach, ich aktywnością antyoksydacyjną i przeciwnowotworową a powinowactwem do HSA zbadano za pomocą spektroskopii w podczerwieni z transformacją fourierowską (FTIR), testów wychwytu wolnych rodników, trójwymiarowej spektroskopii fluorescencyjnej i spektrometrii masowej (ESI-MS). W celu przygotowania wyciągów i identyfikacji antyoksydantów zawartych w wyciągach zastosowano różne rodzaje rozpuszczalników tj. wodę, metanol, octan etylu i eter dietylowy. Polifenole, taniny, antocyjany i kwas askorbinowy analizowano przy użyciu metod spektroskopowych. Całkowitą zdolność antyoksydacyjną (TAC) oznaczono pięcioma metodami analitycznymi: ABTS, FRAP, DPPH polegającej na wychwycie rodnika 2,2-difenylo-1-pikrylohydrazylowego, CUPRAC polegającej na ocenie zdolności redukowania kationów miedzi i testem linolenianowym z β-karotenem. Stwierdzono statystycznie istotne różnice ($P<0,05$) pod względem zawartości związków bioaktywnych i zdolności antyoksydacyjnej wyciągów. Całkowita zawartość polifenoli była największa w wyciągach wodnych z borówki wysokiej (46,6±4,2 mg GAE/gDW). Wyciągi te wykazywały jednocześnie największą aktywność antyoksydacyjną wynoszącą 108,1±7,2 μMTE/gDW w metodzie DPPH i 131±9.6 μMTE/gDW w metodzie CUPRAC z współczynnikami korelacji wynoszącymi odpowiednio 0,9918 i 0,9925. W teście linolenianowym z β-karotenem zawartość polifenoli kształtowała się na poziomie 80,1±6,6% z współczynnikiem korelacji 0,9909. Wyciągi wodne z miechunki peruwiańskiej zawierały: polifenoli 5,37±0,6 mg GAE/gDW, tanin 0,71±0,2 mg CE/gDW, antocyjanów 12,0±1,2 mg CGE/gDW i kwasu

askorbinowego 5,15±0,5 mg/gDW a ich zdolność antyoksydacyjna TAC (µMTE/gDW) wynosiła w zależności od metody oznaczeń ABTS lub FRAP odpowiednio 15,53±1,6 i 6,51±0,7.. Wodne wyciągi z mirteoli zawierały: polifenoli 19,13±0,9 mgGAE/g, flawonoidów 3,12±0,1mgCE/g, antocyjanów 120,23±5,4 mgCGE/g i kwasu askorbinowego 2,20±0,1 mg/g. Ich zdolność antyoksydacyjna oznaczana metodą ABTS lub CUPRAC wynosiła odpowiednio 200,55±8,7 µMTE/gDW i 116,76±5,7 µMTE/gDW. Zawartość polifenoli była wysoko istotnie skorelowana z aktywnością antyoksydacyjną (R^2 od 0,94 do 0,81).

Analiza chemometryczna została przeprowadzona na podstawie danych kinetycznych dwóch zmiennych tj. stężenia i czasu reakcji w metodzie wychwytu rodnika DPPH. Zawartość związków bioaktywnych i aktywność antyoksydacyjna była statystycznie istotnie większa w wyciągach z owoców kiwi odmian uprawowych Bidan i SKK12 niż w innych ocenianych próbkach. Wyciągi z roślin wykazywały duże powinowactwo do albuminy surowicy ludzkiej (HSA) w porównaniu z katechiną i kwercetyną. Spektroskopia fluorescencyja 3-D i spektroskopia FTIR mogą być stosowane jako dodatkowe narzędzia szybkiej analizy jakości produktów żywieniowych. Siedem nowych odmian owoców kiwi można zaszeregować na podstawie profili fluorescencyjnych do trzech grup: Hayward (łącznie z Daheung, Haenam, Hwamei i SKK 12), Bidan i Hort 16A. W badaniach techniką MS stwierdzono różnice w wysokości i rozkładzie pików na spektrogramach między odmianami uprawowymi owoców kiwi i owocami jagodowymi. Wszystkie analizowane owoce mogą być wartościowym uzupełnieniem znanych diet stosowanych w prewencji chorób.

Słowa kluczowe: owoce jagodowe, wyciągi, związki bioaktywne, aktywność antyoksydacyjna i przeciwnowotworowa, oddziaływanie z albuminą, konsumpcja żywności, rośliny lecznicze, metody analityczne, odmiany uprawowe owoców kiwi

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SCIENTIFIC ACHIVEMENTS

List of Kann Vearasilp's Articles

1. Park, Yong-Seo, **Jacek Namiesnik**, **Kann Vearasilp**, Hanna Leontowicz, Maria Leontowicz, Dinorah Barasch, Alina Nemirovski, Simon Trakhtenberg, **Shela Gorinstein**. Bioactive compounds and the antioxidant capacity in new kiwi fruit cultivars. 2014, **Food Chemistry,** accepted, **IF=4.072.**

2. **Namiesnik, J., Vearasilp, K.,** Nemirovski, A., Leontowicz, H., Leontowicz, M., Pasko, P., Martinez-Ayala, A.-L., Gonzalez-Aguilar, G. A., Suhaj, M. and **Gorinstein, S.** 2014. In Vitro studies on the relationship between the antioxidant activities of some berry extracts and their binding properties to serum albumin. **Applied Biochemistry and Biotechnology** 172: 2849-2865, **IF=1.893.**

3. **Namiesnik, J., Vearasilp, K.,** Leontowicz, H., Leontowicz, M., Kyung-Sik Ham, Kang, S.-G., Park, Y.-K., Arancibia-Avila, P., Toledo, F. and **Gorinstein, S.** 2014. Comparative assessment of two extraction procedures for determination of bioactive compounds in some berries used for daily food consumption. **International Journal of Food Science and Technology** 49: 337–346, **IF=1.24**

4. Gorinstein, S., Arancibia-Avila, P., Toledo, F., **Namiesnik, J.,** Leontowicz, H., Leontowicz, M., Ham, K.-S. Kang, S.-G., **Vearasilp, K**, and Suhaj, M. 2013 Application of analytical methods for the determination of bioactive compounds in some berries, **Food Analytical Methods** 6: 432-444, **IF=1.969**

5. **Namiesnik J., Kann Vearasilp**, Magdalena Kupska , Kyung-Sik Ham, Seong-Gook Kang, Yang-Kyun Park, Dinorah Barasch, Alina Nemirovski, **Shela Gorinstein. 2013. Antioxidant activities and bioactive components in some berries. European Food Research and Technology,** 237: 819-829, **IF=1.436**

6. Jang, H.-G., Heo, B.-G., Park, Y. S., **Namiesnik, J**., Barasch, D., Katrich, E., **Vearasilp, K**., Trakhtenberg, S. and **Gorinstein, S.** 2012. Chemical composition, antioxidant and anticancer effects of the seeds and leaves of indigo (*Polygonum tinctorium* Ait.) plant. **Applied Biochemistry and Biotechnology** 67: 1986–2004, **IF=1.943**

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Objective

Position in the field of medical/bio informatics

Summary

Kann graduated MS in Bioinformatics from Medical College of Wisconsin, United States (2008) and bachelor of Pharmacy from Chiang Mai University, Thailand (2004). Following his college years, he went to work in the area of medical informatics for various overseas and domestic research institutions involving Marquette University, Medical College of Wisconsin, and Emory University. His specialties and interests involve implementations of his pharmaceutical and medical background with informatics techniques to solve current medical challenges particularly about tropical diseases. Now Kann is working as a lecturer of school of pharmacy, Srinakharinwirot University, Thailand.

Education

Emory University, Atlanta, GA **2008-2009**

Department of Computer Sciences, Graduate School

- 1 year of training under Computer Sciences Program

Medical College of Wisconsin & Marquette University, Milwaukee WI **2006-2008**

Joint program between Medical School & Dept. of Computer Sciences

- MS in Bioinformatics, concentration in Computer Sciences & Bioinformatics

Chiang Mai University, Thailand **1999-2004**

School of Pharmacy

- Bachelor of Pharmacy, specialized in pharmaceutical sciences and technologies
- Registered Pharmacy license of Thailand, currently active

Experiences

Srinakharinwirot University, Bangkok, Thailand ***2009, 2004-2006***

Lecturer, School of Pharmacy

- Teach undergraduate students and do researches in the field of medical informatics and pharmacy
- Develops a database of Thai's native medicinal compounds & pharmaceutically active agents for the university (currently internally used)
- Royal Thai Government research scholar

Emory University, Atlanta, GA ***2008-2009***

Computer Science Graduate Student

- Implemented algorithms for Dengue viral genome assembling from short-read sequencing technologies.
- Implemented utility-based anonymization techniques for data security course project, a microtable anonymization module with customizable weight for attributes

Medical College of Wisconsin & Marquette University, Milwaukee, WI ***2006-2008***

Research Assistant & Graduate Student

- Managed and administered Bioinformatics center and its computational resources for Dept. of Computer Sciences & Medical School
- Implemented a neural network prediction model for CYP2D6 enzyme and its binding affinities towards thousands of distributed drug & chemical compounds
- Established internal software portals & information systems for Chemical & Proteomics facilities at Marquette University
- Developed an information retrieval system that gathered all semantic & political opinions from US major bloggers to determine potential votes, and built a prediction model that casted the potential winner of US president election.

Institut Pasteur, Paris, France ***2006***

Exchanged scholar (3 months)

- Assisted a project researching on association studies of human genome and patients susceptibilities to Dengue diseases using biostatistics application to perform a genome-wide data mining to search for significant relationship between polymorphisms & genetic traits.
- Managed and curated human genomic database for Centre National de Génotypage via Oracle technologies

Honor and Awards

- Research Assistantship, Bioinformatics program, Marquette University, 2007
- Higher Educational Strategic Scholarships for Frontier Research Network, Royal Thai Government, 2005
- Chiang Mai University honorary alumni in the category of excellency in research, Chiang Mai University, 2005
- Thailand Innovation Award, runner-up prize, The Science Society of Thailand under patronage of His Majesty the King Bumiphol, 2004
- Industrial research for undergraduate student scholarship (IRPUS), Thailand Research Fund, 2003

Skills

- 3.5 years of research & applications in bioinformatics & pharmaceutical sciences technologies
- Programming in C, Objective-C, Java, Perl, SQL, HTML, subversion, Windows & Unix environments
- Now involved in various medical informatics projects at Srinakharinwirot University (Pharmacy) and Mahidol University (Siriraj Hospital)

Language proficiency

- Fluent in Thai and English; TOEFL iBT score of 110/120 (May 15th, 2009)

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Chemical Composition, Antioxidant and Anticancer Effects of the Seeds and Leaves of Indigo (*Polygonum tinctorium Ait.*) Plant

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Abstract Seeds and leaves of indigo (*Polygonum tinctorium Ait.*) plant were investigated and compared with another medicinal plant named prolipid for their properties such as chemical composition, antioxidant, and anticancer effects by Fourier transform infrared, three-dimensional fluorescence spectroscopy, and electrospray ionization-MS in negative mode. It was found that polyphenols, flavonoids, and flavanols were significantly higher in prolipid ($P<0.05$), following by indigo mature leaves, immature leaves, and seeds. Methanol extract of mature indigo leaves in comparison with the ethyl acetate extract showed higher

Prof. S. Trakhtenberg died on 20 November 2011.

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inhibition of proliferation. The interaction between polyphenol extracts of indigo mature leaves and BSA showed that indigo has a strong ability, as other widely used medicinal plants, to quench the intrinsic fluorescence of BSA by forming complexes. In conclusion, indigo mature leaves were compared with prolipid. High content of bioactive compounds, antioxidant, fluorescence, and antiproliferative properties of indigo justifies the use of this plant as a medicinal plant and a new source of antioxidants.

Keywords Indigo · Extracts · Bioactive compounds · Antioxidant and anticancer activities

Introduction

Indigo (*Polygonum tinctorium Ait.*) is an herbaceous subtropical annual plant, belonging to the family Polygonaceae. Within the cells of its leaves, *P. tinctorium* accumulates large amounts of a colorless glycoside, indican (indoxyl beta-D-glucoside), from which the blue dye indigo is synthesized [1, 2]. The composition of the natural dyes was determined after the extraction procedures with different solvent systems [3]. Indigo naturalis is used by traditional Chinese medicine to treat various inflammatory diseases [4]. Some wild indigo species as herbal drugs were evaluated [5]. The data concerning the anticancer activity of indigo are very limited [6]. There are still few data on indigo plant; therefore, it is possible to compare it with other better investigated medicinal plants. Plants are a source of compounds that may be used as pharmacologically active products. *Cytisus multiflorus*, *Filipendula ulmaria* and *Sambucus nigra* have been used as important medicinal plants in the Iberian Peninsula for many years and are claimed to have various health benefits as indigo plant [7]. It was shown using chemical, biochemical, and electrochemical assays that these wild plants are source of phytochemicals and antioxidant potential [7]. Also Dall'Acqua, Cervellati, Loi, and Innocenti [8] examined the antioxidant capacities of 11 botanical species used in the tradition of Sardinia as tea beverages or as decoction for medicinal purposes. The anti-metastasis and immune-stimulating activities of EtOH extracts of fermented Korean red ginseng (FRG-E) in animal and human subjects was investigated [9]. The antioxidant properties of phenolic compounds from olive pulp of *chamlal* variety and those of individual phenolic compounds were evaluated and compared with that of vitamin C [10]. Generalic et al. [11] studied the phenolic profile and antioxidant properties of Dalmatian sage. Another plant as *Lithospermum erythrorhizon* could be a promising rich source of natural antioxidants [12]. It was of interest to know if also methanol and ethyl alcohol extracts of indigo have the same properties as some other plants. Therefore, in addition to determination of the bioactive compounds content, especially phenolics, and antioxidant activity of indigo, also its anticancer properties were examined. As far as we know, there are no published results of such investigations.

Methods and Materials

Chemicals

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); gallic acid; quercetin; Tris, tris(hydroxymethy1)aminomethane; 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS); Folin−Ciocalteu reagent; lanthanum (III) chloride heptahydrate; $FeCl_3 \cdot 6H_2O$; $CuCl_2 \cdot 2H_2O$; 2,9-dimethyl-1,10-phenanthroline (neocuproine); and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St Louis, MO, USA. 2,4,6-Tripyridyl-*s*-

triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland. All reagents were of analytical grade. Deionized and distilled water was used throughout.

Testing Samples Preparation

There are three samples of indigo plant (*P. tinctorium*): seeds and two samples of leaves. The leaves with slight green color (immature leaves) were harvested on April 10, 2010, and the leaves with green brown color (mature leaves) were harvested on July 20, 2010, from the same place. Two samples of leaves were used because one was mature and the other— immature. The most important is to find exact data when the leaves can be harvested. The leaves were dried for 5 days under sunlight. The leaves were pulverized in the laboratory conditions. The particle size was 200 mesh. For comparison, prolipid was used [13]. Prolipid is a mixture of the following plants: *Sonchus* 532 Z. *arvensis* L. from the Compositae (Asteraceae) family, *Guazuma ulmifolia* L. from the Sterculiaceae family and *Murraya paniculata* L. from the Rutaceae family. Prolipid contains extracts of *G. ulmifolia* [20 g/100 g dry weight (dw)], *M. paniculata* (10 g/100 g dw), and *S. arvensis* (10 g/100 g dw). Prolipid capsules were obtained as a gift from the drug importer COWIK (Warsaw, Poland).

Extraction of Polyphenols

The extracts from seeds and leaves were prepared by the same way for all tests (bioactive compounds, antioxidant, and anticancer assays). The phenols were extracted with methanol and ethyl acetate from the indigo powder, seeds, or the prolipid (concentration 25 mg/mL) at room temperature twice for 3 h [14]. The prolipid capsules were opened and the content was dissolved in the same solvents at the same conditions.

Extraction of Phenolic Compounds for MS

The lyophilized sample of indigo plant (1 g) was extracted with 100 mL of methanol/water (1:1) at room temperature and in darkness for 24 h. The extracts were filtered in a Buchner funnel. After removal of the methanol in a rotary evaporator at a temperature below 40 °C, the aqueous solution was extracted with diethyl ether and ethyl acetate, and then the remainder of the aqueous solution was freeze-dried. The organic fractions were dried and redissolved in methanol. These extracts were used for MS, for determination of bioactive compounds and Fourier transform infrared (FTIR) analyses [15].

MS Analysis A mass spectrometer, a TSQ Quantum Access Max (Thermo Fisher Scientific, Basel, Switzerland), was used. Analytes were ionized by electrospray ionization (ESI) in negative mode. Vaporizer temperature was kept at 100 °C. Settings for the ion source were as follows: spray voltage 3,000 V, sheath gas pressure 35 AU, ion sweep gas pressure 0 AU, auxiliary gas pressure 30 AU, capillary temperature 200 °C, and skimmer offset 0 V.

Total Phenolic Content

The polyphenols were determined by Folin–Ciocalteu method with measurement at 750 nm with a spectrophotometer (Hewlett-Packard, model 8452A, Rockville, USA). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram DW [16].

Total Flavonoid Content

Total flavonoid content was determined by an aluminum chloride colorimetric method [17]. Briefly, 0.25 mL of the indigo or prolipid sample extract was diluted with 1.25 mL of distilled water. Then 75 μL of a 5 % $NaNO_2$ solution was added to the mixture. After 6 min, 150 μL of a 10 % $AlCl_3 \cdot 6H_2O$ solution was added, and the mixture was allowed to stand for another 5 min. Half of a milliliter of 1 M NaOH was added, and the total was made up to 2.5 mL with distilled water. The solution was well mixed, and the absorbance was measured immediately against the prepared blank at 510 nm in comparison with the standards prepared similarly with known (+)-catechin concentrations. The results are expressed as milligrams of catechin equivalents.

Total Tannins Content

To 50 μL of methanol extract of the plant sample, 3 mL of a 4 % methanol vanillin solution and 1.5 mL of concentrated hydrochloric acid were added. The mixture was allowed to stand for 15 min. The absorption was measured at 500 nm against methanol as a blank. The amount of total condensed tannins is expressed as (+)-catechin equivalents per gram of the sample [18].

Total Flavanols Content

The total flavanols amount was estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method, and then the absorbance at 640 nm was read. To ensure the presence of flavanols on the nuclei, subsequent staining with the DMACA reagent resulted in an intense blue coloration in plant extract [19]. As it was mentioned previously, (+)-catechin served as a standard for flavonoids, flavanols, and tannins, and the results were expressed as catechin equivalents (CE).

Antioxidant Activity by 2,2-Azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) Diammonium Salt ($ABTS^{\cdot +}$) Method

$ABTS^{\cdot +}$ radical cation was generated by the interaction of ABTS (7 mM/L) and $K_2S_2O_8$ (2.45 mM/L). This solution was diluted with methanol until the absorbance in the samples reached 0.7 at 734 nm [20].

Antioxidant Activity by Ferric-Reducing/Antioxidant Power

This assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripyridyltriazine (Fe^{3+}–TPTZ) to a ferrous form (Fe^{2+}). FRAP reagent (2.5 mL of a 10 mM ferric-tripyridyltriazine solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3 \cdot H_2O$ and 25 mL of 0.3 M acetate buffer, pH 3.6) of 900 μL was mixed with 90 μL of distilled water and 30 μL of plant samples or methanol as the appropriate reagent blank. The absorbance was measured at 595 nm [21].

Antioxidant Activity by Cupric Reducing Antioxidant Capacity

This assay is based on utilizing the copper(II)-neocuproine reagent as the chromogenic oxidizing agent. To the mixture of 1 mL of copper (II)-neocuproine and NH_4Ac buffer

solution, acidified and non-acidified methanol extracts (or standard) solution (x, in milliliters) and H_2O [$(1.1-x)$ in milliliters] were added to make the final volume of 4.1 mL. The absorbance at 450 nm was recorded against a reagent blank [22].

Fluorimetry

Two-dimensional- and three-dimensional (3D-FL) fluorescence measurements were done using a model FP-6500, Jasco Spectrofluorometer, serial N261332, Japan [23]. Fluorescence emission spectra for all indigo samples at a concentration of 0.001 mg/mL were taken at emission wavelength (in nanometers) of 330 and recorded from wavelength of 265 nm to a wavelength of 310 nm, at emission wavelengths of 685 nm from 260 to 750 nm, and at excitation of 350 nm from 250 to 500 nm. 3D-FL spectra of the investigated plant extracts were collected with subsequent scanning emission spectra from 260 to 750 nm at 1.0 nm increments by varying the excitation wavelength from 260 to 500 nm at 10 nm increments. The scanning speed was set at 1,000 nm/min for all measurements. All measurements were performed with emission mode and with intensity up to 1,000. All solutions for protein interaction were prepared in 0.05 mol/L Tris–HCl buffer (pH 7.4) containing 0.1 mol/L NaCl. The final concentration of BSA was 2.0×10^{-5} mol/L. All solutions were kept in dark at 0–4 °C. The BSA was mixed with methanol or with ethyl acetate extracts of 20 μl. The samples were mixed in the properties of BSA/extract 01:1. The samples after the interaction with BSA were lyophilized and subjected to FTIR.

Fourier Transform Infrared Spectra Studies

The presence of polyphenols in the investigated extracts of indigo samples was studied by FTIR spectroscopy. A Nicolet iS 10 FTIR Spectrometer (Thermo Scientific Instruments LLC, Madison, WI, USA), with the smart iTR™ attenuated total reflectance accessory, was used to record IR spectra [24–26].

MTT Assay

Anticancer activity of methanol and ethyl acetate extracts of the studied plants on human cancer cell lines (Calu-6 for human pulmonary carcinoma and SNU-601 for human gastric carcinoma) was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The cell lines were purchased from the Korean Cell Line Bank for MTT assay. Cells were grown in RPMI-1640 medium at 37 °C under 5 % CO_2 in a humidified incubator. Cells were harvested, counted (3×10^4 cells/mL), and transferred into a 96-well plate and incubated for 24 h prior to the addition of test compounds. Serial dilutions of test samples were prepared by dissolving compounds in dimethyl sulfoxide (DMSO) followed by dilution with RPMI-1640 medium to give a final concentration at 25, 50, 100, 200, 400, and 800 μg mL^{-1}. Stock solutions of samples were prepared for cell lines at 90 μL and samples at 10 μL and incubated for 72 h. MTT solution at 5 mg mL^{-1} was dissolved in 1 mL of phosphate buffer solution, and 10 μL of it was added to each of the 96 wells. The wells were wrapped with aluminum foil and incubated at 37 °C for 4 h. The solution in each well containing media, unbound MTT and dead cells were removed by suction, and 150 μL of DMSO was added to each well. The plates were then shaken and optical density was recorded using a micro plate reader at 540 nm. Distilled water

was used as positive control and DMSO as solvent control. Controls and samples were assayed in duplicate for each concentration and replicated three times for each cell line. The anticancer activity was obtained by comparing the absorbance between the samples and the control [27].

Statistical Analyses

To verify the statistical significance, mean ± SD of five independent measurements was calculated. Differences between groups were tested by two-way ANOVA. In the assessment of the antioxidant activity, Spearman correlation coefficients (*R*) were used. Linear regressions were also calculated. *P* values of <0.05 were considered significant.

Results

Bioactive Compounds

The results were summarized in the Table 1. As can be seen, the significantly highest content of polyphenols and flavonoids was in prolipid and mature leaves, flavanols—in seeds, and tannins—in prolipid, immature, and mature leaves ($P<0.05$ in all cases).

Mass Spectra

Gallic acid (Fig. 1a) and quercetin (Fig. 1b) were used as standards. The spectrum shows the main *m*/*z* peaks found in seeds (Fig. 2a) in methanol fraction: at 106, relative abundance (RA)058 %; benzoic acid at 120 has RA0100 %; and methyl vanillate at 180 has RA 018 %, at 214 RA 040 %. The peaks in seeds were not found in immature leaves at the same location (Fig. 2b): at 104, RA020 %; in comparison with the one at 106, RA was higher as twice as in leaves; instead of peak at 120, the peak appeared at 134 (RA075) of *p*-hydroxybenzoic acid; at 192 (RA0100 %) of scopoletin; at 356 (RA040 %) and at 365 (RA018 %). The same fraction for mature leaves showed the following peaks (Fig. 2c): one of the main peaks was located at 134 for *p*-hydroxybenzoic acid with RA095 %, slightly higher than for immature

Table 1 Bioactive compounds in methanol extracts of the studied samples per dry weight (DW)

Sample	Polyphenols mg GAE	Flavonoids mg CE	Flavanols μg CE	Tannins mg CE
Seeds	5.14±0.3 a	3.842±0.2 a	1,568.95±79.1 b	1.14±0.05 a
Immature leaves	11.55±0.5 b	5.175±0.2 a	432±4.4 c	2.56±0.1 b
Mature leaves	14.22±0.7 c	6.079±0.3b	213±2.1 d	2.95±0.2b
Prolipid	16.64±0.7 c	6.566±0.3 b	1,109.65±54.2 a	3.18±0.3 b

Values are means ± SD of five measurements. Values in columns for every bioactive compound with the same solvent bearing different letters are significantly different ($P<0.05$).

CE catechin equivalent, *GAE* gallic acid equivalent

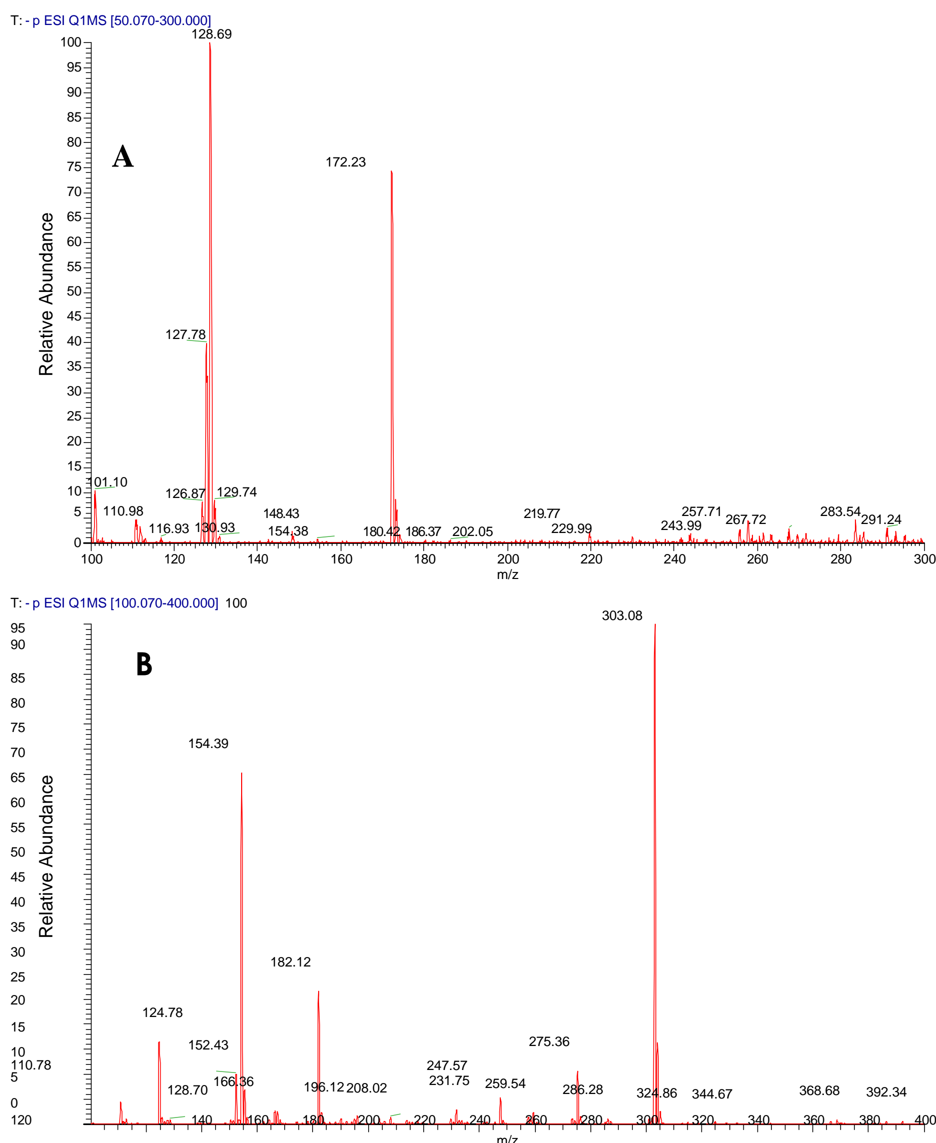
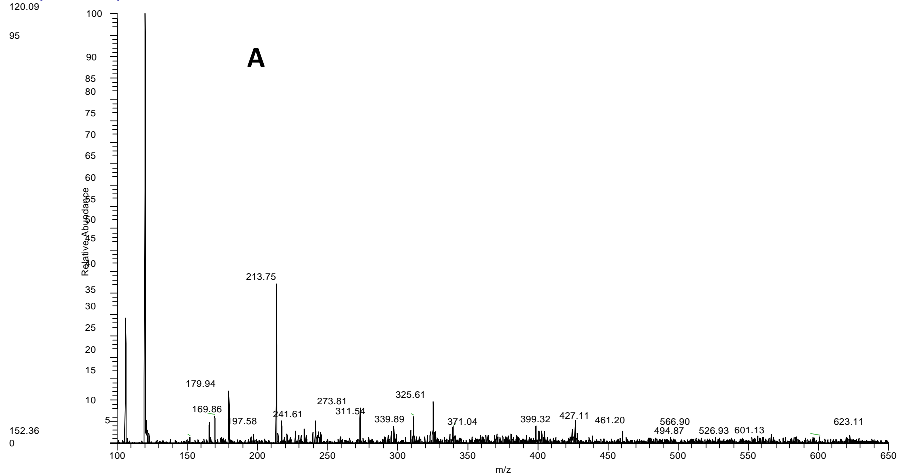
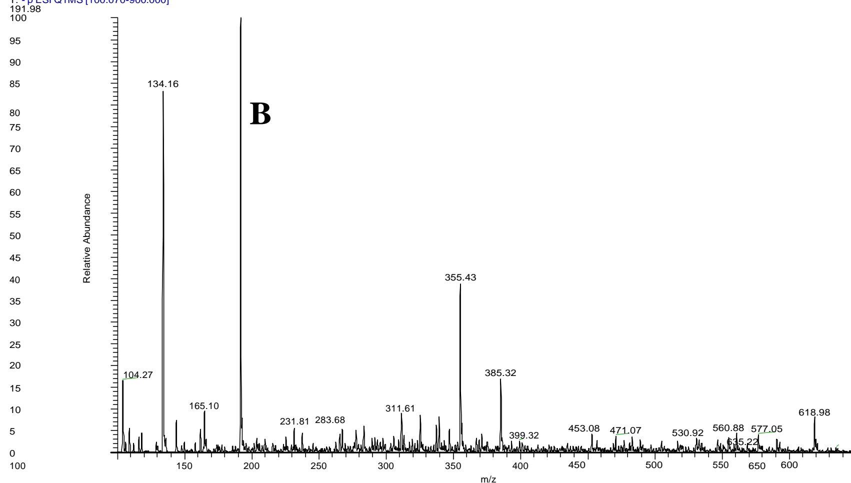


Fig. 1 ESI-MS spectra of a gallic acid; b quercetin

ones; at 192 (RA0100 %) of scopoletin, than the other ones at 355 (RA0100 %), 365 (RA040 %), and 611 (RA020 %).

The spectrum shows the main *m*/*z* peaks found in seeds (Fig. 3a) in ethyl acetate fraction: at 174 (RA020 %) for coniferaldehyde, at 188 (RA0100 %), at 312 (RA045 %), at 330 (RA065 %), and at 340 (RA040 %). Only two peaks were in immature leaves (Fig. 3b) with slightly different abundance such as at 314 with RA of 50 % and at 340 with RA of 75 %. Other peaks were for *p*-hydroxybenzoic acid at 138 (RA020 %); vanillic acid at 166 (RA0100 %); at 266 (RA040 %) for apigenin; at 294 (RA0100 %) for catechin, at


SG-022 #57 RT: 0.52 AV: 1 SB: 36 0.01-0.33 NL: 4.83E6 T: - p ESI
Q1MS [100.070-900.000]
A
Relative Abundance
213.75
179.94
169.86
273.81
325.61
311.54
241.61
339.89
371.04
399.32
427.11
461.20
566.90
494.87
526.93
601.13
623.11
m/z


T: - p ESI Q1MS [100.070-900.000]
191.98
B
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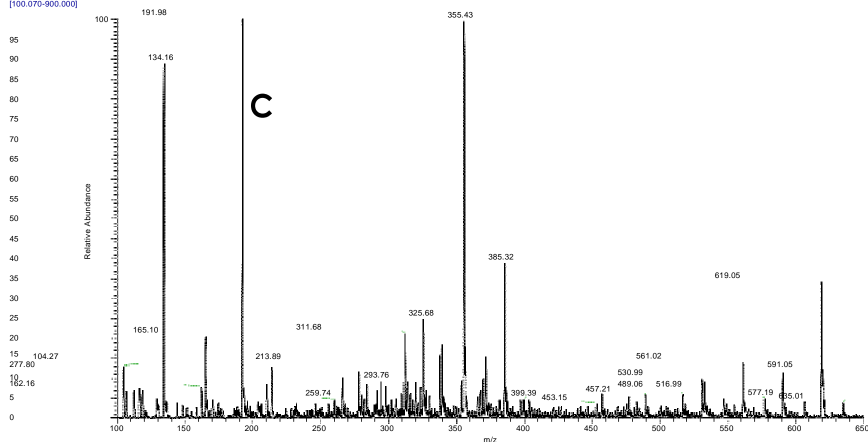
Relative Abundance
355.43
385.32
104.27
165.10
231.81
283.68
311.61
399.32
453.08
471.07
530.92
560.88
577.05
618.98
m/z


SG-010 #40-68 RT: 0.37-0.62 AV: 29 SB: 36 0.01-0.34 SM: 7B NL: 9.20E5 T: - p ESI Q1MS
[100.070-900.000]
191.98
355.43
134.16
C
Relative Abundance
385.32
619.05
165.10
311.68
325.68
213.89
293.76
561.02
591.05
530.99
489.06
516.99
259.74
399.39
453.15
457.21
577.19
104.27
277.80
162.16
m/z

R Fig. 2 ESI-MS spectra of methanol fractions of seeds (a), immature leaves (b), and mature leaves (c) of indigo plant in negative ion mode

326 with RA 070 % and 619 with RA of 15 %. The peaks appeared for mature leaves in the same fraction were the following (Fig. 3c): at 134 (RA 055 %) for *p*-hydroxybenzoic acid, the same peak was in immature leaves with slight shift. Another peaks appeared at 165 (RA 0100 %) for vanillic acid for both leaves; at 192 (RA 050 %) for scopoletin only in immature leaves; at 215 (RA050 %); at 286 (RA050 %); at 294 (RA085 %) for catechin for both leaves; and at 330 (RA060 %) and at 618 (RA0 50 %) which were shown in both leaves. The obtained results showed the same location of the peaks in both leave samples, only with higher amounts of the compounds showing different relative abundances.

The Antioxidant Activity

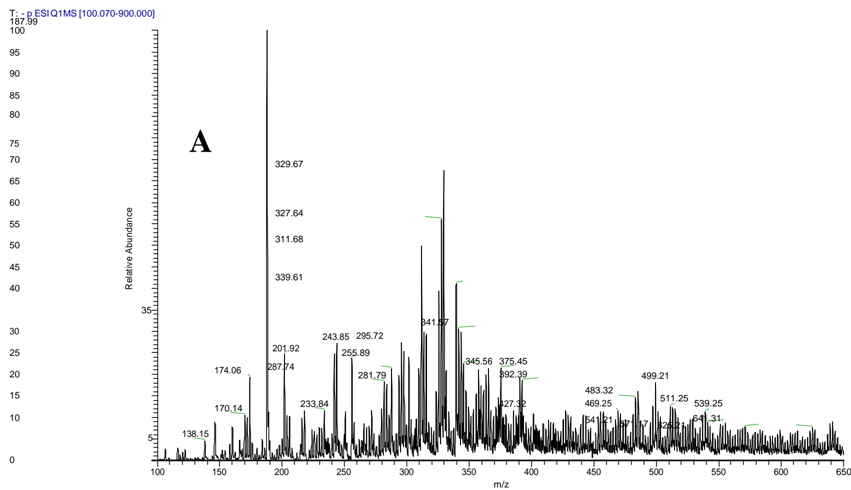
The results of the determination of the antioxidant activity in the studied samples are summarized in the Table 2. As can be seen, according to ABTS test, the significantly higher antioxidant activity was in mature leaves, according to CUPRAC—in prolipid and mature leaves, and according to FRAP—in prolipid, mature, and immature leaves ($P<0.05$ in all cases).

Fluorimetry

3D-FL (Fig. 4A, B) spectra illustrated the elliptical shape of the contour maps (Aa, Ba) and cross maps (Fig. 4Ab, Bb) of the main peaks for indigo methanol and ethyl acetate extracts of mature leaves. The main peaks for methanol extracts appeared at λex/em of 260/310 with fluorescence intensity (FI) of 889.58 and another one at λex/ em of 260/360 nm with FI of 776.07 (Fig. 4Aa, C, Ab). The ethyl acetate fraction had slightly different peaks: at λex/em of 260/320 with FI of 169.59 and at λex/em of 260/360 nm with FI of 165.94 (Fig. 4Ba, D, Bb). One of the main peaks for 2×10^{-5} M/L BSA was found at λex/em of 225–230/335 nm with FI of 877.60 (Fig. 4E, upper curve). The interaction of BSA and ethyl acetate extract of indigo mature leaves (Fig. 4E, middle curve, with FI0715.61) and BSA and indigo (Fig. 4E, lower curve, with FI 0650.81) showed the peak of 335 nm and decrease in the fluorescence intensity (FI). These results are in correspondence with the amount of polyphenols, antioxidant activity, and MS bioactivity data that the methanol extract is more bioactive than the ethyl acetate. The decrease in fluorescence intensity of BSA was about 18.5 % for the ethyl acetate fraction and 25.8 % for methanol fraction, showing higher quenching activity of methanol extracts of polyphenols. The interaction be- tween methanol and ethyl acetate polyphenol extracts of indigo and BSA showed that indigo has a strong ability as other studied medicinal plants to quench the intrinsic fluorescence of BSA by forming complexes.

FTIR Spectra

The FTIR spectra of methanol extract of prolipid (Fig. 5A, upper curve) was compared with ethyl acetate extract of indigo mature leaves (Fig. 5B, middle curve) and with methanol extract of indigo mature leaves (Fig. 5C, lower curve). Noticeably, the presence of wave-
lengths of FTIR spectra of gallic acid at 860, 1,025, 1,100, and 1,654 cm^{-1}, tannic acid at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187.99
A
329.67
327.64
311.68
339.61
341.57
243.85
295.72
201.92
255.89
174.06
287.74
281.79
345.56
375.45
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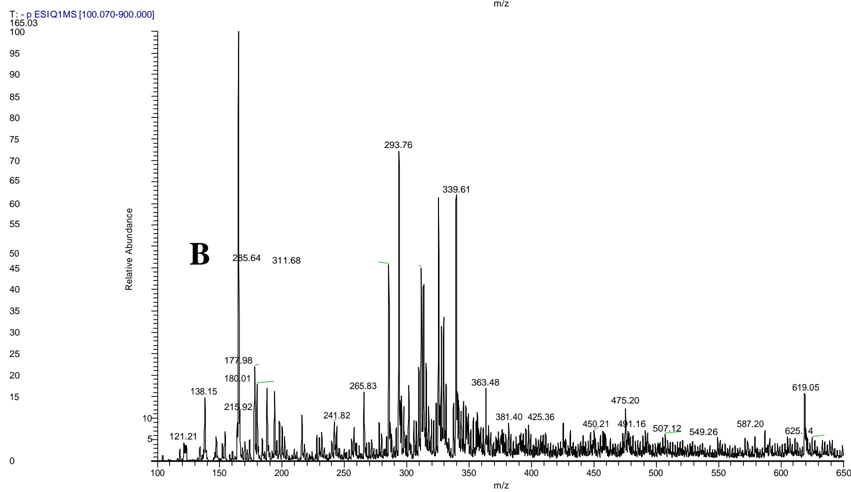
499.21
483.32
469.25
511.25
539.25
170.14
233.84
138.15
Relative Abundance
m/z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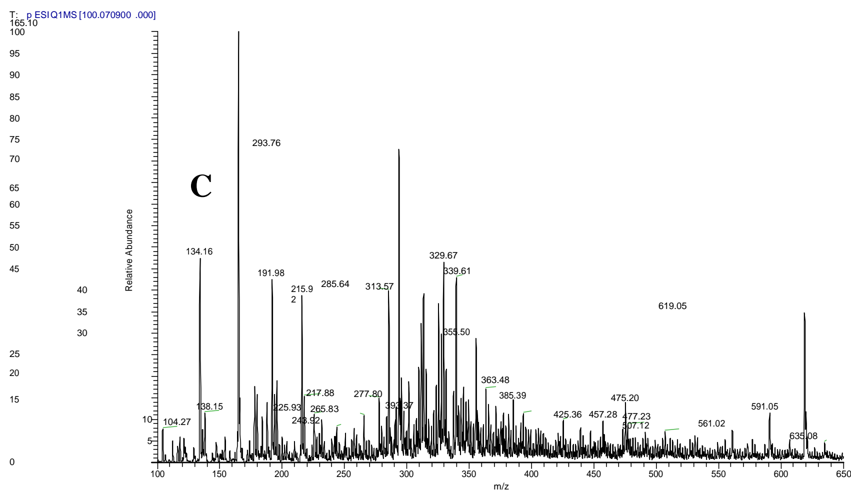
165.03
B
293.76
339.61
285.64
311.68
177.98
180.01
138.15
215.92
265.83
363.48
619.05
475.20
241.82
381.40
425.36
450.21
491.16
507.12
549.26
587.20
121.21
Relative Abundance
m/z


165.10
C
293.76
134.16
191.98
285.64
313.57
329.67
339.61
619.05
355.50
363.48
385.39
217.88
277.80
475.20
425.36
457.28
477.23
507.12
561.02
591.05
104.27
138.15
265.83
Relative Abundance
m/z

R Fig. 3 ESI-MS spectra of ethyl acetate fractions of seeds (a), immature leaves (b), and mature leaves (c) of indigo plant in negative ion mode

1,172, 1,511, and 1,627, and *p*-coumaric acid at 1,124, 1,171, 1,508, and 1,638 cm^{-1} were observed in samples analyzed. The wavelength of FTIR spectra corresponding for vanillin was 1,498, 1,534, 1,617, 1,654, and 3,392 cm^{-1} [24, 26]. The main bands presenting in the samples are the following: the band of 1,029 cm^{-1} (−C-O alcohols) is exactly found in ethyl acetate extract of mature leaves (Fig. 5, line b) with a small shift at 1,017 cm^{-1} for the dry substance (Fig. 5, line a) and for methanol extract of 1,033 cm^{-1} (Fig. 5, line c). The band of 1,280 cm^{-1} (−OH aromatic) appeared in slightly different location of 1,201 cm^{-1}. Other peaks appeared at 1,319–1,397 cm^{-1}. The peak of 1,422 cm^{-1} (−C-O alcohols) appeared only in prolipid. The peak of 1618 cm^{-1} (C0C aromatic and C0C alkenes) appeared in all the samples with a shift at 1,650 and 1,597 cm^{-1} for carbonyl substituents. The broad band of 3,309, 2,925 and 2,917 cm^{-1} belong to glycosidic groups O-H. FTIR of quercetin as a standard showed broad phenolic OH band centered around 3,404 cm^{-1}, characteristic -CO stretching at 1,663 cm^{-1} aromatic bending and stretching around 1,091 and 1,663 cm^{-1}, and -OH phenolic bending around 1,197 and 1,374 cm^{-1} [28]. FTIR spectra of water extracts of mature indigo leaves [28] showed a peak characteristic -CO stretching at 1,634 cm^{-1} aromatic bending and the peaks at 2,925 and 2,852 cm^{-1} are related to the C–H bond of saturated carbons, which are different from our results of methanol and ethyl acetate extracts. Matching between the peaks in the range from 4,000 to 400 cm^{-1} of (prolipid methanol extract)/(indigo ethyl acetate extract)065.08 %, (prolipid methanol extract)/(indigo metha- nol extract)076.52 %, and (indigo ethyl acetate extract)/(indigo methanol extract)069.41 % (Fig. 5). Matching between the peaks of the water extracts of indigo mature leaves and the same substances in the same range of the peaks in prolipid was slightly higher of about 78.38 % [28].

Anticancer Activity

It was observed that the percentage of proliferativity of the methanol and ethyl acetate extracts of mature leaves and prolipid samples on two cell lines (Fig. 6a, Calu-6 for human pulmonary carcinoma and Fig. 6b, SNU-601 for human gastric carcinoma) was different. The proliferativity (in percent) for concentrations of 800 μg/mL for methanol and ethyl acetate extracts of prolipid on Calu-6 were 75.49 and 79.24 %, respectively, and on SNU- 601 were 77.42 and 80.45 %, showing the highest antiproliferative activity in comparison

Table 2 Antioxidant activities (in micromole Trolox equivalents) in methanol extracts of the studied samples per dry weight (DW)

Sample	ABTS	CUPRAC	FRAP
Seeds	68.326±3.4 b	29.27±1.3 a	12.21±0.6 a
Immature leaves	134.438±6.6 c	30.62±1.4 a	19.79±0.9 b
Mature leaves	185.464±9.1 d	59.46±2.8 b	20.91±1.1 b
Prolipid	206.24±20.2a	64.65±3.1 b	22.68±1.2 b

Values are means ± SD of five measurements. Values in columns for every bioactive compound with the same solvent bearing different letters are significantly different ($P<0.05$).

ABTS 2,2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt, *CUPRAC* cupric reducing antioxidant capacity, *FRAP* ferric-reducing/antioxidant power

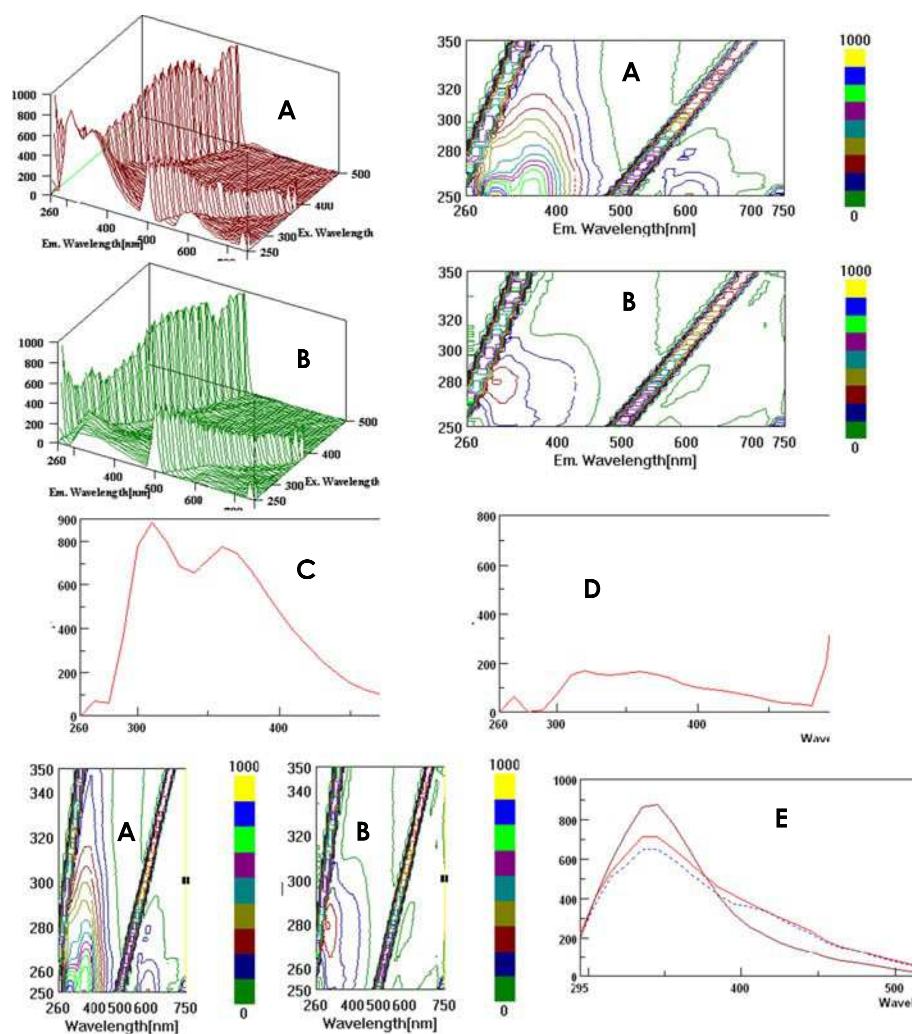


Fig. 4 *A* three-dimensional fluorescence (3D-FL) spectrum of methanol extract (0.001 mg/mL) of indigo mature leaves, *Aa* elliptical shapes of the contours of methanol extracts of indigo mature leaves, *B* 3D-FL spectrum of ethyl acetate extract (0.001 mg/mL) of indigo mature leaves, *Ba* elliptical shapes of the contour map of ethyl acetate extracts of indigo mature leaves, *C* 2D-FL of methanol extract of indigo mature leaves, *D* 2D-FL of ethyl acetate extract of indigo mature leaves, *Ab* cross maps of methanol extracts of indigo mature leaves, *Bb* cross maps of ethyl acetate extracts of indigo mature leaves, *E* change in the fluorescence intensity (ID) as a result of binding affinity of: 2.0×10^{-4} mol/L of BSA (*upper line*); BSA and 40 μg/mL of indigo leave methanol extract (*middle line*); BSA and 40 μg/mL of indigo leave ethyl acetate extract (*lower line*); the 3D-FL were run emission mode and fluorescence intensity up to 1,000, emission wavelengths from 260 to 750 nm and excitation wavelengths from 250 to 500 nm; scanning speed was 1,000 nm/min, For *Aa*, *Ba*, *C*, *D*, *Ab*, *Bb*, and *E*, emission wavelength on x-axis and fluorescence intensity on y-axis for *C*, *D* and *E*; for *Aa*, *Ba*, *Ab* and *Bb*, excitation wavelength on y-axis

with mature leave sample for Calu-6 (76.12 and 80.22 %) and SNU-601 (79.43 and 82.26 %). Our investigation shows that antioxidant activity of the studied samples was highly correlated with their antiproliferative activity.

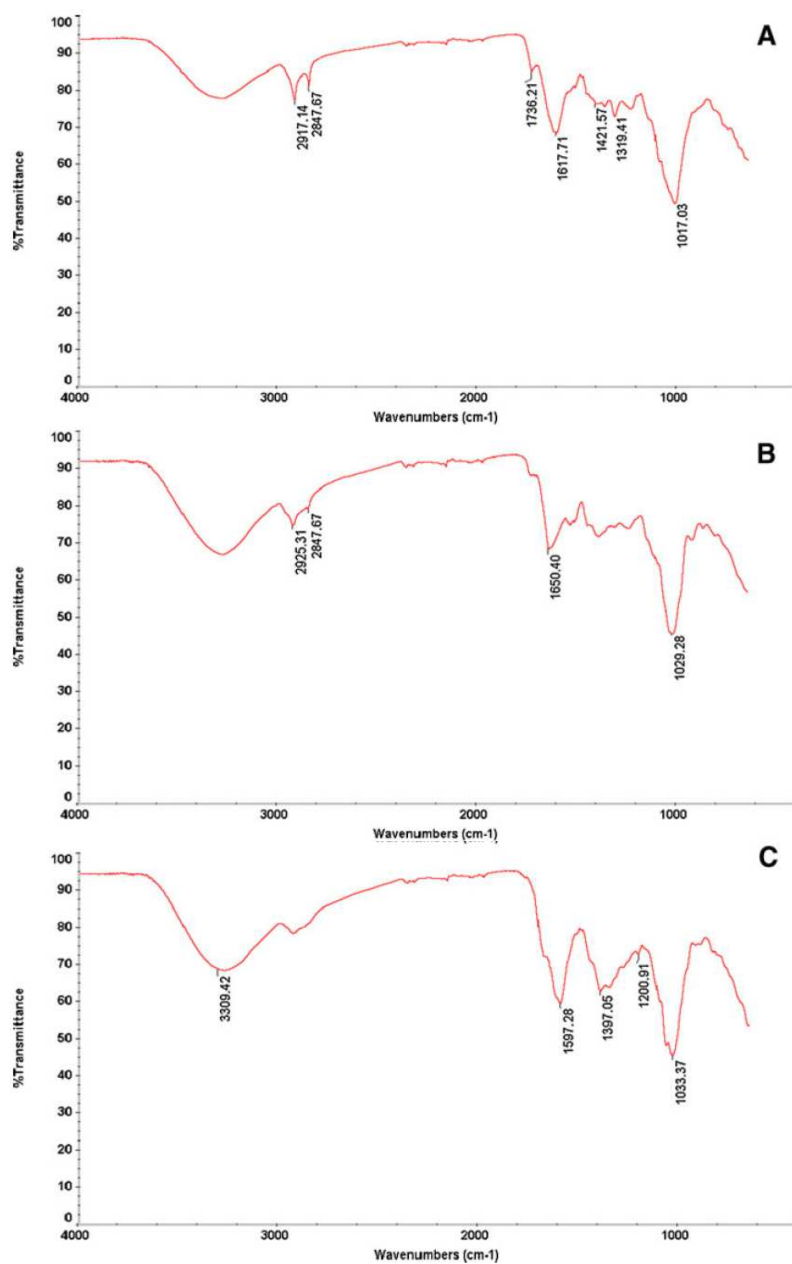


Fig. 5 Infrared study of FTIR spectra of **a** methanol extract of prolipid; **b** ethyl acetate extract of indigo mature leaves; and **c** methanol extract of indigo mature leaves

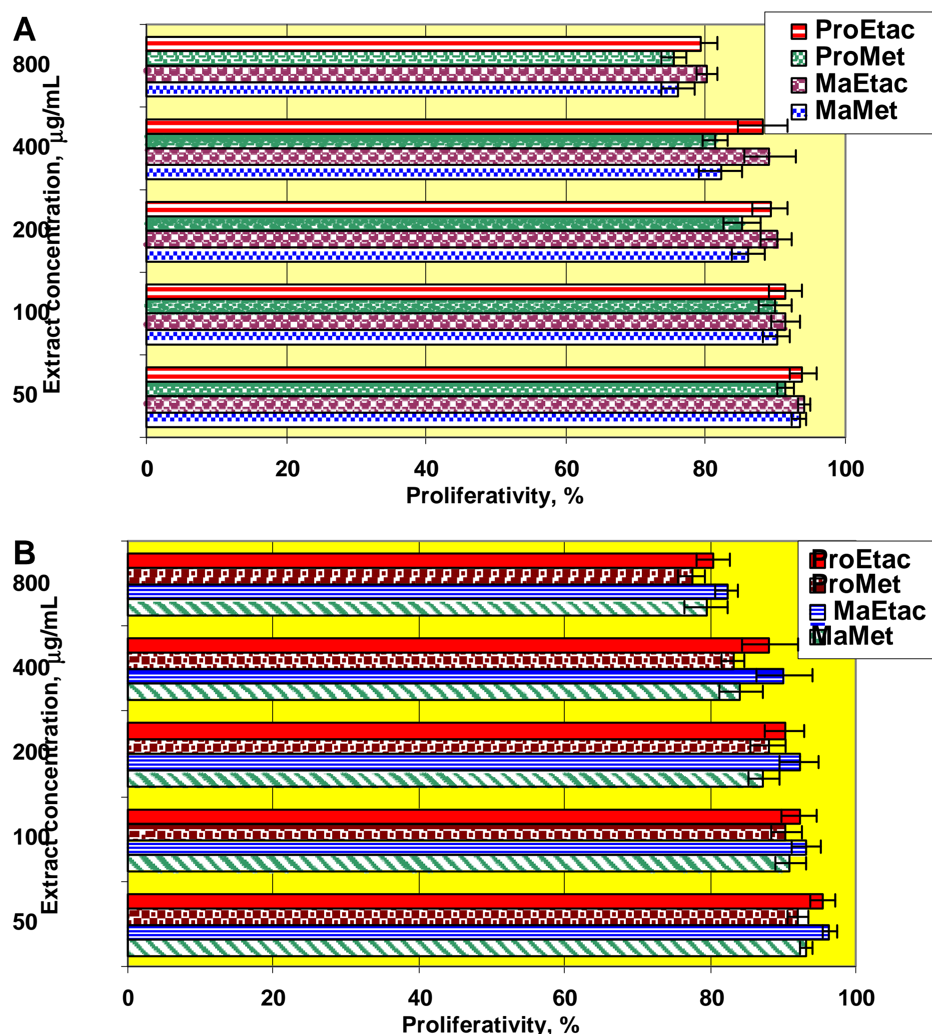


Fig. 6 The proliferativity (in percent) of human cancer cells of the a CALU-6 and b SNU-601 lines, in the presence of methanol and ethyl acetate indigo mature leaves and prolipid. Each point represents the mean ± SD (*n*06). Abbreviations: *Proetac* prolipid ethyl acetate extract, *ProMe* prolipid methanol extract, *MaEtac* mature leaves ethyl acetate extract, *MaMet* mature leaves methanol extract

Discussion

The obtained results of our research can be connected with the recently performed experiments in order to find the scientific basis for the health properties of this plant [29], where the antinociceptive, anti-inflammatory, and antipyretic effects of indigo plant root methanolic extract were evaluated. The indigo plant root extracts significantly and dose-dependently inhibited the writhing responses of mice and decreased the licking time in both the early and late phases of the formalin test. However, as was stated, the research on the content of bioactive compounds and the antioxidant and anticancer activities of indigo was limited. Therefore, some other plants were

reviewed in order to compare the obtained results. So, Dall'Acqua et al. [8] evaluated in vitro antioxidant properties of some traditional medicinal plants: investigation of the high antioxidant capacity of *Rubus ulmifolius* used in Sardinia as tea beverages or as decoction for medicinal purposes. Among the various species, *R. ulmifolius* resulted as the more bioactive with all the used methods. Phytochemical investigation revealed several phenolic compounds as caffeic acid, ferulic acid, quercetin, kaempferol-3-O-glucuronide, kaempferol-3-O-(6''-p-coumaroyl)-β-D-glucopyranoside, kaempferol-3-O-(6''-caffeoyl)-β-D-glucopyranoside, and many others which are in accordance with our MS data, which are responsible for the antioxidant properties. Our results can be compared with Generalic et al. [11], who studied the phenolic profile and antioxidant properties of Dalmatian sage. The results strongly indicate that Dalmatian sage leaves are rich source of valuable phenolics, mainly phenolic acids, with extremely good antioxidant properties. The presence of resveratrol or its derivates was confirmed in all extracts. The authors found that the best results for total phenols and flavonoids, as well as the best antioxidant properties were obtained for May sage. Our results can be compared with the phytochemical composition and antioxidant activity of wild medicinal plants, based on chemical, biochemical, and electrochemical methods. So, *F. ulmaria* was found to be the richest in antioxidant phytochemicals, such as phenolics (228 mg GAE/g DW) and flavonoids (62 mg CE/g DW). The antioxidant activity was found to vary in the order: *F. ulmaria* > *S. nigra* > *C. multiflorus*, irrespective of the analysis method [7]. Seven compounds related to flavonoids and a mixture of two caffeic acid esters were isolated from *L. erythrorhizon* Sieb et. Zucc. and identified by spectroscopic methods with good radical scavenging activities toward ABTS but showed moderate inhibition of DPPH [12]. The presented MS data (Figs. 1, 2, and 3) were in accordance with others [2], where for direct identification of the organic dye compounds quercetin, indigotin, and alizarin in reference materials, in solution, by use of direct analysis in real time ionization and high-resolution time-of-flight mass spectrometry was done. These data are in accordance with Mantzouris et al. [1] that the treatment by the standard HCl dyestuff extraction method revealed different flavonoids and phenolic acids, where some of them are listed: apigenin, ellagic acid, fisetin, indigotin, indirubin, kaempferol, naringenin, quercetin, and others. Our results exactly in accordance with others [3], where the composition of the natural dyes was determined after different extraction procedures. The efficiency of eight different procedures used for the extraction of natural dyes was evaluated using contemporary wool samples dyed with cochineal, madder, woad, weld, brazilwood, and logwood. Comparison was made based on the LC-DAD peak areas of the natural dye's main components which had been extracted from the wool samples. Among the tested methods, an extraction procedure with Na_2EDTA in water/DMF (1:1, *v*/*v*) proved to be the most suitable for the extraction of the studied dyes, which presented a wide range of chemical structures [3].

The present results can be compared with our recent ones [28], where the water extract of indigo plant was analyzed. In water extract, the polyphenols and flavonoids were significantly higher in prolipid, flavanols—in indigo seeds. Our results are in accordance with Fialova et al. [30], where in leaves of *Isatis tinctoria* L. the following indices were determined: total polyphenols (3.03 %), tannins (1.05 %), and total flavonoids (expressed as isoquercitrin 0.3 %). The phenolic compounds showed higher radical scavenging activity that vitamin C [10]. The antioxidant activity was the highest in prolipid, followed by indigo mature leaves. Exactly the same relationship was obtained in methanol and ethyl acetate extracts, but the highest

value was in methanol fraction [28]. The composition of the indigo plant depends on the extraction procedure. Results of the study of five plants, of which four are endemic to Turkish flora [31] showed that the plants were screened for their possible in vitro antioxidant activities by two complementary test systems (DPPH and β-carotene/linoleic acid). In the first case, *Pelargonium endlicherianum* extract exerted greater antioxidant activity with an IC_{50} value of 7.43 ± 0.47 μg/mL, followed by *Hieracium cappadocicum* of 30.0 ± 0.14 μg/mL. When compared to the synthetic antioxidant BHT (18.0 ± 0.40 μg/mL), the methanolic extract of *P. endlicherianum* exhibited more than twofold greater antioxidant activity. In the β-carotene/linoleic acid test system, the most active plant was *P. endlicherianum* with 72.6 %± 2.96 inhibition rate, followed by *H. cappadocicum* (55.1 %± 2.33) and *Verbascum wiedemannianum* (52.5 %± 3.11). The results of antioxidant activities of indigo plant (Table 2) are in agreement with the above-cited data. A strong correlation between TEAC values and those obtained from CUPRAC assay implied that antioxidants in these plants were capable of scavenging free radicals and reducing oxidants. A high correlation between antioxidant capacities and their total phenolic contents indicated that phenolic compounds were a major contributor of antioxidant activity of these plants. Our results on cytotoxicity are in accordance with others [32–34]. The antioxidant activity of maca (*Lepidium meyenii*) was assessed by the inhibition of peroxynitrite. Maca (1 mg/mL) protected RAW 264.7 cells against peroxynitrite-induced apoptosis and increased ATP production in cells treated with H_2O_2 (1 mM). The concentration of catechins in maca was lower than in green tea (2.5 vs. 145 mg/g). Maca has the capacity to scavenge free radicals and protect cells against oxidative stress. Our results can be compared with the recent work of Lin et al. [4]. The extract of indigo naturalis (QD) and its main components indirubin, indigo, and tryptanthrin in human neutrophils were investigated for their anti-inflammatory effects. QD showed the significant inhibition of superoxide anion, attenuated the formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP)-induced phosphorylation of extracellular regulated kinase; QD inhibited calcium mobilization caused by FMLP. On the other hand, neither indirubin, indigo, nor tryptanthrin produced similar changes in human neutrophils.

The plant extracts were tested for cytotoxicity by the brine shrimp lethality assay, sea urchin eggs assay, hemolysis assay, and MTT assay, using tumor cell lines [32]. The extract of *Oroxylum indicum* showed the highest toxicity on all tumor cell lines tested, with an IC50 of 19.6 μg/mL for CEM, 14.2 μg/mL for HL-60, 17.2 μg/mL for B-16 and 32.5 μg/mL for HCT-8. On the sea urchin eggs, it inhibited the progression of cell cycle since the first cleavage (IC50013.5 μg/mL). As was recently shown by Heo et al. [27] that the Korean medicinal plants, which were used for a long time as traditional seasoned salads, possess anticancer activity. Our studies on cytotoxicity are in correspondence with Iwaki and Kurimoto [6], where it was shown that tryptanthrin and indirubin, both compounds originating from indican in the leaves of *P. tinctorium*, are responsible for many of the biological activities of this plant. Tryptanthrin has a potent anti-inflammatory activity and shows growth inhibitory activity against cancer cell lines in vitro. The effect of this substance on azoxymethane-induced intestinal tumorigenesis in rats with carcinogenesis in the intestines is closely associated with inflammation. Tryptanthrin inhibited the incidence of intestinal tumors. Indirubin has been reported to possess an anti-leukemic activity and *P. tinctorium* also contains various anti-oxidative substances, such as gallic acid and caffeic acid, with potential anti-tumor activity. We consider it likely

that *P. tinctorium* shows cancer preventive activity as a consequence of the integral effects of these substances. Our results are in accordance with others [35], where diploid leaf extracts of *Gynostemma pentaphyllum* Makino, which is used in tea and food, had strongest inhibition on inflammation and HT-29 proliferation, but these extracts had different order of antiproliferative properties in the LNCaP cells. The interaction between water polyphenol extracts of indigo mature leaves and BSA showed that indigo has a strong ability, as other medicinal plants, to quench the intrinsic fluorescence of BSA by forming complexes [28]. Better ability is shown by methanol extract. The application of IR spectroscopy and fluorescence in herbal
analysis is still limited when compared to other areas. The representative IR spectra from the mid-IR region (4,000–800 cm^{-1}) for ethyl acetate and methanolic extracts were observed. The three extracts in the region of polyphenols showed slight variation in bands than the standards.

Conclusions

It was found that polyphenols, flavonoids, and flavanols were significantly higher in prolipid, following by indigo mature leaves, immature leaves, and seeds. The ability of indigo to quench the intrinsic fluorescence of BSA, relatively high content of polyphenols compounds and anticancer properties can be used as a new source of antioxidants.

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Application of Analytical Methods for the Determination of Bioactive Compounds in Some Berries

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Abstract Fluorometry, ESI-MS, FTIR, and radical scavenging assays were used for characterization of bioactive compounds and the levels of their antioxidant activities. Polyphenols, flavonoids, anthocyanins, and ascorbic acid and the level of antioxidant activity of water extracts of "Murtilla-like" [*Myrteola nummularia* (Poiret) Berg.], and other widely consumed berries were determined and compared. The contents of bioactive compounds and the levels of antioxidant activities in water extracts differed significantly in the investigated samples ($P<0.05$). "Murtilla-like" extracts contained polyphenols (mg GAE/g)−19.13±0.9, flavonoids, (mg CE/g)−3.12±0.1, anthocyanins (mg CGE/g)−120.23±5.4, and ascorbic acid (mg/g)−2.20±0.1; and antioxidant activities (μmolTE/g) by ABTS and CUPRAC assays were 200.55±8.7 and CUPRAC 116.76±5.7, respectively. Chemometrical processing was done on the basis of kinetic data of two variables (concentration and reaction time) by DPPH scavenging reaction. Polyphenol content highly correlated with antioxidant capacity (R^2 from 0.96 to 0.83). The quenching properties of berries were studied by the interaction of water polyphenol extracts with a small protein such as BSA by 3-D fluorescence and FTIR spectroscopy. These methods were used as additional tools for the characterization of polyphenols. Wild-grown non-investigated berries were compared with widely consumed ones, using their bioactive composition, antioxidant activities, and antiproliferative and fluorescence properties. In conclusion, the antioxidant properties of "Murtilla-like" can be used as a new source for consumption. The bioactivity of "Murtilla-like" is comparable with blueberries and raspberries. 3-D fluorescence and FTIR

This article was written in memory of my dear brother Prof. Simon Trakhtenberg, who died in November 2011, who encouraged me and our entire scientific group during all his life.

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spectroscopy can be applied as additional analytical tools for rapid estimation of the quality of food products.

Keywords Analytical methods · Berries · Bioactive compounds · Antioxidant activity

Introduction

Consumption of berries and fruits has become popular among health-conscious consumers due to the high levels of valuable antioxidants, such as plenolics (Wolfe et al. 2008). These phytochemicals include flavonoids, stilbenes, tannins, phenolic acids, and anthocyanins (Céspedes et al. 2010; Paredes-Lopez et al. 2010; You et al. 2011; Ahmad et al. 2012; Kang et al. 2012; Yadav et al. 2012). Administration of a freeze-dried powder of mulberry (*Morus alba* L.) fruit (MFP) to rats on a high-fat diet resulted in a significant decline in levels of serum and liver triglyceride, total cholesterol, serum low-density lipoprotein cholesterol, and a decrease in the atherogenic index. Oppositely, the serum high-density lipoprotein cholesterol was significantly increased (Yang et al. 2010). Berry phenolics may also act as antimicrobials which may be of help in the control of the wild spectra of pathogens, in view of recent problems associated with antibiotic resistance (Paredes-Lopez et al. 2010). Recent studies in vitro and in vivo have been improved the scientific understanding of how berries and fruits promote human health and prevent chronic illnesses such as some cancers, heart and neurodegenerative diseases (Prior et al. 2008; Seeram 2010). The purpose of some studies was to investigate and to compare the composition, stability, antioxidant and anticancer properties, and mechanisms of anthocyanin-containing berries extracts from selected cultivars and using different extraction methods. The influence of water content in the extraction system was evaluated. A 90-day stability study of the extract and a 48-h stability study of the extract in biologically relevant buffers were completed (Dai et al. 2009). Potential benefits of polyphenolic compounds from raspberry seeds of three different extracts as efficient antioxidants was studied (Gođevac et al. 2009). The use of blackberry showed also its different properties: blackberry administration minimized the toxic effects of fluoride (Hassan and Abdel-Aziz 2010). Berries contain powerful antioxidants, potential allergens, and other bioactive compounds (Battino et al. 2009). Anthocyanins are water-soluble plant pigments that have important functions in plant physiology as well as possible health effects (Valcheva-Kuzmanova et al. 2005; Wu et al. 2006). Antioxidant capacity and phenolic compounds (phenolic acids and anthocyanins) of four berry fruits (strawberry, Saskatoon berry, raspberry, and wild blueberry), chokecherry, and seabuckthorn were compared (Li et al. 2009). Different fractions of mature wild blackberry *Aristotelia chilensis* (Mol) Stuntz (Elaeocarpaceae) were analyzed. Cranberry was investigated as chemotherapeutic agent (Elberry et al. 2010; Cuevas-Rodriguez et al. 2010). Some wild Jamaica-grown species and the Michigan-grown *Rubus acuminatus*, *Rubus idaeus* cv. Heritage, and *Rubus idaeus* cv. Golden were analyzed for their anthocyanin contents, and lipid peroxidation, cyclooxygenase enzyme, and human tumor cell proliferation inhibitory activities. The high anthocyanin contents and biological activities of these fruits indicate that their consumption would be beneficial to health. This may be useful in the production of functional foods containing an efficacious dose of anthocyanins (Bowen-Forbes et al. 2010). The subject of different berries was investigated intensively, and it was shown in the cited literature, including the studies of Chilean berries (Céspedes et al. 2010). *Ugni molinae Turcz*, also known as "Murtilla", is a plant that grows in the south of Chile. Infusions of their leaves have long been used in traditional native herbal medicine (Rubilar et al. 2006; Suwalsky et al. 2006). The bioactivity of "Murta" ("Murtilla") was investigated by Rufino et al. (2010). It was interesting to compare different extraction procedures in some fruits and plants (Chanda and Kaneria 2012; Khoo et al. 2012). In our recent research the methanol extracts of different berries was investigated and compared (Arancibia-Avila et al. 2011). We were interested to investigate water extracts of a new kind of Chilean berry known by the name of "Myrteola" or "Murtilla-like" and to compare its composition with the widely consumed berries. The water extracts of berries are important from the point of tea consumption. To meet this aim the contents of bioactive compounds (polyphenols, flavonoids, anthocyanins, and ascorbic acid) and the level of antioxidant activities (AA) were determined and compared. In order to receive reliable data the AA was determined by three assays: cupric-reducing antioxidant capacity (CUPRAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Apak et al. 2004; Ozgen et al. 2006; Re et al. 1999). In order to compare the fluorescence properties of the extracted bioactive compounds, in vitro studies were performed by interaction of protein with flavonoids. Human serum albumin is the drug carrier's protein and serves to greatly amplify the capacity of plasma for transporting drugs. It is interesting to investigate in vitro how this protein interacts with flavonoids extracted from berry samples in order to get useful information of the properties of flavonoid-protein complex. Therefore the functional properties of a new kind of berry were studied by the interaction of water polyphenol extracts with a small protein such as bovine serum albumin (BSA) (Zhang et al. 2009). The advanced analytical methods such as 3D-FL and FTIR spectroscopy were applied in this research.

As far as we know no results of such investigations were published.

Material and Methods

Reagents

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 1,1-diphenyl-2-picrylhydrazyl, Folin-Ciocalteu reagent, urea, catechin, $CuCl_2 \times 2H_2O$, 2,9-dimethyl-1,10-phenanthroline (neocuproine), and lanthanum (III) chloride heptahydrate were purchased from Sigma Chemical Co., St Louis, MO, USA. All reagents were of analytical grade. Deionized and distilled water was used throughout.

Samples

Chilean "Murtilla", "Murta" *(U. molinae* Turcz) and "Myrteola" berries (*Myrtaceae*, *Myrteola nummularia* (Poiret) Berg.), Chilean and Polish blueberries (*Vaccinium corymbosum*), raspberries (*R. idaeus*), and black chokeberry (*Aronia melanocarpa*) were investigated. "*Myrteola*" *nummularia* (Poiret) Berg. *Myrtaceae*, (Daudapo) is distributed geographically from Valdivia to Magallanes. The fruit is edible. The fruits were harvested at their maturity stage and "Murtilla" and "Myrteola" berries were in two stages of ripening. "Myrteola" ripe was harvested in May 2008. "Myrteola" non-ripe was harvested in February 2010, in Chiloé. "Murtilla" non-ripe was collected in Puerto Varas, Chile, in February 2010 (Fig. 1). Arandano (blueberries) and raspberries were purchased at the local market in Chillan, Chile; and blueberries and chokeberries were purchased at the local market in Warsaw, Poland. For the investigation five replicates of five berries each were used. Their edible parts were prepared manually without using steel knives. The prepared berries were weighed, chopped, and homogenized under liquid nitrogen in a high-speed blender (Hamilton Beach Silex professional model) for 1 min. A weighed portion (50-100 g) was then lyophilized for 48 h (Virtis model 10-324), and the dry weight was determined. The samples were ground to pass through a 0.5-mm sieve and stored at −20 °C until the bioactive substances were analyzed.

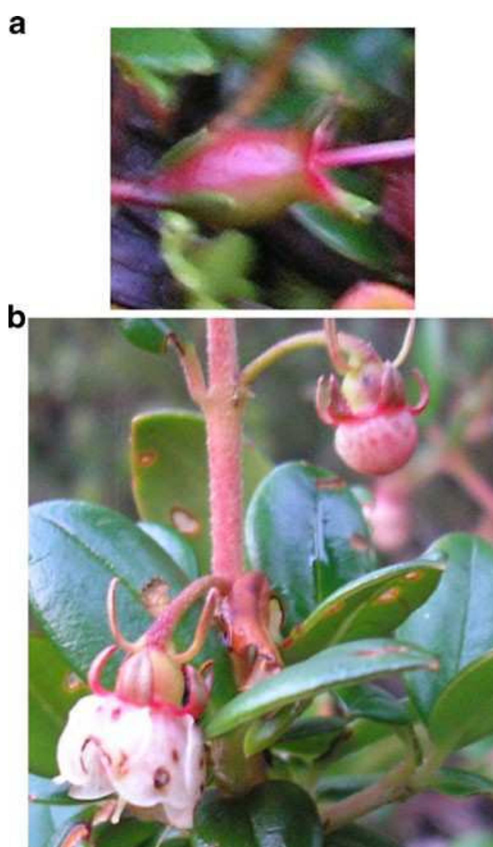


Fig. 1 Chilean berries: a "Myrteola", b "Murtilla"

Determination of Bioactive Compounds and Antioxidant Activity

The contents of polyphenols, flavonoids, anthocyanins, and ascorbic acid in water extracts of the studied samples were determined as previously described (Gorinstein et al. 2009). Phenols were extracted from lyophilized berries with water (concentration 25 mg/ml) at room temperature twice during 3 h. The polyphenols were determined by Folin-Ciocalteu method with measurement at 750 nm with spectrophotometer (Hewlett-Packard, model 8452A, Rockville, USA). The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of DW (Singleton et al. 1999). Flavonoids, extracted with 5 % $NaNO_2$, 10 % $AlCl_3 \times 6H_2O$ and 1 M NaOH, were measured at 510 nm. The extracts of condensed tannins (procyanidins) with 4 % methanol vanillin solution were measured at 500 nm. (+)-Catechin served as a standard for flavonoids, and the results were expressed as catechin equivalents (CE). Total ascorbic acid was determined by CUPRAC assay (Ozyurek et al. 2007) in water extract (100 mg of lyophilized sample and 5 ml of water). The absorbance of the formed bis (Nc)-copper (I) chelate was measured at 450 nm. The total anthocyanins were measured by a pH differential method. Absorbance was measured in a Beckman spectrophotometer at 510 nm and at 700 nm in buffers at pH 1.0 and 4.5, using the following equation: A 0 $[(A_{510}\text{-}A_{700})_{\mathrm{pH1.0}} - (A_{510}\text{-}A_{700})_{\mathrm{pH4.5}}]$. Results were expressed as milligrams of cyanidin-3-glucoside equivalent (CGE) per gram of DW (Cheng and Breen 1991).

The AA was determined by three assays:

- 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^+$) method for the screening of

antioxidant activity is reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant activity. ABTS $^{\bullet+}$ radical cation was generated by the interaction of ABTS (7 mM/L) and $K_2S_2O_8$ (2.45 mM/L). This solution was diluted with methanol until the absorbance in the samples reached 0.7 at 734 nm (Re et al. 1999).

- Cupric-reducing antioxidant capacity: This assay is based on utilizing the copper (II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidizing agent. The absorbance at 450 nm was recorded against a reagent blank (Apak et al. 2004).
- 1,1-diphenyl-2-picrylhydrazyl antioxidant activity assay (Ozgen et al. 2006) was used for kinetic studies with extracts of different berries.

Fluorometry and Fourier Transform Infrared Spectra Studies

Two dimensional (2D-FL) and three dimensional (3D-FL) fluorescence measurements were done using a model FP-6500, Jasco Spectrofluorometer, serial N261332, Japan. Fluorescence emission spectra for all berries samples at a concentration of 0.25 mg/ml in water were taken at emission wavelength (nm) of 330, and recorded from wavelength of 265 to a wavelength of 310 nm, at emission wavelengths of 685 nm from 300 to 750 nm; and at excitation of 350 nm from 370 to 650 nm. Catechin was used as a standard. 3D-FL spectra of the investigated berries extracts were collected with subsequent scanning emission spectra from 250 to 750 nm at 1.0 nm increments by varying the excitation wavelength from 230 to 350 nm at 10 nm increments. The scanning speed was set at 1,000 nm/min for all measurements. All measurements were performed with emission mode and with intensity up to 1,000 (Wulf et al. 2005; Zhang et al. 2009). All solutions for protein interaction were prepared in 0.05 mol/L Tris-HCl buffer (pH 7.4), containing 0.1 mol/l NaCl. The final concentration of BSA was 2.0×10^{-4} mol/L. All solutions were kept in dark at 0-4 °C. The BSA was mixed with catechin. The samples were mixed in the proportion of BSA: extract01:1. Denaturation with 2.4 M and 4.8 M urea was carried out as well. The samples after the interaction with BSA were lyophilized and subjected to FTIR.

The presence of polyphenols in the investigated berries samples and the interaction between polyphenols and bovine serum albumin was studied by Fourier transform infrared spectroscopy. A Nicolet iS 10 FT-IR Spectrometer (Thermo Scientific Instruments LLC, Madison, WI, USA), with the smart iTR^{TM} ^{ATR} (attenuated total reflectance) accessory was used to record IR spectra (Sinelli et al. 2008).

Chemometrical Processing

Samples with different concentrations of berry water extracts (1, 2.5, 5, 10, 15, 20, and 30 mg/mL) were analyzed by DPPH antioxidant activity assay (Ozgen et al. 2006). In the kinetic studies two variables were used: the change in the concentration of the samples and the change in time of the reaction with scavenging radical: 1, 10, 30, 60, and 90 min. The DPPH data (μmol Trolox equivalent TE)/g DW) set consisted of a 25×7 matrix in which rows represent the different extract concentrations and columns the seven berry species. Basic chemometric characterization of the investigated berry extract samples according to their ability to reduce the DPPH was carried out by summary, descriptive (normal probability, box/whisker, and dot plots) statistics and multisample median testing using the statistical program Unistat® (London, UK).

Extraction of Phenolic Compounds for MS

The lyophilized samples of berries (1 g) were extracted with 100 mL of methanol/water (1:1) at room temperature and in darkness for 24 h. The extracts were filtered in a Buchner funnel. After removal of the methanol in a rotary evaporator at a temperature below 40 °C, the aqueous solution was then freeze-dried. These extracts were used for MS.

MS Analysis A mass spectrometer, a TSQ Quantum Access Max (Thermo Fisher Scientific, Basel, Switzerland) was used. Analytes were ionized by electrospray ionoization in positive mode. Vaporizer temperature was kept at 100 °C. Settings for the ion source were as follows: spray voltage 3,000 V, sheath gas pressure 35 AU; ion sweep gas pressure 0 AU; auxiliary gas pressure at 30 AU; capillary temperature at 200 °C, and skimmer offset 0 V (Gómez-Romero et al. 2011).

MTT Assay

Anticancer activity of water extracts of the studied berries on human cancer cell lines (Calu-6 for human pulmonary carcinoma and SNU-601 for human gastric carcinoma) were measured using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The cell lines were purchased from Korean Cell Line Bank (KCLB) for MTT assay. Cells were grown in RPMI-1640 medium at 37 °C under 5 % CO_2 in a humidified incubator. Cells were harvested, counted (3×10^4 cells/mL), and transferred into a 96-well plate, and incubated for 24 h prior to the addition of test compounds. Serial dilutions of test samples were prepared by dissolving compounds in water followed by dilution with RPMI-1640 medium to give final concentration at 25, 50, 100, 200, 400, and 800 and 1,000 μg mL^{-1}. Stock solutions of samples were prepared for cell lines at 90 μL and samples

at 10 μL, and incubated for 72 h. MTT solution at 5 mg mL^{-1} was dissolved in 1 mL of phosphate buffer solution, and 10 μL of it was added to each of the 96 wells. The wells were wrapped with aluminum foil and incubated at 37 °C for 4 h. The solution in each well containing media, unbound MTT, and dead cells were removed by suction and 150 μL of DMSO was added to each well. The plates were then shaken and optical density was recorded using a micro plate reader at 540 nm. Distilled water was used as positive control and DMSO as solvent control. Controls and samples were assayed in duplicate for each concentration and replicated three times for each cell line. The cytotoxicity was obtained by comparing the absorbance between the samples and the control (Heo et al. 2007).

Statistical Analyses

To verify the statistical significance, mean ± SD of five independent measurements were calculated. Differences between groups were tested by two ways ANOVA. In the assessment of the antioxidant activity, Spearman correlation coefficients (R) were used. Linear regressions were also calculated. P values of <0.05 were considered significant.

Results

Bioactive Compounds

The results of the determination of the contents of the bioactive compounds in all studied samples are summarized in Fig. 2a. As can be seen, the significant highest content ($P<0.05$) of polyphenols, flavonoids, anthocyanins, and ascorbic acid was in "Murtilla" non-ripe sample (84.81±3.9 mg GAE/g, 11.47±0.6 mg CE/g, 16.7±0.9 mg CGE/g, and 9.12±0.4 mg/g, respectively, Fig. 2a and b). Only the content of anthocyanins (Fig. 2b) was significantly higher ($P<0.05$) in blueberries from Poland (323.2±16.1 mg CGE/g). The following order of the value of polyphenols was obtained (Fig. 2a): "Murtilla" non-ripe (MNR) > *Aronia* (ARON) > Polish blueberry (POLBB) > Chilean blueberry (CHBB) > "Murtilla-like" non-ripe (M-LNR) > raspberry (RASB) > "Murtilla-like" ripe (M-LR).

Antioxidant Activity

The results of the determination of the level of antioxidant activity of all studied samples are shown in the Fig. 2c. As can be seen, the AA of Murtilla non-ripe as determined by ABTS and CUPRAC assays was 620.74±30 and 600.52± 27 μmol TE/g, respectively) was significantly higher than in other studied berries ($P<0.05$). The antioxidant activity of blueberries was higher than that of raspberries, and comparable with AA of "Murtilla" non-ripe (Fig. 2c). As was calculated, a very good correlation was found between the antioxidant activity and the contents of total polyphenols and other bioactive compounds (R^2 from 0.96 to 0.83) in water extracts. Flavonoids showed lower correlation. The correlation between the antioxidant activity and ascorbic acid (Fig. 1c and b) was lower than with polyphenols (R^2 from 0.84 to 0.50).

Anticancer Activity

It was observed that the percentage of proliferativity of the water extracts of berries samples on two cell lines (Fig. 2d, Calu-6 for human pulmonary carcinoma and Fig. 2e, SNU-601 for human gastric carcinoma) were different. The proliferativity (%) for concentrations of 1,500 μg/mL for water extracts of "Murtilla" on Calu-6 and SNU-601 were 41.76 and 42.12 %, respectively, and for "Murtilla-like" were 73.43 % and 71.23 % on Calu-6 and SNU-601, showing the higher antiproliferative activity of "Murtilla" in comparison with all other samples. Our investigation showed that antioxidant activity of the studied samples was correlated with their antiproliferative activity directly: the highest antioxidant activity was matching the highest antiproliferative activity.

Fluorometric Data

Fluorometric data showed the characterization of bioactive compounds in different berries with their specific fluorescence intensity and the location of the main peak and its shift. In addition the quenching ability of bioactive compounds in extracts was compared with pure catechin by the interaction with BSA in the presence of urea. The 3-D FL was used to determine the peak situation and the picture of the full peak. The 2-D FL was used for the determination of the fluorescence properties and for the change in the fluorescence intensity.

In three-dimensional fluorescence spectra and contour maps of berries one main peak can easily be observed in water extracts at the location of 1 em/ex 340/275 nm in "Murtilla-like" non-ripe with fluorescence intensity (FI) of 680 and the average second peak at em/ex 430/310 nm with FI0480; and one very small peak at em/ex 620/280 nm with FI 80 (Fig. 3a). "Murtilla-like" ripe (Fig. 3b) showed nearly the same two peaks at em/ex 330/280 nm with intensity of 507; and the second peak at em/ex 420/310 nm with FI0 400; one very small peak at em/ex 620/280 nm with FI 60. The difference was only in a small shift in the case of the ripe sample and higher fluorescence intensity of "Murtilla" non-ripe sample. "Murtilla" non-ripe (Fig. 3c) showed only one main peak at 1 em/ex 420/320 nm with FI0800; raspberry (Fig. 3d) showed the following peaks: an average one at 1 em/ex 290/280 nm with FI0200, and a small one at

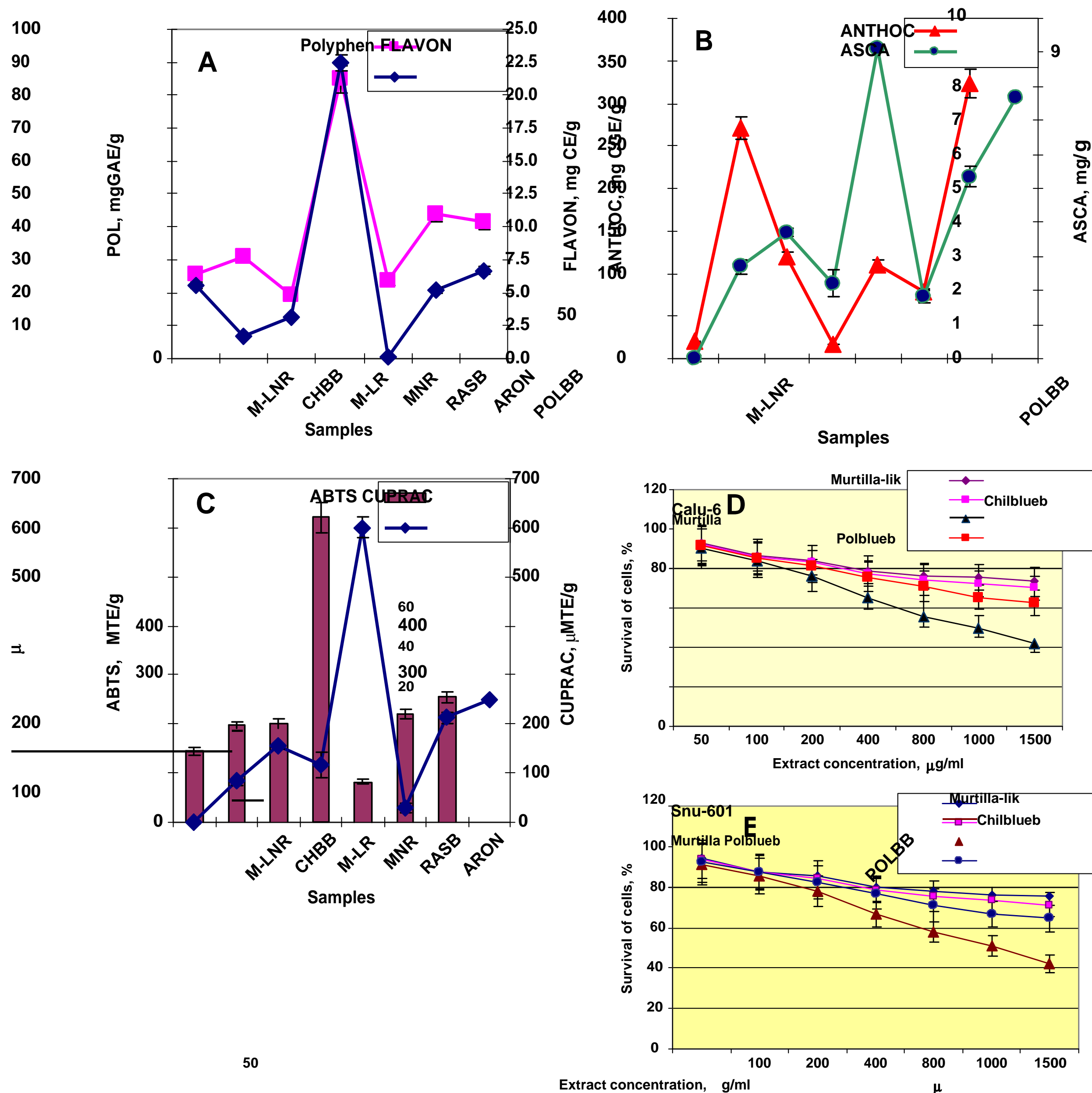


Fig. 2 a Total polyphenols (*Polyphen*, mg GAE/g) and flavonoids (*FLAVON*, mg CE/g); b anthocyanins (*ANTHOC*, mg CGE/g) and ascorbic acid (*ASCA*, mg/g); c antioxidant activities (μMTE/g) by *ABTS* and *CUPRAC* in the following berries: "Murtilla-like" non-ripe (*M-LNR*), Chilean blueberry (*CHBB*), "Murtilla-like" ripe (*M-LR*), "Murtilla" non-ripe (*MNR*), raspberry (*RASB*), *Aronia* (*ARON*), Polish blueberry (*POLBB*). *Abbreviations*: *GAE* gallic acid equivalent, *CE* catechin equivalent, *CGE* cyanidin-3-glucoside equivalent, *ABTS* 2, 2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt, *CUPRAC* cupric-reducing antioxidant capacity, *TE* trolox equivalent. The survival of cells (%) of human cancer cells of the d Calu-6 and e SNU-601 in the presence of water extracts of "Murtilla-like" (*Murtilla-lik*); Chilean blueberries (*Chilblueb*); "Murtilla", Polish blueberries (*Polblueb*). Each point represents the mean±SD (*n*06)

em/ex 620/280 nm with FI 78. Chilean blueberries (Fig. 3e) showed one peak at l em/ex 420/325 nm with FI0468, and a small one at l em/ex 640/270 nm with FI027. *Aronia* (Fig. 3f) showed one big peak at λ em/ex 420/310 nm with FI0580, and Polish blueberries (Fig. 3g) - two peaks: one small at l em/ex 380/275 nm with FI011, and another bigger one at l em/ex 400/330 nm. There are not too many applications of 3D fluorescence spectra, therefore our present conclusions that 3-D fluorescence can be used as an additional tool for the characterization of the polyphenol extracts during different stages of ripening and different berries cultivars correspond with the previous data (Gorinstein et al. 2010). The interaction between BSA, urea, catechin, and berry extract is shown in Fig. 4 by the changing of fluorescence intensity and shift of the main peak. Two different concentrations of urea were used: 2.4 M at 37 °C during 1 h

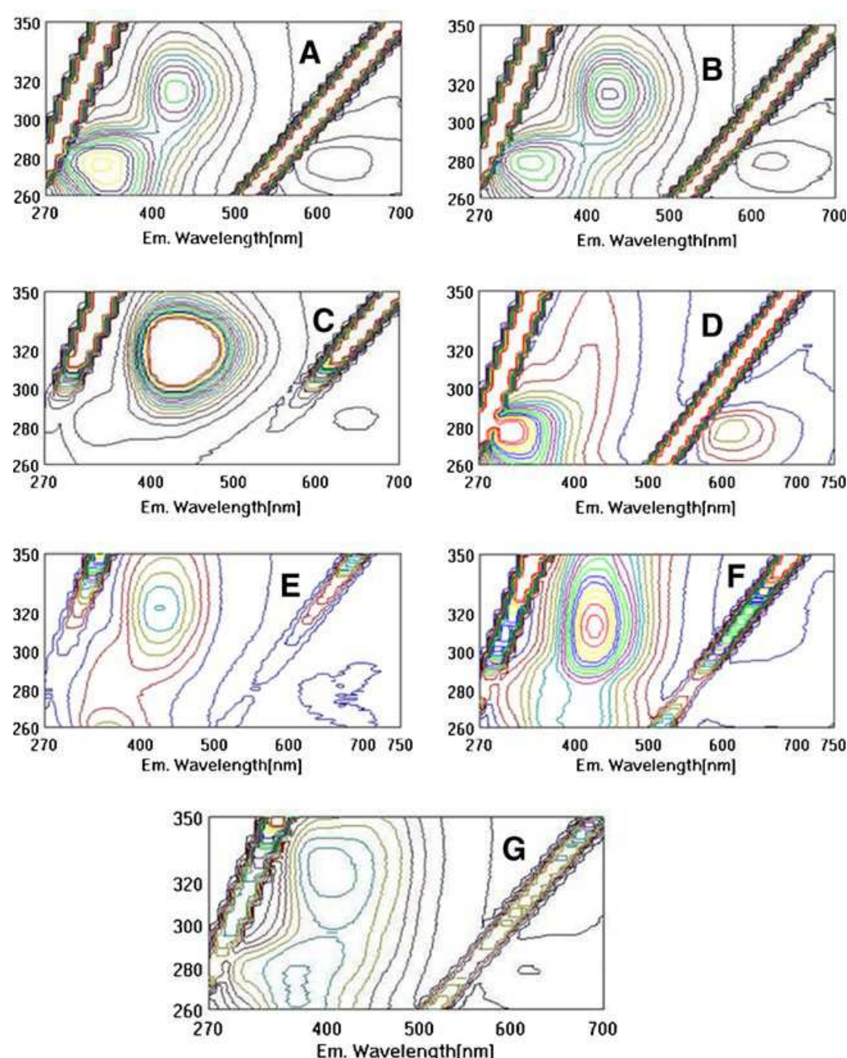


Fig. 3 Contour maps in three-dimensional fluorescence of water extracts (2.5 mg/mL) of "Murtilla-like" non-ripe, "Murtilla-like" ripe, "Murtilla" non-ripe, raspberry, Chilean blueberries, *Aronia*, Polish blueberries (a-g). The 3D-FL were run emission mode and fluorescence intensity up to 1,000, emission wavelengths from 270 to 750 nm and excitation wavelengths from 260 to 350 nm; scanning speed was 1,000 nm/min, emission wavelength on *x*-axis and excitation wavelength on *y*-axis

and the FI of BSA decreased from 878 to 605 (Fig. 4a and c). Oppositely at 4.8 M urea at the same conditions of time and temperature the FI of BSA decreased till 97, nearly full denaturation (Fig. 4e). Partly the same binding was obtained with 2.4 M urea and addition of catechin (Fig. 4b) and water extract of "Murtilla" non-ripe (Fig. 4c). The binding of catechin was higher (Fig. 4d, FI0646) than under the same conditions of the extract of "Murtilla" non-ripe (Fig. 4f, FI0 731.2). The main peak has changed in the region of lex/em of 225-230/335 nm.

The decrease of the intensity of the main peak of BSA with berry extract was about 16.7 % in comparison with catechin of 26.4 %. Other berry samples showed the decrease from 15 to 8 %. The decrease in the fluorescence intensity is the indicator of the quenching of berries extracts in interaction with BSA.

Fourier Transform Infrared Spectra Studies

The FTIR spectra of BSA and catechin (Fig. 5a, first line from the top) were compared with BSA and "Murtilla" non-ripe (Fig. 5a, second line from the top) and BSA (Fig. 5a, third line from the top). The amide I and amide II peaks of BSA (Fig. 5a, third line from the top) were shifted from 1,548 to 1,544 cm^{-1} and from 1,650 to 1,627 cm^{-1} upon interaction with catechin (Fig. 5a, first line from the top) and to 1,552 and 1,630 cm^{-1} upon interaction with "Murtilla" non-ripe extract (Fig. 6a, second line from the top). The FTIR wave numbers of catechin (Fig. 5a, third line from the top) shows broad phenolic OH band centered around 3,183 cm^{-1}, characteristic -CO stretching at 1,650 cm^{-1} aromatic bending and stretching around 1,040 and 1,650 cm^{-1}, -OH phenolic bending around 1,205 and 1,393 cm^{-1}. The FTIR spectra of BSA (Fig. 5b, third line from the top) were compared with BSA-urea (Fig. 5b, third line from the top). The amide I and amide II peaks of BSA (Fig. 5b, third line from the top) disappeared under denaturation with urea and with urea and addition of "Murtilla" non-ripe extract (Fig. 5b, second line from the top). The phenolic OH corresponding to catechin appeared around 3,400 cm^{-1} for the catechin-BSA complex was at

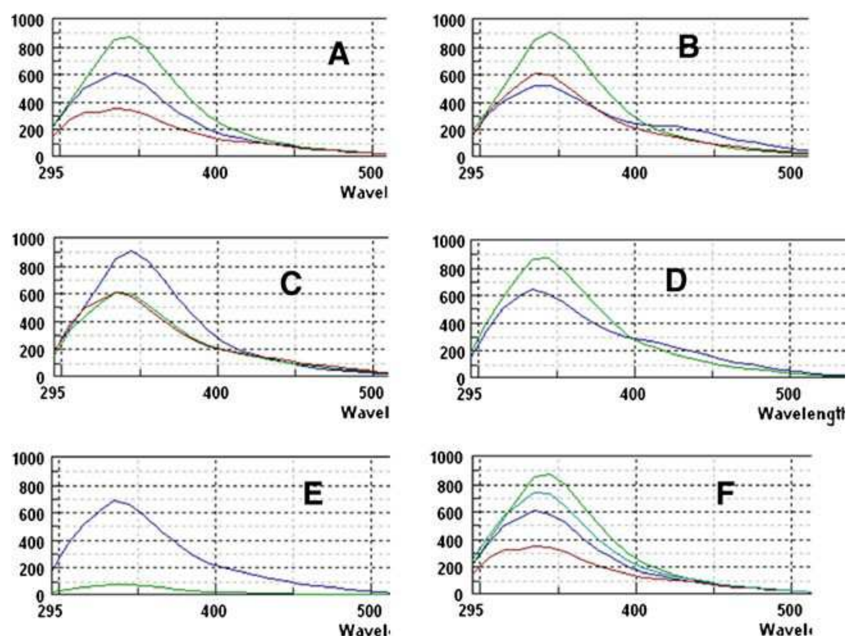


Fig. 4 Two-dimensional fluorescence spectra illustrate the interaction between BSA, catechin, urea, and water extracts of studied berries. a change in the fluorescence intensity as a result of binding affinity: 0.0132 μM BSA [*upper line* with fluorescence intensity of 877.8]; 0.0132 μM BSA+2.4 M urea during 1 h at 25 °C (*middle line* with FI 0 605); 0.0132 μM BSA+2.4 M urea+30 μM catechin during 1 h at 37 °C (*lower line* with FI0341); b 0.0132 μM BSA (*upper line* with FI of 877.8]; 0.0132 μM BSA+2.4 M urea during 1 h at 37 °C (*middle line* with FI 0600); 0.0132 μM BSA+2.4 M urea+ 50 μg/ml of water extract of "Murtilla" non-ripe during 1 h at 37 °C (*lower line* with FI0525); c 0.0132 μM BSA (*upper line* with FI of 900), 0.0132 μM BSA+2.4 M urea during 1 h at 25 °C (*middle line* with FI0 605.3); 0.0132 μM BSA+2.4 M urea+50 μg/ml of water extract of "Murtilla" non-ripe (*lower line* with FI0604.2) during 1 h at 25 °C; d 0.0132 μM BSA (*upper line* with FI of 878), 0.0132 μM BSA+30 μM catechin during 1 h at 25 °C (*lower line* with FI0646); e 0.0132 μM BSA+ 4.8 M urea at 0 time (*upper line* with FI of 686.4), 0.0132 μM BSA+ 4.8 M urea during 1 h at 25 °C (*lower line* with FI of 97); f 0.0132 μM BSA (*first line from the top* with FI0878), 0.0132 μM BSA+50 μg/ml of water extract of "Murtilla" non-ripe at 0 h time (*second line from the top* with FI0731.2), 0.0132 μM BSA+2.4 M urea+30 μM catechin during 1 h at 25 °C (*third line from the top* with FI0600); .0132 μM BSA+2.4 M urea+30 μM catechin during 1 h at 37 °C (*fourth line from the top* with FI0341). Fluorescence intensities are on *y*-axis and emission wavelengths on *x*-axis (*Wavel*, *Wavelength)*

3,188 cm^{-1}. Matching between the peaks in the range from 4,000 to 400 cm^{-1} between (BSA + urea + "Murtilla" non-ripe)/(BSA + urea)099.8 %; (BSA + catechin)/(BSA + "Murtilla" non-ripe)098.05 %; (BSA + catechin)/BSA0 47.38 %; and (BSA + "Murtilla" non-ripe)/BSA 048 % (Fig. 5a and b).

Chemometrical Processing

Chemometrical processing is an additional method to show the similarities and the differences in the investigated berries based on their bioactive compounds. The comparison of the DPPH antiradical activity (μmol TE/g DW) of investigated berries is shown in the Fig. 6a, where the highest values were in *Aronia* and "Murtilla" non-ripe. In order to exactly compare the quenching ability of the examined berries, the half maximal inhibitory concentration (IC_{50}), which is the concentration of the extract that inhibited DPPH free radical by 50 %, was calculated for a widely used scavenging reaction time of 30 min shown in Fig. 6b. The lower the IC_{50} value, the higher the radical-scavenging activity of the berries. By comparing the IC_{50} value of the berries water extracts, we found that the highest radical scavenging effect was observed in "Murtilla" non-ripe and *Aronia* berries with IC_{50} of about 6 mg ml^{-1}. The potency of radical scavenging effect of these two extracts was about ten times greater than in raspberry extract with the lowest antiradical activity. The scavenging activity of the extracts in decreasing order was: *Aronia* > "Murtilla" non-ripe > "Murtilla-like" ripe > blueberry (Chile) > "Murtilla-like" non-ripe ≥ blueberry (Poland) and raspberry (Fig. 6b). After the PCA, the dimensionality of data was reduced from 15 measured, calculated, and partially correlated original variables to the new set of uncorrelated variables—principal components, from which first two components accounted for 91.3 % of the total variability. These new variables highly correlate with the original antiradical descriptors of absorbance reading and inhibition at 60 min in the first principal component (PC1) and DPPH scavenging activity (μmol TE/g DW) at 60 and 90 min in the second PC. Plot of these PCs (Fig. 6c) shows not very strong clustering tendency among all berry water extracts according to scavenging ability data, but some similarities between fruit groups are evident. Clusters of water extracts of *Aronia* and "Murtilla" non-ripe fruits, both

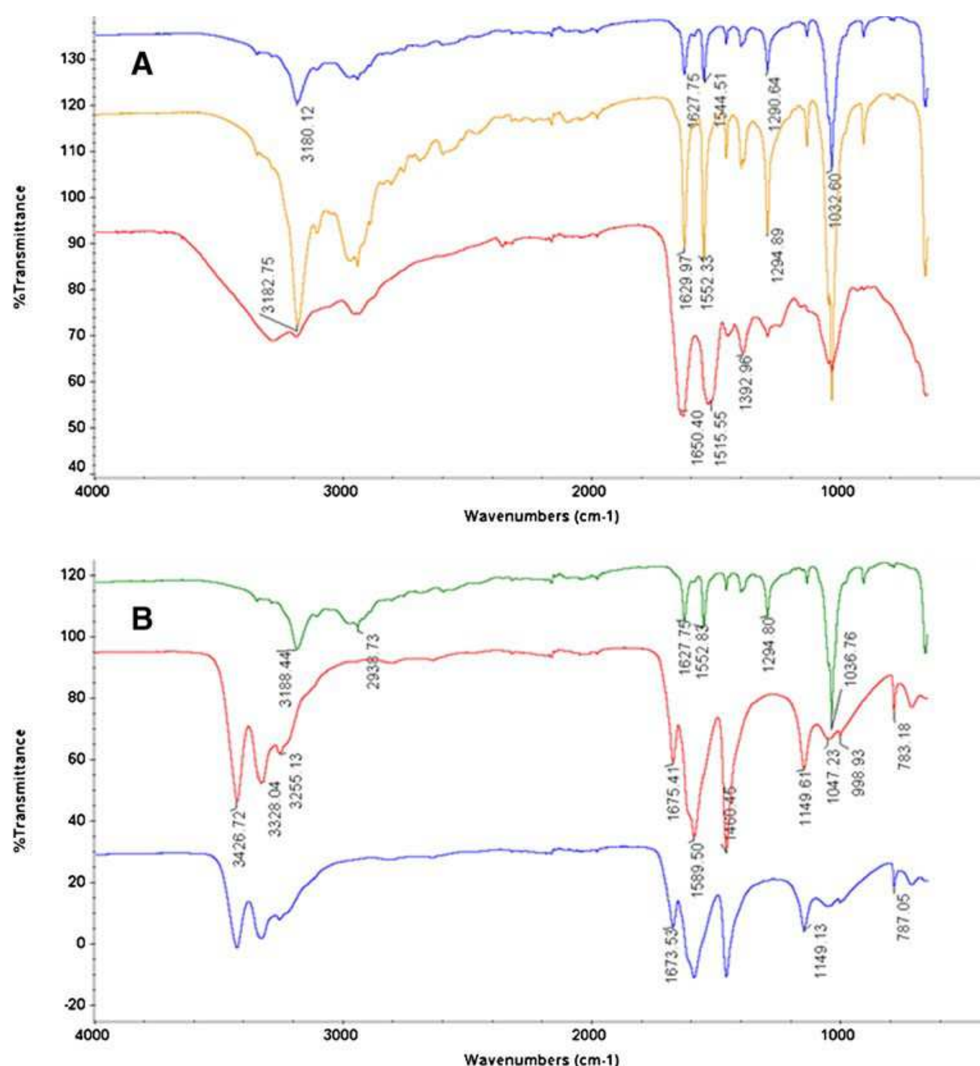


Fig. 5 Infrared study of FTIR spectra of a 0.0132 μM BSA+ 30 μM catechin during 1 h at 25 °C (*upper line from the top*), 0.0132 μM BSA+50 μg/ml of water extract of "Murtilla" non-ripe at 0 h time (*second line from the top*), 0.0132 μM BSA (*third line from the top*); b 0.0132 μM BSA+30 μM catechin during 1 h at 25 °C (*upper line from the top*), 0.0132 μM BSA+2.4 M urea+50 μg/ml of water extract of "Murtilla" non-ripe during 1 h at 25 °C (*second line from the top*), 0.0132 μM BSA+2.4 M urea during 1 h at 25 °C (*third line from the top*)

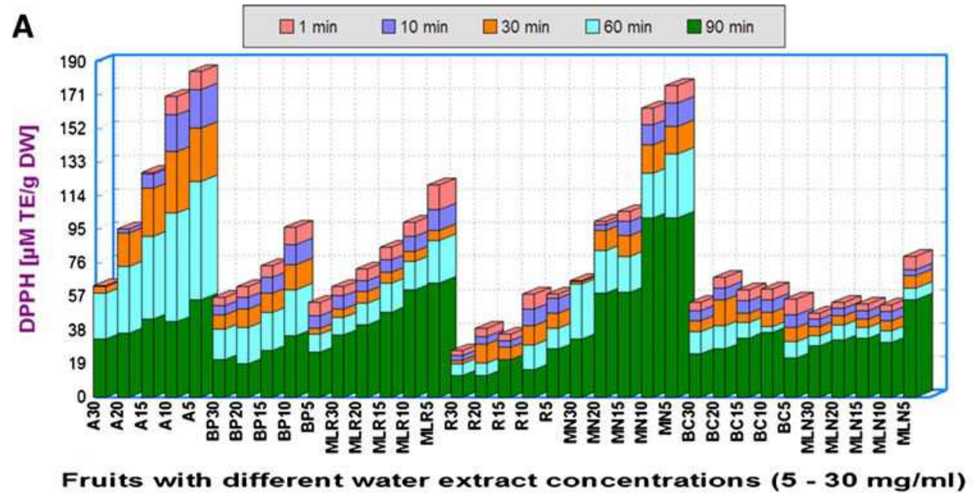
with the relatively very high antiradical activity are well separated from "Murtilla-like" ripe and rest fruits as well as from raspberries with the lowest antioxidant activity. A multiparametric approach of canonical discrimination analysis (CDA) was carried out in order to evaluate the influence of all DPPH antiradical parameters in the classification and differentiation of examined water fruit extracts according to their scavenging ability. Main seven fruit species were totally and correctly separated into relevant clusters. CDA based on the selected antiradical variables indicated that the first two significant canonical discriminant functions with eigenvalues > 1, Wilk's lambda ~ 0, and Chi-square test significance $P<0.0001$ explained 98.9 % of cumulative variance (first function 93.8 %). Taking into account the coefficients of canonical discriminant functions (data not presented here), the most significant contribution to discrimination in the first function was obtained from absorbance readings and inhibition value in reaction time 30 min and in the second function absorbance reading and inhibition in time 60 min. The stepwise discrimination found the DPPH antiradical activity after 1 min of reaction time as the most discriminant variables. Furthermore, the classification matrix gave evidence that the studied water extracts were correct, with 100 % success rate, classified to their fruit classes according to their DPPH scavenging ability.

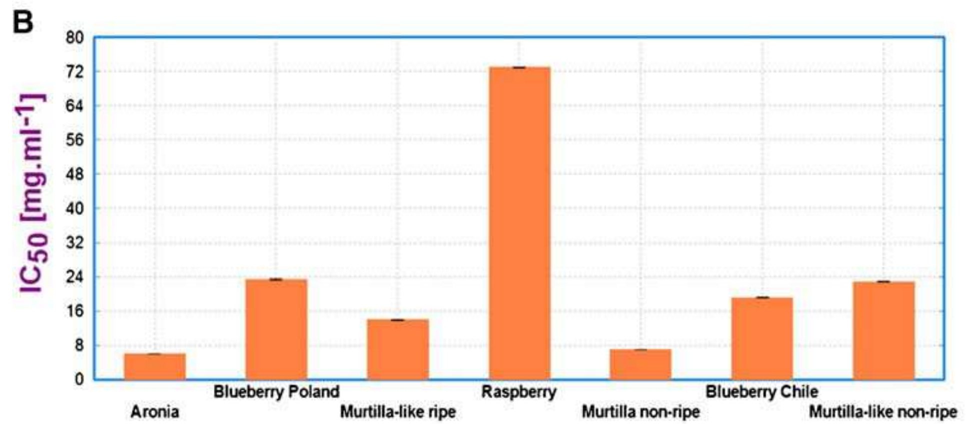
Mass Spectra Data

The spectrum shows the main *m/z* peaks found in berries (Fig. 7a-d) in water fraction with relative abundance (RA, %) from 20 to 100 %. The main peak was about 192-193, which mostly belongs to ferulic acid (Gómez-Romero et al. 2011). The RA of the obtained peaks showed the difference in the amount of polyphenol compounds in these samples.

Discussion

It was of great interest to compare "Murtilla-like" with "Murtilla" in order to find out if the "Murtilla-like"





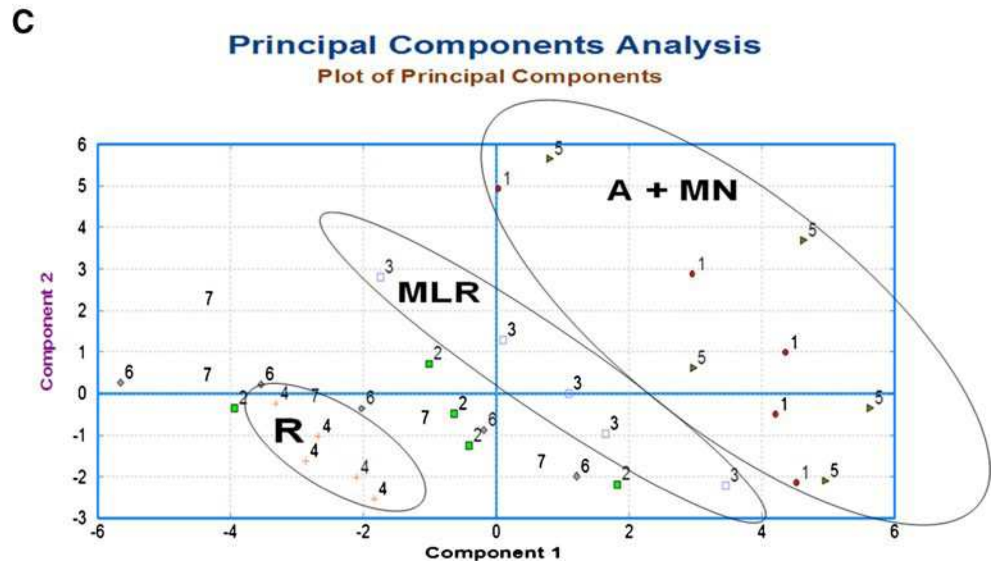


Fig. 6 a Overlap bar chart comparing the water extracts by DPPH antiradical activity (μM TE/g DW) of investigated berries (*A Aronia*, *BP* blueberry Poland, *MLR* "Murtilla-like" ripe, *R* raspberry, *MN* "Murtilla" non-ripe, *BC* blue- berry Chile, *MLN* "Murtilla- like" non-ripe) according to re- action time contribution at 1, 10, 30, 60, and 90 min. b IC_{50} bar chart of DPPH-radical scavenging activity in the water extract of berries. The lower the IC_{50} values the higher antiradi- cal activity. Data were per- formed in triplicates (*n*03) for a reaction time of 30 min, and in the range of extract concentra- tion was from 5 to 30 mg ml^{-1}. c Differentiation of the berry water extracts by the principal component analysis. Score plot on the first two components of the DPPH scavenging parame- ters (*1 Aronia* (*A*), *2* blueberry Poland, *3* "Murtilla-like" ripe (*MLR*), *4* raspberry (*R*), *5* "Murtilla" non-ripe (*MN*), *6* blueberry Chile, *7* "Murtilla- like" non-ripe); extract concen- trations: 30, 20, 15, 10, and 5 mg ml^{-1}; reaction times: 1, 10, 30, 60, and 90 min)

bioactivity is on the same level as of original "Murtilla". Therefore, the contents of the bioactive compounds and AA were determined and compared with the widely consumed blueberries, red raspberries, and chokeberries. A number of reviewed articles showed that the main bioactive compounds determining the nutritional quality of berries are polyphenols, anthocyanins, and flavonoids (Battino et al. 2009; Bowen-Forbes et al. 2010; Cuevas-Rodriguez et al. 2010; Dai et al. 2009). As was declared in "Results", the contents of bioactive compounds (polyphenols, flavonoids, anthocyanins, and ascorbic acid) in water extracts was determined and compared, and the significantly highest were

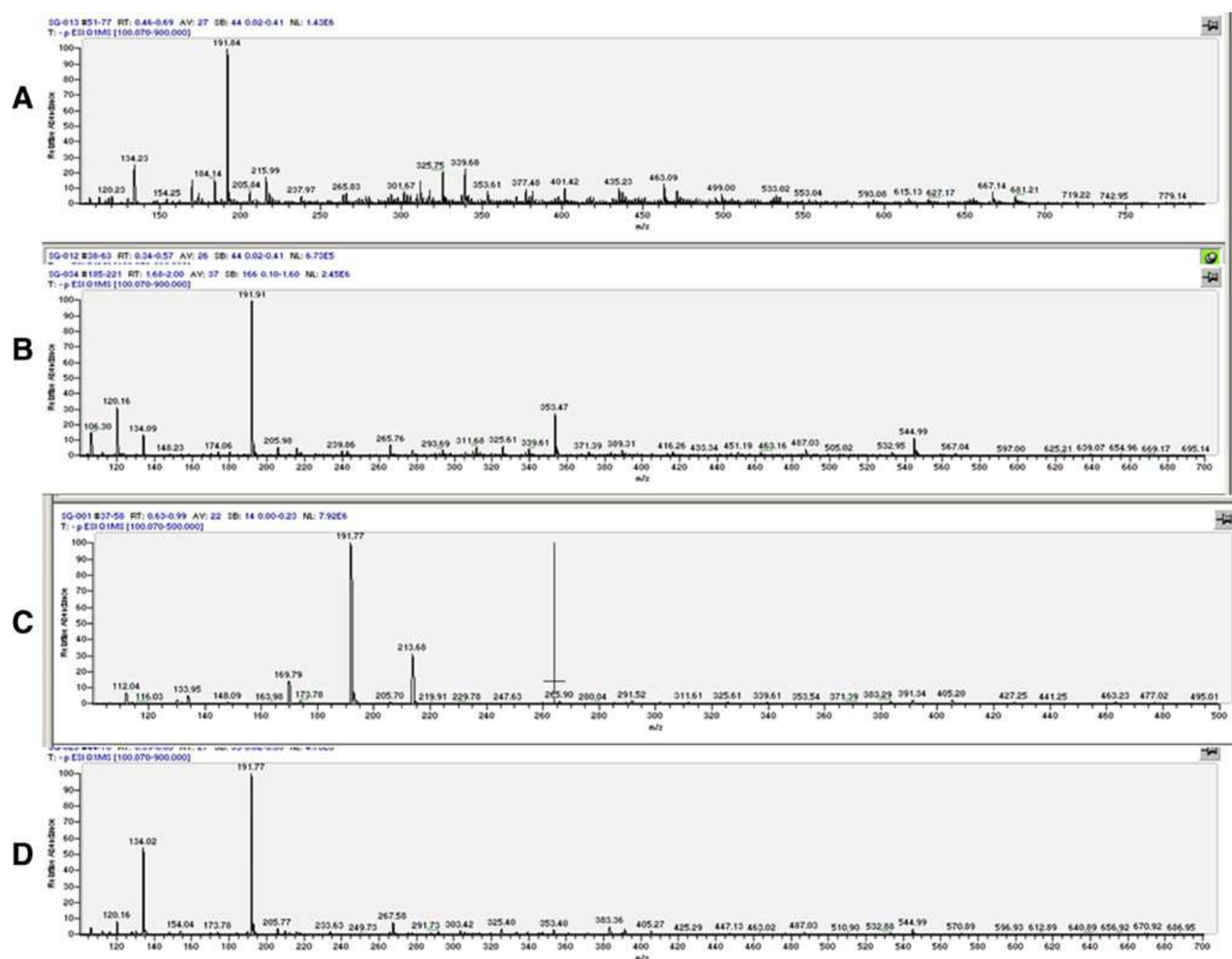


Fig. 7 ESI-MS spectra of water fractions of the following berries: a, b, c, d "Murtilla-like" non-ripe, Chilean blueberry, "Murtilla" non-ripe, Polish blueberry, respectively, in negative ion mode

in water extract of non-ripe "Murtilla". Also the antioxidant activity according to ABTS and CUPRAC was significantly higher in water extract of non-ripe "Murtilla". Our results were in agreement with others, showing that water extracts of blackberries contain high amounts of anthocyanins (Dai et al. 2009). The results show promising perspectives for the exploitation of non-traditional tropical fruit species with considerable levels of nutrients and antioxidant capacity. Our data add valuable information to current knowledge of the nutritional properties of tropical fruits, such as the considerable antioxidant capacity found for acerola—*Malpighia emarginata* and camu-camu—*Myrciaria dubia* (ABTS, DPPH, and FRAP) and for puçá-preto—*Mouriri pusa* (all methods). "Murtilla" in comparison with other 18 non-traditional tropical fruits from Brazil has an average value of antioxidants (Rufino et al. 2010). For dry matter the order observed was: bacuri > carnauba > yellow mombin > java plum > umbu > cashew apple > mangaba > assai > murta > gurguri > puçá-coroa-de-frade > uvaia > nance > jaboticaba > jussara > puçá-preto > acerola > camu-camu. When evaluated by the ABTS method, our fruits ranged from 6.3 to 153 μmol TE/g FW and from 16.4 to 1,237 μmol TE/g DW. FRAP values were 11.8-279 and 16.1-2,502 μmol $FeSO_4$/g, respectively. Our data are in agreement with these results. The order of increasing antioxidant capacity, measured by the ABTS method, was: umbu < yellow mombin < carnauba < cashew apple < mangaba < assaí < uvaia < java plum < gurguri < jaboticaba < puçá-coroa-de-frade < murta (Peña-Neira et al. 2007). Vitamin C of "Murta" was 181 mg/100 g FW (6.98 mg/g DW) which is approximately equal to our results (Fig. 2b). The anthocyanins were about 143 mg/100 g FW (5.52 mg/g DW) and this number is lower than our results (Fig. 2b). The polyphenols in "Murtilla" were 20.55 mg GAE/g DW and this number is lower than our results (Fig. 2a). The antioxidant activity (μmol TE/g DW) by ABTS was about 166. A positive and significant correlation was found in this study between vitamin C-extractable polyphenols and ABTS ($R^2$0

0.70). Polyphenols and DPPH results expressed as antioxidant concentrations corresponding to 50 % scavenging activity were negatively and significantly correlated (R^2 00.72; P<0.05); this is due to the fact that the DPPH method yields inversely proportional results. There was also a positive and significant correlation of polyphenols (P<0.05) and ABTS (R^2 00.92) assay (Rufino et al. 2010). These data are in agreement with our results. Our results correspond also with the research approach of Wu et al. (2006), where concentrations of total anthocyanins varied considerably from 0.7 to 1,480 mg/100 g FW in gooseberry ('Careless' variety) and chokeberry, respectively. Total phenolic content and total anthocyanin content of four berry fruits (strawberry, Saskatoon berry, raspberry, and wild blueberry), chokecherry, and seabuckthorn ranged from 22.83 to 131.88 g/kg and 3.51 to 13.13 g/kg, respectively, which corresponds with our results. Our data can be comparable with another report (Cuevas-Rodriguez et al. 2010), where the proanthocyanidins (condensed tannins) were present in the blackberry fruits. The average anthocyanin concentration was 49.2 mg/g in the commercial cultivar Tupy, while in the wild genotypes and the breeding line, the range was 361.3-494.9 mg/g (cyanidin 3-*O*-glucoside equivalent). The proanthocyanidin concentration varied widely among wild genotypes (417.5-1,343.6 mg/g CE). Comparison of different fractions of water extracts from of wild blackberry *A. chilensis* (Mol) Stuntz (Elaeocarpaceae), corresponded with our results. Wu et al. (2006) showed that in chokeberry the amount of anthocyanins was 1,480 mg/100 g FW (52.54 mg/g DW), for red raspberry- 92.1 mg/100 g FW (6.48 mg/g DW). Also other authors reported similar results. So, Ruiz et al. (2010) found the highest total polyphenol content in maqui, followed by calafate and "Murtilla". Reported high anthocyanin content in calafate berries berries (17.81±0.98 μmol g^{-1}) are comparable with those indices found in maqui (17.88±1.15 μmol g^{-1}). The AA of "Murtilla-like", blueberries and red raspberries was comparable. Also other reported different AA data in different cultivars harvested in different seasons (Ruiz et al. 2010). According to these authors the means of AA for calafate, maqui, and Murtilla were 74.4±15.9, 88.1±21.5, and 11.7± 2.3 μmol TE/g FW, respectively. Seeram (2010) discussed also that phytonutrients ranged from fat-soluble/lipophilic to water-soluble/hydrophilic compounds. Conclusions in the report of Elberry et al. (2010) are in line with our results about the high antioxidant activity of berries. Our results are in accordance with You et al. (2011), where four Rabbiteye blueberry cultivars (Powderblue, Climax, Tifblue, and Woodward) grown organically and conventionally were compared regarding their chemical profiles and antioxidant capacity in terms of total phenolic content, total anthocyanin content, and antioxidant values by ABTS, DPPH, FRAP, and CUPRAC. Total phenolics, flavonoids, and anthocyanins (mg/g FW) were in blueberry 261-585, 50, and 25-495 and in raspberry - 121, 6, and 99; antioxidant activity (μmol Trolox/g FW) for blueberry 14 by ABTS and 25.3 - by DPPH assays (Li et al. 2009). The result from this study indicated that blueberries had very high ORAC values, and higher antioxidant capacity than other selected fruits and vegetables (Wulf et al. 2005). The comparison of the results of different solvents in Dabai fruit parts (methanol, ethanol, ethyl acetate, acetone, and water) and total phenolics, total flavonoids, total anthocyanins, and antioxidant capacity (ABTS $^{•+}$ and FRAP assays) were in accordance with our data (Khoo et al. 2012). The acetone extract had maximum phenol and flavonoid content and showed best DPPH free radical scavenging activity and reducing capacity assessment. Ethyl acetate extract showed best superoxide radical scavenging activity, while aqueous extract showed best hydroxyl radical scavenging activity (Chanda and Kaneria 2012).

In conclusion, the bioactivity of Chilean "Murtilla" berries is significantly higher than the bioactivity of other studied samples; however, this index in the "Murtilla-like" berries is comparable with blueberries and raspberries. The antiproliferative properties of the investigated samples are in correlation with the antioxidant activity. 3-D fluorescence and FTIR spectroscopy was used as an additional tool for the characterization of the polyphenol extracts during different stages of ripening and in different berries cultivars. It is a necessity of discovering new plant breeding and genetic studies of berries with the expression of compounds for human health. The analytical methods used in this study can be applied for any of the food analysis.

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ORIGINAL PAPER

Antioxidant activities and bioactive components in some berries

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Abstract The objective of this study was to evaluate the antioxidant and binding effects of gooseberry, a less-studied berry, and to compare with blueberry and cranberry in the model of interaction with human serum albumin (HSA). The relationship between the scavenging properties of dietary polyphenols of the selected berries and their affinities for HSA were investigated by fluorescence analysis. In order to perform the extraction and identification of the antioxidants present in the samples, different types of extraction solvents were used, such as water, ethyl acetate, and diethyl ether. The polyphenols, tannins, anthocyanins and ascorbic acid contents, and the total antioxidant capacities (TACs) of the berry extracts were assessed by using ESI–MS, FTIR, and radical scavenging assays. The contents of bioactive compounds and the levels of TACs in water extracts differed significantly and were the highest in water extracts in comparison with other extracts in all the investigated berries ($P \setminus 0.05$). Gooseberry water extracts contained: polyphenols (mg GAE/g DW)—5.37 ± 0.6, tannins (mg CE/g DW)—0.71 ± 0.2, anthocyanins (mg CGE/g DW)—12.0 ± 1.2, ascorbic acid (mg AA/g DW)—5.15 ± 0.5, and TACs (lMTE/g DW) by ABTS and FRAP assays were 15.53 ± 1.6 and 6.51 ± 0.7, respectively. In conclusion, the bioactivity of gooseberry was lower than blueberries and cranberries. The antioxidant and binding properties of gooseberries in comparison with widely consumed blueberries and cranberries can be used as a new source for food supplementation.

Keywords Berries · Bioactive compounds · Antioxidant activity · Binding properties

S. Gorinstein: This article was written in memory of my dear brother Prof. Simon Trakhtenberg, who died in November 2011, who encouraged me and all our scientific group during all his life.

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Introduction

Berries contain powerful antioxidants, potential allergens, and other bioactive compounds. Genetic and environmental factors affect production and storage of such compounds, where the anthocyanins (water-soluble plant pigments) have important functions in plant physiology as well in health effects [1]. The high anthocyanin contents and biological activities of these fruits indicate that their consumption would be beneficial to health. It was revealed that the fruits contained superior levels of anthocyanins (146–2,199 mg/100 g fresh weight) to those previously reported for other raspberry and blackberry species, and their hexane, EtOAc, and MeOH extracts showed good antioxidant activity. The majority of the extracts exhibited

over 50 % lipid peroxidation inhibitory activity at 50 mg/mL. This may be useful in the production of functional foods containing an efficacious dose of anthocyanins [2]. The presence of predominantly phenolic compounds (ellagic and gallic acids, and corilagin) demonstrated varying degrees of antioxidative efficacy [3]. Cranberries with high content of polyphenols have been associated with several cardiovascular health benefits [4]. Borges et al. [5] identified the content of bioactive compounds in different berries. A complex spectrum of anthocyanins was the major contributor to the TAC of blueberries, whereas the lower TAC of cranberries was due mainly to reduced anthocyanin content. Vitamin C was responsible for 18–23 % of the TAC of cranberries and did not contribute to the TAC of the blueberry extract [5]. Puente et al. [6] studied the physicochemical and nutritional properties of the *Physalis peruviana* L. fruit and their relation of active components with beneficial effects on human health. The food industry has used cape gooseberry in different products including beverages, yogurts and jams, nutraceutical, and pharmaceutical industries [7, 8]. All the evaluated gooseberries extracts presented detectable amounts of phenolic, flavonoid, and tannin. Different extraction procedures reported in the literature and used to extract antioxidants in fruit were compared and analyzed [9, 10]. In our recent research, polyphenols, flavonoids, flavanols, and tannins and the level of antioxidant activity by ABTS, FRAP, and CUPRAC radical scavenging assays of methanol extract of studied berry samples were determined and compared. It was found that the contents of the polyphenol compounds and the level of antioxidant activity in extracts of berries differ significantly [11]. We were interested to investigate extracts of gooseberry (*Physalis peruviana)* and to compare its composition with the widely consumed berries. The water extracts of berries are important from the point of tea consumption all year around, outside of the season of growing. To meet this aim, the contents of bioactive compounds (polyphenols, tannins, anthocyanins, and ascorbic acid) and the levels of total antioxidant capacities (TAC) were determined and compared. Two radical scavenging assays ABTS and FRAP were carried out in order to determine the TAC [12, 13]. Human serum albumin is the drug carrier's protein and serves to greatly amplify the capacity of plasma for transporting drugs. In order to compare the fluorescence properties of the extracted bioactive compounds, in vitro studies were performed by interaction of proteins with polyphenols. It is interesting to investigate in vitro how this protein interacts with polyphenols extracted from berry samples in order to get useful information of the properties of polyphenol–protein complex. Different aspects of berries phenolics activity were studied in individual papers [7–10, 14], but complex study in this matter is missing. Therefore, the aim of this study was to determine the antioxidant and binding properties of the water extracts of gooseberry in comparison with blueberry and cranberry on the basis of interaction with HSA. As far as we know, no results of such investigations were published.

Materials and methods

Reagents

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), caffeic acid, 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Folin–Ciocalteu reagent (FCR), lanthanum (III) chloride heptahydrate; $CuCl_2$ 9 $2H_2O$; and 2,9-dimethyl-1,10-phenanthroline (neocuproine); $FeCl_3$ 9 $6H_2O$ were purchased from Sigma Chemical Co., St Louis, MO, USA. 2, 4, 6-Tripyridyl-*s*-triazine (TPTZ) was from Fluka Chemie, Buchs, Switzerland. All reagents were of analytical grade. Deionised and distilled water was used throughout.

Samples

Cape gooseberries (*Physalis peruviana*), blueberries (*Vaccinium corymbosum*), and cranberries (*Vaccinium macrocarpon*) were investigated. All berries were purchased at the local market in Gdansk and Warsaw, Poland. For the investigation, five replicates of five berries each were used. Their edible parts were prepared manually without using steel knives. The prepared berries were weighed, chopped, and homogenized under liquid nitrogen in a high-speed blender (Hamilton Beach Silex professional model) for 1 min. A weighed portion (50–100 g) was then lyophilized for 48 h (Virtis model 10–324), and the dry weight was determined. The samples were ground to pass through a 0.5 mm sieve and stored at -20° C until the bioactive substances were analyzed.

Determination of bioactive compounds and total antioxidant capacity (TAC)

The contents of polyphenols, tannins, anthocyanins, and ascorbic acid in extracts of the studied berries were determined as previously described [15]. The lyophilized samples of berries (1 g) were extracted with 100 mL of methanol/water (1:1) at room temperature and in darkness for 24 h. The extracts were filtered in a Buchner funnel. After removal of the methanol in a rotary evaporator at a temperature below 40 °C, the aqueous solution was extracted with diethyl ether and ethyl acetate, and then, the remainder of the aqueous solution was freeze-dried. The organic fractions were dried and redissolved in methanol.

These extracts were used for MS, for determination of bioactive compounds [16].

The polyphenols were determined by Folin–Ciocalteu method with measurement at 750 nm with spectrophotometer (Hewlett-Packard, model 8452A, Rockvile, USA). The results were expressed as mg of gallic acid equivalents (GAE) per g DW [17]. The extracts of condensed tannins (procyanidins) with 4 % methanol vanillin solution were measured at 500 nm. (?)-Catechin served as a standard for flavonoids, and the results were expressed as catechin equivalents (CE). Total ascorbic acid was determined by CUPRAC assay [18] in water extract (100 mg of lyophilized sample and 5 ml of water). The absorbance of the formed bis (Nc)-copper (I) chelate was measured at 450 nm. The total anthocyanins were measured by a pH differential method. Absorbance was measured in a Beckman spectrophotometer at 510 nm and at 700 nm in buffers at pH 1.0 and 4.5, using A = [(A_{510}–A_{700}) $_{pH1.0}$–(A_{510}–A_{700}) $_{pH4.5}$]. Results were expressed as mg of cyanidin-3-glucoside equivalent (CGE)/g of DW [19].

MS analysis: A mass spectrometer, a TSQ Quantum Access Max (Thermo Fisher Scientific, Basel, Switzerland), was used. Analytes were ionized by electrospray ionization (ESI) in positive mode. Vaporizer temperature was kept at 100 °C. Settings for the ion source were as follows: spray voltage 3,000 V, sheath gas pressure 35 AU; ion sweep gas pressure 0 AU; auxiliary gas pressure at 30 AU; capillary temperature at 200 °C; skimmer offset 0 V [20].

The TAC was determined by two assays:

- 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{·?}$) method for the screening of antioxidant activity is reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxi- dants. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant capacity. $ABTS^{·?}$ radical cation was generated by the interaction of ABTS (7 mM/L) and $K_2S_2O_8$ (2.45 mM/L). This solution was diluted with methanol until the absorbance in the samples reached 0.7 at 734 nm [13].
- Ferric-reducing/antioxidant power (FRAP) assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripiridyltriazine ($Fe^{3?}$-TPTZ) to a ferrous form (Fe^{2}). FRAP reagent (2.5 mL of a 10 mmol ferric-tripiridyltriazine solution in 40 mmol HCl plus 2.5 mL of 20 mmol $FeCl_3xH_2O$ and 25 mL of 0.3 mol/L acetate buffer, pH 3.6) of 900]L was mixed with 90]L of distilled water and 30]L of berry samples as the appropriate reagent blank. The absorbance was measured at 595 nm [12].

Fluorometry and Fourier transform infrared (FT-IR) spectra studies

Two-dimensional (2D–FL) fluorescence measurements for all berries extracts at a concentration of 0.01 mg/mL were recorded on a model FP-6500, Jasco spectrofluorometer, serial N261332, Japan, equipped with 1.0 cm quartz cells and a thermostat bath. The 2D–FL was taken at emission wavelengths from 310 to 500 nm, and at excitation of 295 nm [11]. Caffeic acid was used as a standard. All solutions for protein interaction were prepared in 0.05 mol/L Tris–HCl buffer (pH 7.4), containing 0.1 mol/L NaCl. The final concentration of HSA was $2.0x10^{-6}$ mol/L. The HSA was mixed with caffeic acid in the proportions of HSA: extract = 1:1 [14, 21, 22]. The presence of polyphenols in the investigated berries samples was studied by Fourier transform infrared (FT-IR) spectroscopy. A Nicolet iS 10 FT-IR Spectrometer (Thermo Scientific Instruments LLC, Madison, WI, USA), with the smart iTR™ ATR (Attenuated Total Reflectance) accessory, was used to record IR spectra [23].

Statistical analyses

To verify the statistical significance, mean ± SD of five independent measurements were calculated. Differences between groups were tested by two-ways ANOVA. In the assessment of the antioxidant capacity, Spearman correlation coefficients (R) were used. Linear regressions were also calculated. P-values of \0.05 were considered significant.

Results

Bioactive compounds

The amounts of bioactive compounds in all studied samples are summarized in Table 1 and Fig. 1. As can be seen, the significant highest content (P \ 0.05) of bioactive compounds was in blueberries water extract. Gooseberries showed average results in water extracts for polyphenols, tannins, anthocyanins, and ascorbic acid (Table 1, Fig. 1).

Total antioxidant capacity (TAC)

The results of the determination of the level of TAC of all studied samples are shown in Table 1. As can be seen, the TAC (lMTE/g) by ABTS and FRAP assays for

Table 1 Bioactive compounds and antioxidant capacities in water, ethyl acetate, and diethyl ether extracts of gooseberries (*Physalis peruviana*), cranberries (*Vaccinium macrocarpon*), and blueberries (*Vaccinium corymbosum*)

Extracts	Indices			
	POLYPHEN, mg GAE	TANNINS, mg CE	ABTS, lM TE	FRAP, lM TE
Goberry, H_2O	5.37 ± 0.6^{c}	0.71 ± 0.2^{c}	15.53 ± 1.6^{c}	6.51 ± 0.7^{c}
Crberry, H_2O	22.13 ± 2.5^{b}	5.12 ± 0.7^{b}	72.76 ± 6.5^{b}	26.97 ± 2.7^{b}
Blberry, H_2O	46.56 ± 4.2^{a}	13.04 ± 1.3^{a} 0.31	199.41 ± 18.6^{a} 1.47	94.10 ± 9.3^{a} 0.42
Goberry, EtOAc	0.29 ± 0.1^{e}	± 0.1^{d}	± 0.3^{e}	± 0.1^{d}
Crberry, EtOAc	3.14 ± 0.4^{c}	0.51 ± 0.1^{c}	13.50 ± 1.3^{c}	5.10 ± 0.6^{c}
Blberry, EtOAc	3.87 ± 0.4^{c}	0.62 ± 0.2^{c}	17.73 ± 1.8^{c}	7.53 ± 0.8^{c}
Goberry, DEE	0.14 ± 0.01^{e}	0.30 ± 0.1^{d}	0.88 ± 0.1^{e}	0.084 ± 0.01^{e}
Crberry, DEE	2.11 ± 0.2^{d}	0.32 ± 0.1^{d}	10.72 ± 1.8^{d}	3.28 ± 0.4^{d}
Blberry, DEE	4.13 ± 0.4^{c}	0.62 ± 0.3^{c}	20.42 ± 2.3^{c}	9.59 ± 0.9^{c}

Values are mean ± SD of 5 measurements

Per g dry weight

Values in columns for every bioactive compound bearing different superscript letters are significantly different (P \ 0.05)

POLYPHEN polyphenols, *CE* catechin equivalent, *GAE* gallic acid equivalent, *ABTS* 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt, *FRAP* Ferric-reducing/antioxidant power, *Goberry* gooseberries (*Physalis peruviana*), *Crberry* cranberries (*Vaccinium macrocarpon*), *Blberry* blueberries (*Vaccinium corymbosum*), *EtOAc* ethyl acetate, *DEE* diethyl ether

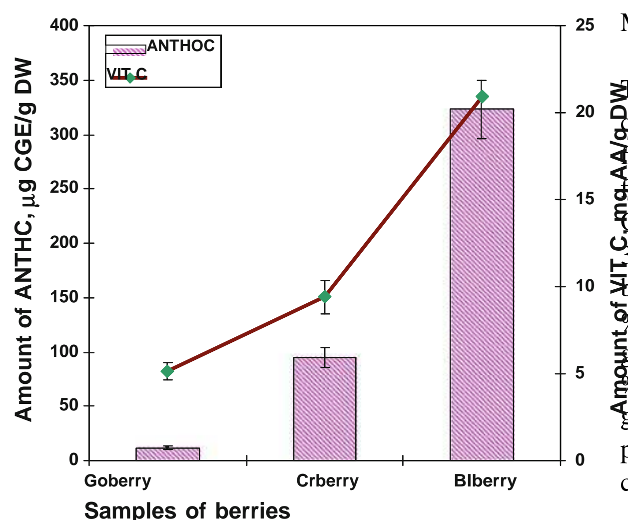

Fig. 1 Total anthocyanins (lg CGE/g DW) and vitamin C (mg AA/g DW) in gooseberries, blueberries and cranberries. *CGE* cyanidin-3-glucoside equivalent, *AA* ascorbic acid, *Goberry* gooseberries, *Crberry* cranberries, *Blberry* blueberries, *ANTHOC* anthocyanins, *VIT* C, vitamin C

gooseberries was 15.53 ± 1.6 and 6.51 ± 0.7, respectively. The TAC of blueberries was higher than that of gooseberries and cranberries. A very good correlation was found between the TAC and the contents of total polyphenols (R^2 from 0.96 to 0.83) in water extracts. The correlation between the antioxidant capacity and ascorbic acid (Fig. 1) was lower than with polyphenols (R^2 from 0.84 to 0.50, Table 1).

Mass spectra data

The spectrum shows the main *m/z* peaks found (Fig. 2a, b, c) in water extract of berries with relative abundance (RA %) from 5 to 100 %. The main peaks were about 393, 381, and 290 for cranberries, gooseberries, and blueberries, respectively [20]. Other peaks appeared for cranberry at 335 (87 %), for blueberries at 241 (70 %), for cranberry and blueberry were found at 104 with RA = 40 %, and RA = 66 %. Common peaks at 266 of 50 % and 32 % were in gooseberries and cranberries, respectively. The peaks of 116 (45 %), of 146 (25 %), and 219 (32 %) were estimated only in gooseberries. The RA of the obtained peaks corresponded with the amount of total polyphenols contents in these samples.

Fluorometry spectra studies and FTIR

Water extracts showed the highest antioxidant properties; therefore, only water extracts were subjected to binding studies with HSA. The scavenging properties of the berries samples in comparison with caffeic acid are shown in two-dimensional fluorescence spectra (2D–FL). One of the main peaks for HSA was found at kex/em of 220/360 nm. The second main peak appeared for these samples at kex/ em of 280/350 nm (Fig. 3b–d). The interaction of HSA and the water extracts of berries, HSA, water extracts and caffeic acid (Fig. 3b–d) showed slight change in the posi- tion of the main peak at the wavelength of 360 nm and the decrease in the relative fluorescence intensity (RFI). The

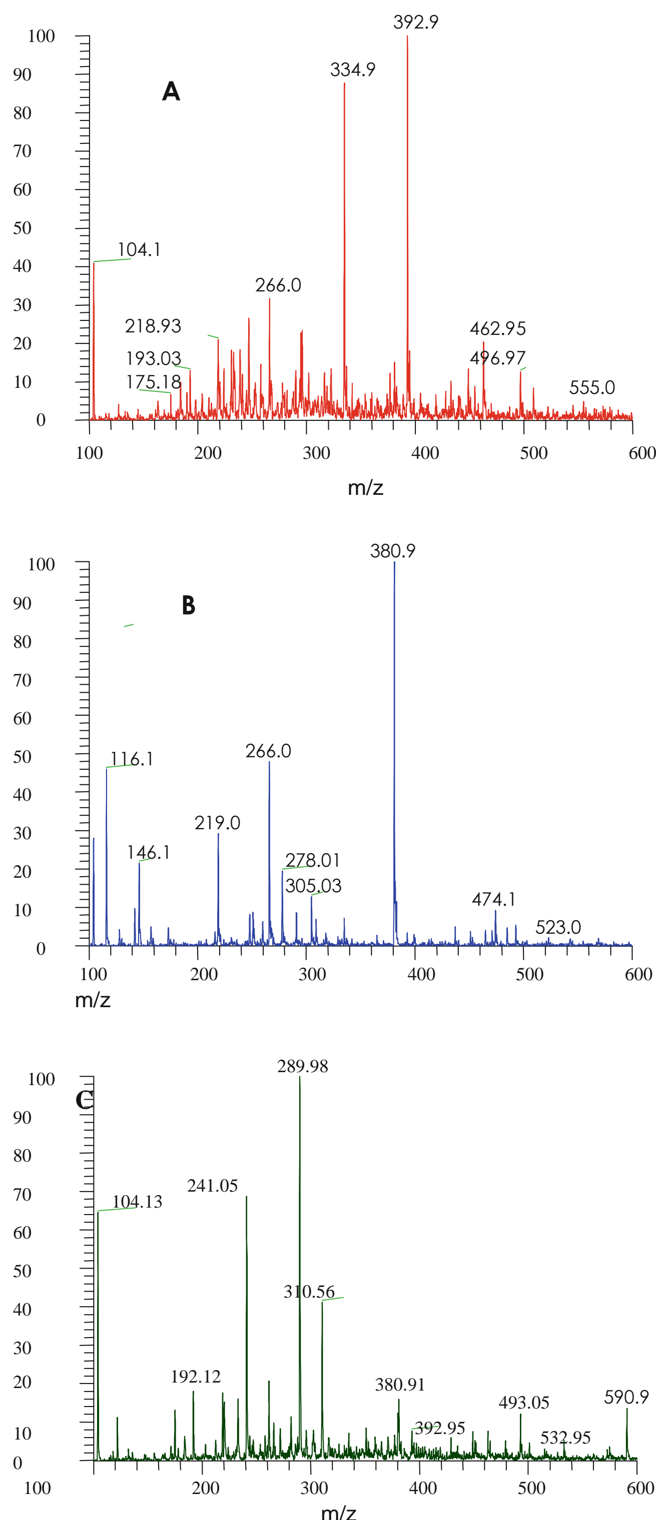

Fig. 2 ESI–MS spectra in positive ion mode of water fractions of the following berries: a cranberries; b gooseberries; c blueberries

following changes appeared when the water extracts of berries were added to HSA [initially the main peak at emission 360 nm and FI of 904.26 (Figs. 3a, b, and 4a, the upper line is HSA). The addition of blueberry extracts and caffeic acid decreased the RFI of HSA (Fig. 3b, lines from the top to the bottom). The decrease in the RFI (%) was proportional to the concentration of the polyphenols and showed 23.3, 58.3, and 67.5 during interaction of 20, 100, and 200 lg/mL blueberry water extract with HSA. Oppositely, in the case of addition of caffeic acid, the decrease was of 29.0, 71.1, and 73 %, respectively. Cranberry extracts showed the following results (Fig. 3c): HSA with 20 lg/mL decreased the RFI on 12.5 % and CaA -23.1 %; with 100 lg/mL 28.5 % and CaA -42.4 %; and with 200 lg/mL of extract decreased the RFI on 35.9 % and with CaA on 47.7 %. Gooseberry extracts showed decrease in fluorescence (Fig. 3d): with 20 lg/mL on 3.1 % and addition of CaA -16.2 %; with 100 lg/mL on 10.8 % and 22.0 %, respectively; and with 200 lg/mL on 16.5 % and with CaA -27.6 %. The decrease in the RFI of HSA with 200 lg/mL gooseberry, cranberry, and blueberry extracts was 16.5, 35.9, and 67.5 %, and when caffeic acid was added, the decrease was 27.6, 47.7, and 73 %, respectively (Fig 3b–d).

FTIR spectra of water extracts of gooseberries, blueberries, and cranberries (A), ethyl acetate extracts of blueberries, gooseberries, and cranberries (B), and diethyl ether extracts (C) of gooseberries, blueberries, and cranberries are presented in Fig. 4 (lines from the top to the bottom). The comparison between the berries, the extracts, and some standards in the range of common peaks is shown in Table 2 A, B, C. The best matching in the common range of the peaks was in water extracts of the berries samples in the range of 3,300–3,000 cm^{-1} (Table 2 A) of 75 % with hesperidin and 85 % with tannic acid. Caffeic acid showed the matching in the range of 2,500–2,000 cm^{-1} (Table 2 A) of 42 %. In ethyl acetate extract, similar matching in the range of 3,500–3,200 cm^{-1} of the peaks was found with tannic acid and quercetin (Table 2 B). In the range of 2,400–2,300 cm^{-1} (Table 2 B), gallic acid, fisetin, tannic, and caffeic acids showed about 70–78 % of common peaks.

Discussion

It was of great interest to compare gooseberries in order to find out if their bioactivity is on the same level as in other widely consumed berries and to use this kind of berries as a daily diet supplement. Therefore, the contents of the bioactive compounds and TAC were determined and compared with the widely consumed blueberries and cranberries. As it was declared in Results, the contents of bioactive compounds (polyphenols, tannins, anthocyanins, and ascorbic acid) in three extracts were determined and compared. The significantly highest amounts of bioactive compounds were

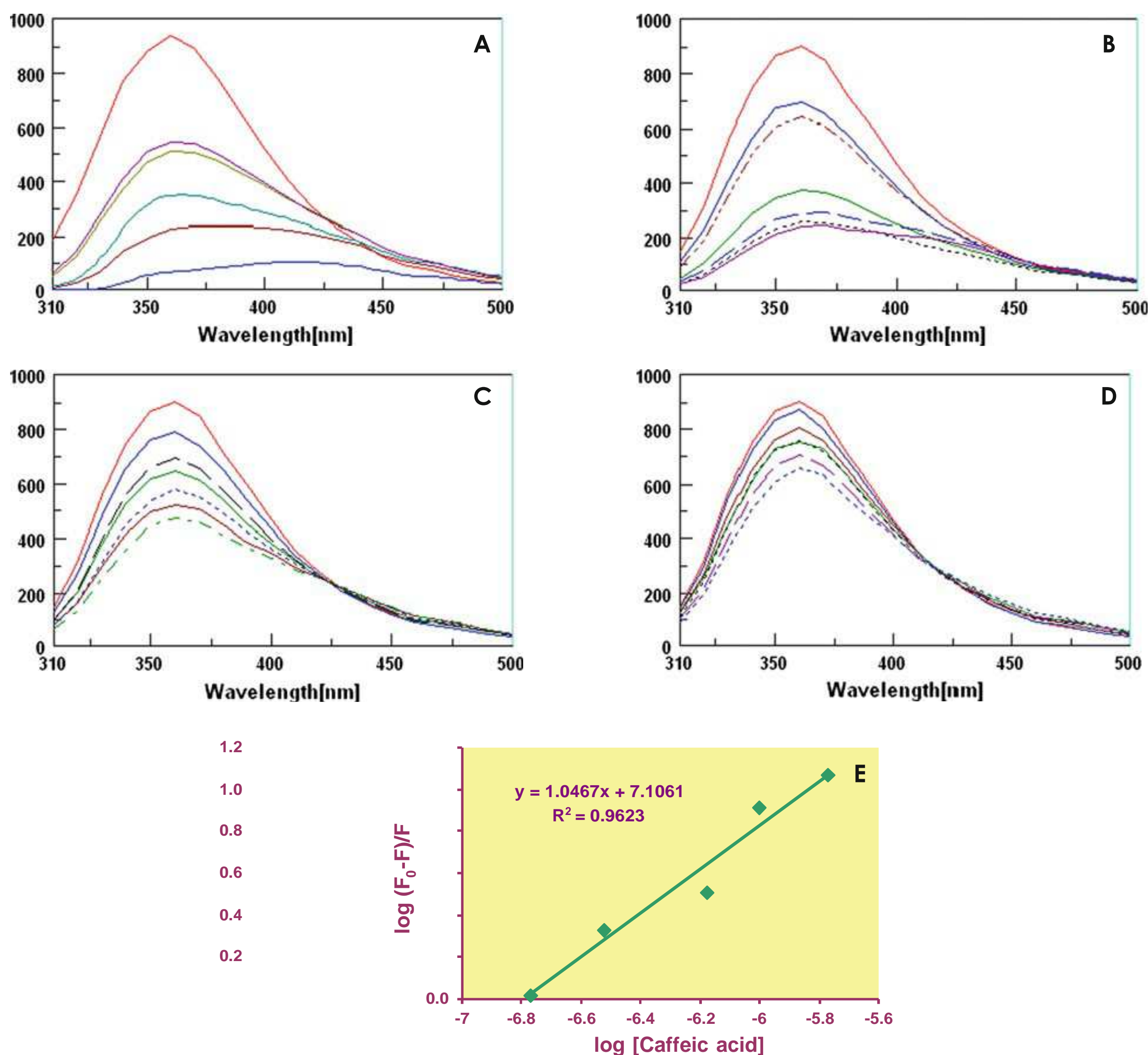

Fig. 3 Change in the relative fluorescence intensity (RFI) in two-dimensional fluorescence (2D-FL) spectra as a result of binding affinity of HSA, caffeic acid (CaA, 0.79 9 10^{-6} mol/L), and water extracts of berries: a fluorescence spectra of water extract of HSA (2.0 9 10^{-6} mol/L) in the presence of different concentrations of CaA 0, 0.17, 0.30, 0.67, 1.0, and 1.7 9 (10^{-6}) mol/L at pH 7.4 at excitation wavelength of 290 nm (lines from the top to the bottom with RFI of 947.37, 545.49, 509.67, 352.50, 237.02, 107.70). b Lines from the top to the bottom with RFI of 904.26, 693.69, 640.57, 376.89, 294.08, 261.65, and 244.51 of HSA, HSA and 20 lg/mL Blberry, HSA and 20 lg/mL Blberry and CaA, HSA and 100 lg/mL Blberry, HSA and 200 lg/mL Blberry, HSA and 100 lg/mL Blberry and CaA, HSA and 200 lg/mL Blberry and CaA. c Lines from the top to the bottom with RFI of 904.26, 791.65, 695.64, 646.56, 579.72, 520.84, and 472.82 for HSA; HSA and 20 lg/mL Crberry, HSA and 20 lg/mL Crberry, and CaA, HSA and 100 lg/mL Crberry, HSA and 200 lg/mL Crberry, HSA and 100 lg/mL Crberry and CaA, HSA and 200 lg/mL Crberry and CaA. d Lines from the top to the bottom with RFI of 904.26, 876.48, 806.60, 757.74, 755.29, 705.17, and 654.84 for HSA, HSA and 20 lg/mL Goberry, HSA and 100 lg/mL Goberry, HSA and 20 lg/mL Goberry and CaA, HSA and 200 lg/mL Goberry; HSA and 100 lg/mL Goberry and CaA, HSA and 200 lg/mL Goberry and CaA. e The linear plot for log $(F_0–F)/F$ versus log [caffeic acid], where F_0, and F represent the fluorescence intensity of HSA in the absence and in the presence of caffeic acid. Abbreviations: HSA, human serum albumin; Go, gooseberry, Crberry, cranberry and Blberry, blueberry

in water extract of all investigated berries, and the highest between the investigated berries was in blueberries. Also, the TAC according to ABTS and FRAP was significantly higher in water extract of blueberries. Our results correspond also with the data of Wu et al. [1], where concentrations of total anthocyanins varied considerably from 0.7 to 1,480 mg/ 100 g FW in gooseberry ('Careless' variety) and chokeberry, respectively. Total phenolic content and total anthocyanin content of four berry fruits (strawberry, Saskatoon berry, raspberry and wild blueberry), chokecherry, and

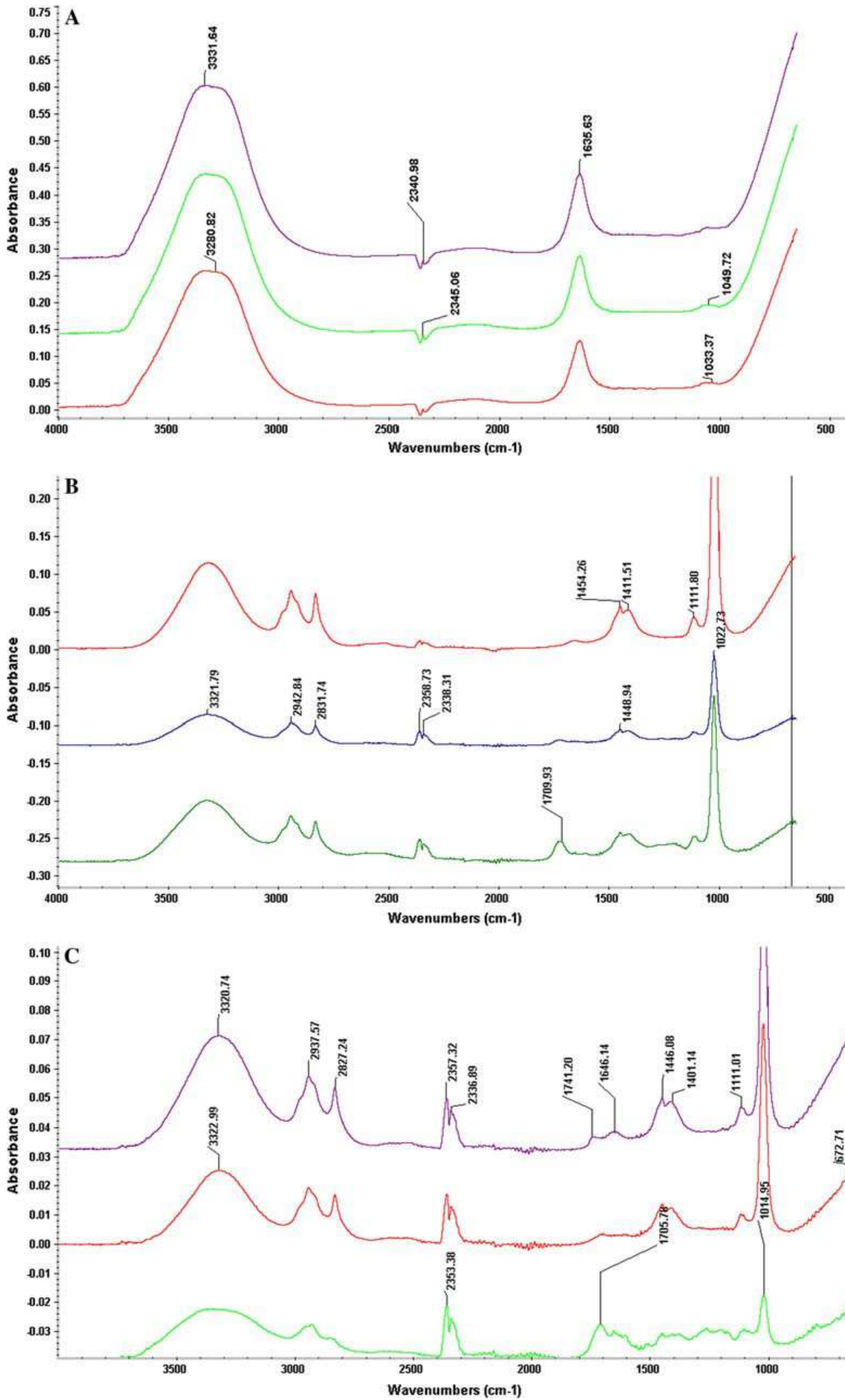

Fig. 4 FTIR spectra of: a water extracts of gooseberries, blueberries, and cranberries from the *top* to the *bottom*. b ethyl acetate extracts of blueberries, gooseberries, and cranberries from the *top* to the *bottom*. c diethyl ether extracts of gooseberries, blueberries, and cranberries from the *top* to the *bottom*

Table 2 Matching of the peaks (%) in the FTIR spectra of polyphenols and standards in water (A), ethyl acetate (B) and diethyl ether (C) extracts from

Range of bands	3,300–3,000 cm^{-1}			2,500–2,000 cm^{-1}			1,800–1,500 cm^{-1}			1,200–900 cm^{-1}		
Standards	Matching of standards/samples (%)											
	Gob	Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb
A												
Gallic acid	42	44	42	37	38	38	0	1	1	23	18	20
Ferulic acid	21	22	22	26	28	28	2	2	2	2	0	0
Fisetin	20	20	25	35	37	37	4	4	4	6	6	6
Hesperedin	75	75	75	5	7	7	29	29	29	19	15	15
Tannic acid	85	85	85	41	44	44	6	6	6	17	12	12
Caffeic acid	26	26	25	41	42	42	26	26	26	8	3	7
Quercetin	73	73	73	18	19	19	4	4	4	7	5	5
Range of bands	3,500–3,200 cm^{-1}			3,000-2,800 cm^{-1}			2,400-2,300 cm^{-1}			1,800-900 cm^{-1}		
Standards	Matching of standards/samples (%)											
	Gob	Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb
B												
Gallic acid	48	51	46	8	9	11	75	73	72	45	46	43
Ferulic acid	22	19	22	15	15	16	57	55	57	15	13	19
Fisetin	17	26	16	10	11	15	71	70	70	17	13	8
Hesperedin	57	55	61	28	23	24	1	1	0	5	4	5
Tannic acid	79	77	78	13	13	15	77	77	78	51	48	57
Caffeic acid	18	15	17	36	39	39	77	75	76	12	16	10
Quercetin	73	72	71	0	6	9	36	34	36	4	6	9
Range of bands	3,300-3,100 cm^{-1}			3,000-2,800 cm^{-1}			2,500-2,200 cm^{-1}			1,800-600 cm^{-1}		
Standards	Matching of standards/samples (%)											
Gob		Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb	Gob	Blb	Crb
C												
Gallic acid	50	47	48	12	10	26	61	59	61	30	29	22
Ferulic acid	15	9	31	15	16	4	38	37	37	6	37	9
Fisetin	25	25	22	9	9	4	51	49	51	3	2	3
Hesperedin	90	87	73	30	27	31	5	5	5	4	4	6
Tannic acid	85	78	75	16	14	27	67	66	67	41	42	32
Caffeic acid	8	3	21	36	37	9	58	58	58	2	3	9
Quercetin	69	65	59	3	3	4	29	28	29	3	1	12

Gob gooseberries, *Blb* blueberries, *Crb* cranberries

seabuckthorn ranged from 22.83 to 131.88 g/kg and 3.51 to 13.13 g/kg, respectively, which corresponds with our results. A number of reviewed articles showed that the main bioactive compounds determining the nutritional quality of berries are polyphenols, anthocyanins, and flavonoids [2, 4, 23–28]. The high anthocyanin content and biological activities of these fruits indicate that their consumption would be beneficial to health. The berries may be useful in the production of functional foods containing an efficacious dose of anthocyanins [2].

Our results were in accordance with the studies of Basu et al. [4], based on the high amount of phenolics in cranberries. As it was mentioned above, Borges et al. [5] showed that FRAP, vitamin C, and polyphenolic compounds have similar results, especially in the relationship between the anthocyanins which were the major contributor to the antioxidant capacity of blueberries, whereas the lower TAC of cranberries was due mainly to a reduced anthocyanin content. Vitamin C was responsible for 18–23 % of the TAC of cranberries, but did not contribute

to the TAC of the blueberry extract. Our results on antioxidant capacity of gooseberry correspond with Puente et al. [6] and Erkaya et al. [25], who showed that addition of gooseberry in the concentration of 15 % to ice cream positively influenced the chemical, sensory, and mineral characteristics of the mixture.

The comparison of the results of different solvents in Dabai fruit parts (methanol, ethanol, ethyl acetate, acetone, and water) and total phenolics, total flavonoids, total anthocyanins, and antioxidant capacity (ABTS$^{\cdot ?}$ and FRAP assays) were in accordance with our data [10]. The acetone extract had maximum phenol and flavonoid content and showed best DPPH free radical scavenging activity and reducing capacity assessment. Ethyl acetate extract showed best superoxide radical scavenging activity, while aqueous extract showed best hydroxyl radical scavenging activity [9]. Our present results correspond with the previous ones where the amount of polyphenol compounds and their antioxidant capacities of Murtilla berries were significantly higher than in other studied berries and are comparable with blueberries [11]. In our recent research, *Myrteola nummularia*, Murtilla, blueberries, raspberries, and black chokeberries were compared [11].

It was evaluated that the ability to inhibit LDL oxidation and total polyphenol content were consistent in classifying the antioxidant capacity of the polyphenol-rich beverages in the following order: blueberry juice [black cherry juice, acai juice, cranberry juice [orange juice [29]. This order is in agreement with the polyphenol and antioxidant ability of blueberry and cranberry data in our investigation. Some studies contribute to the pharmacologic knowledge of *Physalis peruviana* regarding a remedy commonly used in Colombian traditional medicine [30]. Our results in vitro studies were compared with Faria et al. [31] and Hurst et al. [32], where anthocyanin-derived blueberry extracts were analyzed for the contents of polyphenols, flavonoids, anthocyanins, and anthocyanin-derived pigments. All of the extracts provided the protection of membranes against peroxyl radicals by increasing the induction time of oxidation. This effect increased with the polyphenol content and with the structural complexity of the anthocyanin-derived pigments of the extracts. Our results are in correspondence with Burdulis et al. [24], where it was shown that the strongest antioxidant capacity possesses blueberry cultivar ''Berkeley'' (82.13 ± 0.51 %). Our results about the investigated berries like cranberries are in full correspondence with other reports that it is an excellent source of high-quality antioxidants and should be examined in human supplementation studies [33]. Bog bilberry water extracts contained polyphenol, anthocyanin-rich (pigment), and sugar/acid fractions by using ethyl acetate, acidic methanol (MeOH) and

36. N HCl. The crude extract and fractions containing polyphenol and pigment exhibited the greatest antioxidant activities with 50 % inhibitory concentration IC(50) values of 85.8, 33.2, and 16.7 lg/mL, respectively, for the DPPH assay, and 48.1, 83.8, and 51.9 lg/mL for the nonenzymatic superoxide radical assay. In our case, the highest antioxidant capacity was shown in water; therefore, for binding properties were used only these extracts [34]. The amount of total phenolics, anthocyanins, and ascorbic acid varied in berries depending on their maturity and varieties. Our results were in agreement with others [35], who showed the variation in their composition. The phenolic compounds were about 504 mg/100 g. The biggest quantities of ascorbic acid were found in the ripe berries of ''Ben Lear'' cultivar (15.8 mg/100 g). Based on the data reported by Wolfe and Liu [36], quercetin had the highest cellular antioxidant activity (CAA) value, followed by kaempferol, epigallocatechin gallate (EGCG), myricetin, and luteolin among the pure compounds tested. Among the selected fruits tested, blueberry had the highest CAA value, followed by cranberry [apple = red grape [green grape. Our results are similar to Kusznierewicz et al. [37], where the antioxidant activities of different blue-berried honeysuckle cultivars were similar to that of wild-growing bilberries (ranging from 170 to 417 lmol TE/g DW in ABTS and from 93 to 166 lmol TE/g DW in DPPH and Folin-Ciocalteu tests). The major anthocyanin in the blue-berried honeysuckle was cyanidin-3-glucoside, which constituted 84–92 % of the total anthocyanins. Our data can be comparable with another report [38], where the proanthocyanidins (condensed tannins) were present in the blackberry fruits. The average anthocyanin concentration was 49.2 mg/g in the commercial cultivar 'Tupy' while in the wild genotypes and the breeding line, the range was 361.3–494.9 mg/g (cyanidin 3-O-glucoside equivalent). The proanthocyanidin concentration varied widely among wild genotypes (417.5–1,343.6 mg/g CE). Comparison of different fractions of water extracts from wild black- berry *Aristotelia chilensis* (Mol) Stuntz (Elaeocarpaceae) corresponded with our results. Also, other authors reported similar results [39]. Total phenolics, flavonoids, and anthocyanins (mg/g FW) were in blueberry 261–585, 50, 25–495; raspberry 121, 6, 99; antioxidant activity (lmol Trolox/g FW for blueberry 14 by ABTS and 25.3 by DPPH) [39]. The result from this study indicated that blueberries had very high ORAC values and higher antioxidant capacity than other selected fruits and vegetables.

In the present report, the best binding ability to the HSA was with water extracts of berries. It is interesting that in Faria et al. [31], the antiradical properties and the reducing power of the extracts by using DPPH and FRAP methods, respectively, were in agreement with those obtained with the liposome membranes. This is in accordance with our present data that the binding properties and the antioxidant capacities are in correlation. A blueberry fruit extract displayed a potent and significant dose-dependent protective

capacity as it was shown in fluorescence studies with binding with HSA [32]. The obtained results by fluorescence are in direct relationship with the antioxidant properties of the berries extracts. The synergism of bioactive compounds is shown when to the mixture of HSA and berries extracts caffeic acid was added. Our very recent results showed that the fluorescence is significantly quenched, because of the conformation of the HSA changes in the presence of phenolic acids and berries extracts. This interaction between phenolic acids and HSA was investigated using tryptophan fluorescence scavenging. Other results [14, 22] differ from the reported by us, probably because of the variety of antioxidant abilities of pure phenolic acids and different ranges of fluorometry scanning used in a similar study. *In vitro* results of interaction of HSA and caffeic acid shown in the present study can be compared with other reports [22]. The displacement experiments confirmed that caffeic acid could bind to the site I of HSA, which was in agreement with the result of the molecular modeling study [22]. There are not too many applications of 3D fluorescence spectra; therefore, our present conclusions that 3-D fluorescence can be used as an additional tool for the characterization of the extracts of berries cultivars correspond with the previous data [15]. The matching results of common peaks for the first time showed that FTIR spectra can be used for a rapid estimation of extracted bioactive compounds. Quercetin and hesperidin exhibited the highest matching of the peaks in the investigated berries extracts in comparison with fisetin, caffeic, and gallic acids. In our previous study, the FTIR spectra data showed that the main bands in the berries samples slightly shifted [15]. A shift in the difference between the standards and the investigated samples can be explained by the extraction procedures of the main polyphenols.

In conclusion, the bioactivity of blueberries is significantly higher than the bioactivity of other studied samples; however, this index in the gooseberries is comparable with blueberries and cranberries. The binding properties of the investigated samples are in correlation with the antioxidant capacity. 3-D fluorescence and FTIR spectroscopy were used as an additional tool for the characterization of the polyphenol extracts in different berries cultivars. The analytical methods used in this study can be applied for any of the food analysis.

Conflict of interest None.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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In Vitro Studies on the Relationship Between the Antioxidant Activities of Some Berry Extracts and Their Binding Properties to Serum Albumin

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Abstract The aim of this study was to investigate the possibility to use the bioactive components from cape gooseberry (*Physalis peruviana*), blueberry (*Vaccinium corymbosum*), and cranberry (*Vaccinium macrocarpon*) extracts as a novel source against oxidation in food supplementation. The quantitative analysis of bioactive compounds (polyphenols, flavonoids,

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flavanols, carotenoids, and chlorophyll) was based on radical scavenging spectrophotometric assays and mass spectrometry. The total phenolic content was the highest ($P<0.05$) in water extract of blueberries (46.6±4.2 mg GAE/g DW). The highest antioxidant activities by 2,2-diphenyl-*1*-picrylhydrazyl radical scavenging assay and Cupric reducing antioxidant capacity were in water extracts of blueberries, showing 108.1±7.2 and 131.1±9.6 μMTE/g DW with correlation coefficients of 0.9918 and 0.9925, and by β-carotene linoleate assay at 80.1±6.6 % with correlation coefficient of 0.9909, respectively. The water extracts of berries exhibited high binding properties with human serum albumin in comparison with quercetin. In conclusion, the bioactive compounds from a relatively new source of gooseberries in comparison with blueberries and cranberries have the potential as food supplementation for human health. The antioxidant and binding activities of berries depend on their bioactive compounds.

Keywords Berries · Bioactive compounds · Antioxidant activity · Binding properties

Introduction

It is well known that antioxidants present in various fruits, vegetables, juices, and wines have the potential to protect the urinary bladder, prevent cholesterol in blood, and protect the liver from free radical damage [1–3]. The various health benefits of berries are well documented and have been attributed mainly to their antioxidant capacity. There is a growing public interest for cranberry, blueberry, and relatively new gooseberry as a functional food because of the potential health benefits linked to phytochemical compounds [4] responsible for secondary plant metabolites (flavonols, flavan-3-ols, proanthocyanidins, and phenolic acid derivatives). Several different mechanisms have been proposed to explain the possible role of cranberries, blueberries, and gooseberries in the prevention of atherosclerosis [4–6].

Fractions responsible for the antioxidant action were identified and seem promising for phytomedicinal development [7]. Recent advances have been made in scientific understanding of how berries promote human health and prevent chronic illnesses such as some cancers, heart disease, and neurodegenerative diseases [8]. In fact, 90-day and 48-h stability of the blackberry extract in biologically relevant buffers has been investigated in studies [9]. Blackberry administration could minimize the toxic effects of fluoride, indicating its free radical scavenging and potent antioxidant activities. The induced oxidative stress and the alterations in antioxidant system were normalized by the oral administration of 1.6 g/kg body weight of blackberry juice [10]. Consumption of cranberries is known to exert positive health effects, especially against urinary tract infections. Cranberry was investigated as a chemotherapeutic agent [11]. For this reason, presumably, they are used in folk medicine [12]. *Physalis peruviana* (PP) is a widely used medicinal herb for treating cancer, malaria, asthma, hepatitis, dermatitis, and rheumatism [13–16]. Kusznierewicz et al. [17] analyzed different Polish cultivars of blue-berried honeysuckles and wild and bog bilberry for bioactive compounds. Potential benefits of polyphenolic compounds from raspberry seeds of three different extracts as efficient antioxidants were studied [18]. Infusions of *Ugni molinae* Turcz, also known as "Murtilla", have long been used in traditional native herbal medicine [19] and investigated as well. However, the mechanisms behind the functions of berries with proteins are poorly understood. The interactions between polyphenols, especially flavonoids and plasma proteins, have attracted great interest among researchers. Few papers, however, have focused on the structure–affinity relationship of polyphenols on their affinities for plasma proteins [7, 20, 21],

especially from berries. We were interested to investigate relatively new kind of cape gooseberries (*P. peruviana*) and to compare its composition with that of the widely consumed blueberries and cranberries. To meet this aim, the contents of bioactive compounds (polyphenols, flavonoids, flavanols, carotenoids, and chlorophylls) and the level of antioxidant activity (AA) were determined and compared. In order to receive reliable data, AA was determined by three assays: CUPRAC, DPPH, and β-carotene linoleate model system [22–24]. Human serum albumin is the drug carrier's protein and serves to greatly amplify the capacity of plasma for transporting drugs. It is interesting to investigate in vitro how this protein interacts with flavonoids extracted from berry samples in order to get useful information of the properties of flavonoid–protein complex. Therefore, the functional properties of a new kind of berry will be studied by the interaction of water polyphenol extracts with a small protein such as HSA, using 3D-FL. As far as we know, no results of such investigations were published.

Materials and Methods

Chemicals

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picrylhydrazyl (DPPH), β-carotene, linoleic acid, quercetin, human serum albumin, Tris, tris(hydroxymethy1)aminomethane, Folin–Ciocalteu reagent, lanthanum (III) chloride heptahydrate, $CuCl_2 \cdot 2H_2O$, and 2,9-dimethyl-1,10-phenanthroline (neocuproine) were purchased from Sigma Chemical Co., St Louis, MO, USA. All reagents were of analytical grade. Deionized and distilled water was used throughout.

Samples

Cape gooseberries (*P. peruviana*), blueberries (*Vaccinium corymbosum*), and cranberries (*Vaccinium macrocarpon*) were investigated. All berries were purchased at the local market in Gdansk and Warsaw, Poland. For the investigation, five replicates of five berries each were used. Their edible parts were prepared manually without using steel knives. The prepared berries were weighed, chopped, and homogenized under liquid nitrogen in a high-speed blender (Hamilton Beach Silex professional model) for 1 min. A weighed portion (50–100 g) was then lyophilized for 48 h (Virtis model 10-324), and the dry weight was determined. The samples were ground to pass through a 0.5-mm sieve and stored at −20 °C until the bioactive substances were analyzed.

Extraction of Phenolic Compounds

The lyophilized samples of berries (1 g) were extracted with 100 mL of methanol/water (1:1) at room temperature and in darkness for 24 h. The extracts were filtered in a Buchner funnel. After removal of the methanol in a rotary evaporator at a temperature below 40 °C, the aqueous solution was extracted with diethyl ether and ethyl acetate, and then the remainder of the aqueous solution was freeze-dried. The organic fractions were dried and redissolved in methanol. These extracts were submitted to MS analysis for determination of bioactive compounds [25].

The polyphenols were determined by Folin−Ciocalteu method with measurement at 750 nm with a spectrophotometer (Hewlett-Packard, model 8452A, Rockville, MD, USA). The results were expressed as mg of gallic acid equivalents (GAE) per g DW [26].
Flavonoids, extracted with 5 % $NaNO_2$, 10 % $AlCl_3 \cdot 6H_2O$, and 1 M NaOH, were measured at 510 nm. The total flavanols amount was estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method, and then the absorbance at 640 nm was read. To ensure the presence of flavanols on the nuclei, subsequent staining with the DMACA reagent resulted in an intense blue coloration in the plant extract [27]. As was mentioned previously, (+)-catechin served as a standard for flavonoids and flavanols, and the results were expressed as catechin equivalents (CE). Total chlorophyll, chlorophylls *a* and *b*, and total carotenoids were extracted with 100 % acetone and determined spectrophotometrically at different absorbances (nm) such as at 661.6, 644.8, and 470, respectively [28].

MS Analysis A mass spectrometer, TSQ Quantum Access Max (Thermo Fisher Scientific, Basel, Switzerland), was used. Analytes were ionized by electrospray ionoization (ESI) in negative mode. Vaporizer temperature was kept at 100 °C. All samples were done by direct infusion in the mass spectrometer by using ESI source at negative ion mode, full scan analysis, ranging between 100 and 900 m/z. For optimization of the acquisition parameters and for identity confirmation, only a part of the standards was employed, not for all compounds that were found in the investigated samples. Settings for the ion source were as follows: spray voltage 3,000 V, sheath gas pressure 35 AU, ion sweep gas pressure 0 AU, auxiliary gas pressure at 30 AU, capillary temperature at 200 °C, and skimmer offset 0 V [29−31]. The AA was determined by the following assays:

- Cupric reducing antioxidant capacity (CUPRAC): This assay is based on utilizing the copper (II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidizing agent. To the mixture of 1 ml of copper (II)-neocuproine and NH_4Ac buffer solution, acidified and non-acidified methanol extracts of berry (or standard) solution (x, in ml) and H_2O [$(1.1-x)$ ml] were added to make a final volume of 4.1 ml. The absorbance at 450 nm was recorded against a reagent blank [22].
- Scavenging free radical potentials were tested in solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH). In its radical form, DPPH has an absorption band at 515 nm which disappears upon reduction by an antiradical compound. DPPH solution (3.9 mL, 25 mg/L) in methanol was mixed with the sample extracts (0.1 mL), and then the reaction progress was monitored at 515 nm until the absorbance was stable [23].
- β-Carotene linoleate model system: A mixture of β-carotene (0.2 mg), linoleic acid (200 mg), and Tween-40 (200 mg) was prepared. Chloroform was removed at 40 °C under vacuum. The resulting mixture was diluted with 10 mL of water. To this emulsion was added 40 mL of oxygenated water. Four-milliliter aliquots of the emulsion were added to 0.2 mL of berry extracts (50 and 100 ppm). The absorbance at 470 nm was measured. The AA of the extracts was evaluated in terms of bleaching of the β-carotene: $AA=100\,[1-(A_0-A_t)/(A_0^\circ-A_t^\circ)]$, where A_0 and A_0° are the absorbance values measured at zero time of the incubation for test sample and control, respectively, and A_t and A_t° are the absorbance values measured in the test sample and control, respectively, after incubation for 180 min [24].

Fluorometric Measurements

Fluorometric measurements were used for the evaluation of the antioxidant activity of berries extracts and their in vitro binding properties to human serum albumin. Two-dimensional (2D-FL) and three-dimensional (3D-FL) fluorescence measurements for all berry extracts at a concentration of 0.01 mg/mL were recorded on a model FP-6500, Jasco spectrofluorometer, serial N261332, Japan, equipped with 1.0 cm quartz cells and a thermostat bath. The 2D-FL was taken at emission wavelengths from 310 to 500 nm and at excitation of 295 nm.
The 3D-FL spectra were collected with subsequent scanning emission spectra from 250 to 500 nm at 1.0-nm increments by varying the excitation wavelength from 200 to 350 nm at 10- nm increments [32]. Quercetin (QUE) was used as a standard. All solutions for protein interaction were prepared in 0.05 mol/l Tris-HCl buffer (pH 7.4), containing 0.1 mol/l NaCl. The final concentration of HSA was 2.0×10^{-6} mol/l. The HSA was mixed with quercetin in the proportion HSA/extract=1:1. Statistical

Analyses

To verify the statistical significance, mean ± SD of five independent measurements were calculated. Data groups' distribution character was tested by Shapiro–Wilk normality test and the homogeneity of variance by Levene's *F* test, both at 0.95 confidence level. Multiple comparisons also known as post hoc tests to compare all possible pairs of means of a group of berries extracts were performed by Student–Newman–Keuls method based on the studentised data range. *P*-values of <0.05 were considered significant. Linear regressions were also calculated and Pearson correlation coefficients (*R*) were used.

Results and Discussion

Bioactive Compounds and Antioxidant Activities

It was interesting to use different solvent systems such as diethyl ether, ethyl acetate, and water in order to find out the best extraction conditions and the maximum antioxidant activities of gooseberries in comparison with blueberries and cranberries. The results of the determination of the contents of the bioactive compounds in the extracts of three solvents of all studied samples are summarized in the Table 1. As can be seen, the significant highest contents ($P<0.05$) of polyphenols and flavanols were in the water fraction of blueberries (46.56± 4.2 mg GAE/g and 1.75±0.3 mg CE/g, respectively). The contents of flavonoids are comparable with the data in cranberries. The contents of chlorophylls and carotenoids (Fig. 1) were the highest in blueberries as well ($P<0.05$). The weight ratio of Chl *a* and Chl *b* is an indicator of the functional pigments. The ratios of chlorophylls *a*/*b* were the following: 0.68, 1.17, and 2.55 for gooseberries (GOOSEB), cranberries (CRAN), and blueberries (BLUEB), respectively. The ratio of total chlorophylls to total carotenoids is an indicator of the greenness of plants (Fig. 1).
It was mentioned earlier that the main purpose was to compare gooseberry with other berries in order to find out if its bioactivity is on the same level as in other kinds of berry. Therefore, the contents of the bioactive compounds and AA were determined and compared with widely consumed blueberries and cranberries. A number of reviewed articles show that the main bioactive compounds determining the nutritional quality of berries are polyphenols, anthocyanins, and flavonoids [1, 9]. Carotenoids and chlorophylls are important in the

Table 1 Bioactive compounds in water, ethyl acetate, and diethyl ether extracts of gooseberries (*P. peruviana*), cranberries (*V. macrocarpon*), and blueberries (*V. corymbosum*) per gram dry weight

Extracts	Indices		
	POLYPHEN, mg GAE	FLAVON, mg CE	FLAVAN, μg CE
GOOSEB, H_2O	5.37±0.6	0.22±0.04	nd
CRAN, H_2O	22.13±2.5	3.83±0.4	467.36±14.5
BLUEB, H_2O	46.56±4.2	3.89±0.6	1,751.51±25.6
GOOSEB, EtOAc	0.29±0.1	0.11±0.01	nd
CRAN, EtOAc	3.14±0.4	0.66±0.1	44.14±4.3
BLUEB, EtOAc	3.87±0.4	0.74±0.1	112.06±7.4
GOOSEB, DETETHR	0.14±0.01	0.08±0.01	1.21±0.1
CRAN, DETETHR	2.11±0.2	0.10±0.01	7.66±0.8
BLUEB, DETETHR	4.13±0.4	0.39±0.1	32.55±3.9

Values are means ± SD of five measurements. All statistical data are presented in Table 4

POLYPHEN polyphenols, *CE* catechin equivalent, *GAE* gallic acid equivalent, *FLAVON* flavonoids, *FLAVAN* flavanols, *nd* not determined, *GOOSEB* gooseberries (*P. peruviana*), *CRAN* cranberries (*V. macrocarpon*), *BLUEB* blueberries (*V. corymbosum*), *EtOAc* ethyl acetate, *DETETHR* diethyl ether

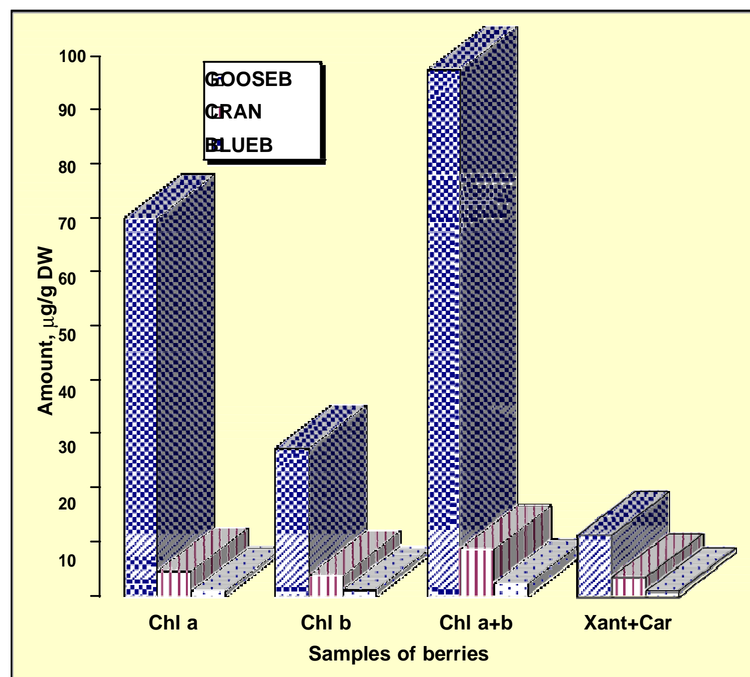


Fig. 1 Chlorophyll and carotenoid levels in berries. Values are means ± SD: ±7.15, ±0.48, and ±0.01 for Chl a in BLUEB, CRAN, and GOOSEB, respectively; ±2.45, ±0.43, and ±0.01 for Chl b in BLUEB, CRAN, and GOOSEB, respectively; ±10.08, ±0. 86, and ±0.12 for Chl a + b in BLUEB, CRAN, and GOOSEB, respectively; ±1.25, ±0. 34, and ±0.08 for Xant + Car in BLUEB, CRAN, and GOOSEB, respectively. *Chl* chlorophyll, *Xant* xanthophylls, *car* carotenes, *GOOSEB* gooseberries, *CRAN* cranberries, *BLUEB* blueberries

composition of berries. The ratio of total chlorophylls to total carotenoids was 2.15, 2.47, and 8.67 for gooseberries, cranberries, and blueberries, respectively. The two ratios were in the range which shows that the berries were grown and collected at optimal growing conditions [33]. The obtained contents of chlorophylls and carotenoids were in acceptable range, showing their sensitivity to seasonal variation in climatic conditions [34]. Our data can be compared with other reports [35], where different carotenoids in seabuck thorn berries increased in concentration during ripening and comprised from 120 to 1,425 μg/g DW of total carotenoids (1.5–18.5 mg/100 g of FW), depending on the cultivar, harvest time, and year. The content of chlorophyll can act as a marker of the degree of ripening.

We investigated the properties of quercetin, the major phenolic phytochemical present in berries, in aqueous media using UV spectroscopy, fluorometry, and ESI-mass spectrometry. As was declared in "Results and Discussion", the contents of bioactive compounds (polyphenols, flavonoids, and flavanols) in three different extracts was determined and compared, and the significantly highest amounts were in water extract of blueberries. Gooseberries showed a moderate amount of bioactive compounds. Our results were in agreement with others, showing that water extracts of blueberries contain high amounts of polyphenols [9]. The amount of phenolics for blueberry and cranberry was reported as 261–585 and 315 mg/g FW and for flavonoids as 50 and 157 mg/g FW [36, 37]. The ESI-MS in negative ion mode (Table 2; Fig. 2a) of water extracts differs between berries. The water extract of gooseberry (Table 2;

Table 2 Mass spectral data (molecular ion and the major fragment ions of polyphenols extracted from berries)

Extracts in ESI, (% in MS)	Berries	$[M-H]^-$ and fragmentation	Compound
Water	Gooseberries	190.79 (100)	Quinic acid
	Cranberries	352.77 (40), 190.79 (100)	Chlorogenic acid, quinic acid
		294.74 (15)	*p*-Coumaroyl tartaric acid
		212.6 (20)	2,3 Dihydroxy-*1*-guaiacyl propanone
	Blueberries	404.85 (60)	Piceatannol 3-*O*-glucoside
		346.68 (40), 190.93 (100)	5-Heptadecylresorcinol, quinic acid
Ethyl acetate	Gooseberries	444.40 (35)	Apigenin 7-*O*-glucuronide
		190.79 (30)	Quinic acid
		212.6 (100)	2,3 Dihydroxy-*1*-guaiacyl propanone
	Cranberries	444.5 (10)	Apigenin 7-*O*-glucuronide
		190.79 (100)	Quinic acid
		212.6 (50)	2,3 Dihydroxy-*1*-guaiacyl propanone
	Blueberries	346.68 (20)	5-Heptadecylresorcinol
		190.79 (100)	Quinic acid
Diethyl ether	Gooseberries	444.33 (40)	Apigenin 7-*O*-glucuronide
		212.6 (100)	2,3 Dihydroxy-*1*-guaiacyl propanone
		168.81 (30)	Gallic acid
	Cranberries	444.47 (40)	Apigenin 7-*O*-glucuronide
		300.83 (40)	quercitin
		212.6 (100)	2,3 Dihydroxy-*1*-guaiacyl Propanone
		190.7 (55)	Quinic acid
	Blueberries	366.9 (50), 190.8 (80)	3-Feruloylquinic acid, quinic acid
		212.7 (100)	2,3 Dihydroxy-*1*-guaiacyl propanone

Fig. 2—Aa) showed that the molecular ion at *m/z* 190.79 corresponded to quinic acid. Oppositely, cranberry (Table 2; Fig. 2—Ab) water extract was characterized by chlorogenic acid of the $[M\text{-}H]^-$ deprotonated molecule (*m/z* 353) and the ion corresponding to the

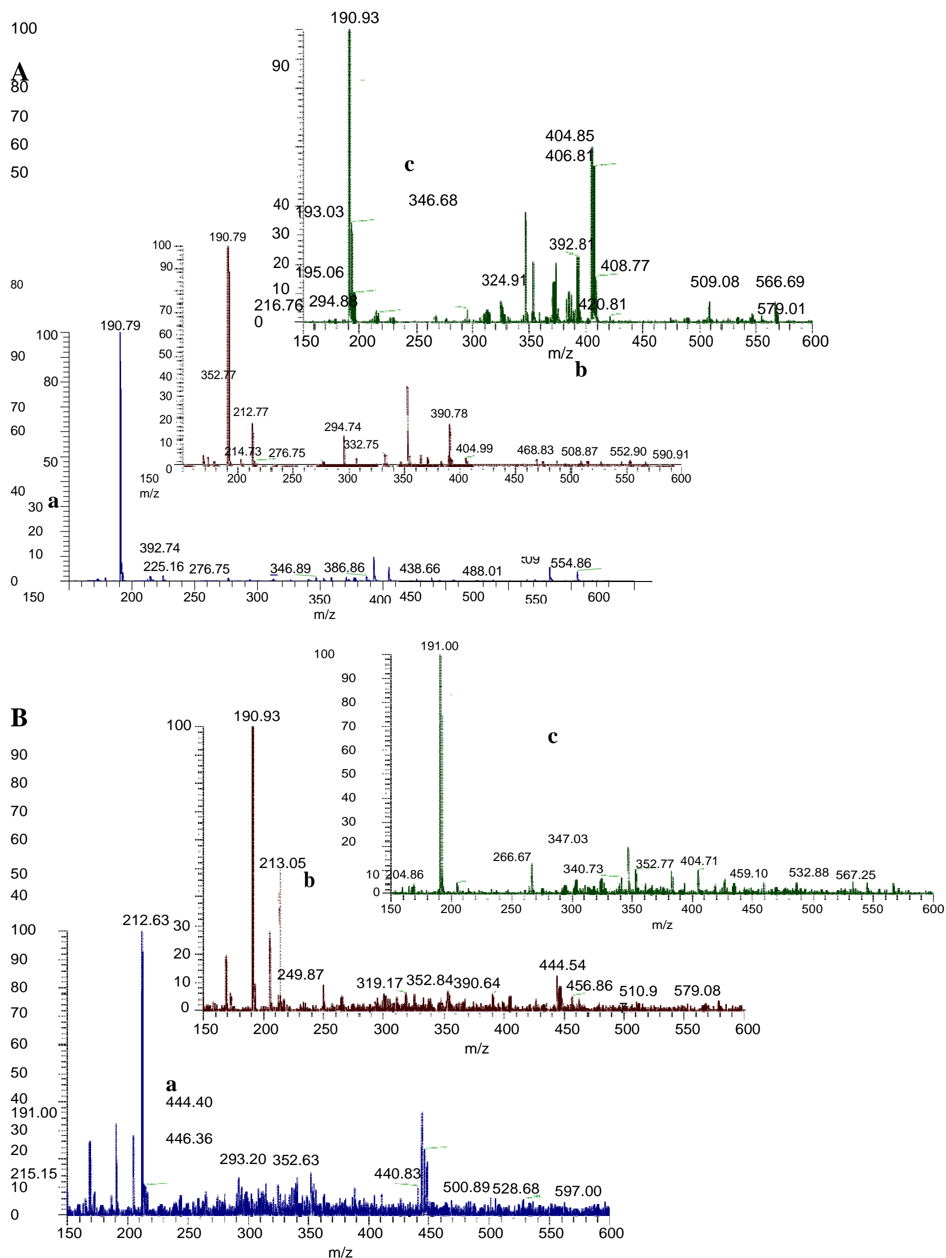


Fig. 2 ESI-MS spectra of extracted fractions from three studied berries. a Aqueous, b ethyl acetate, and c diethyl ether of *a* gooseberries, *b* cranberries, and *c* blueberries in negative ion mode. Phenolic compounds were identified at *m/z* based on the mass spectra data

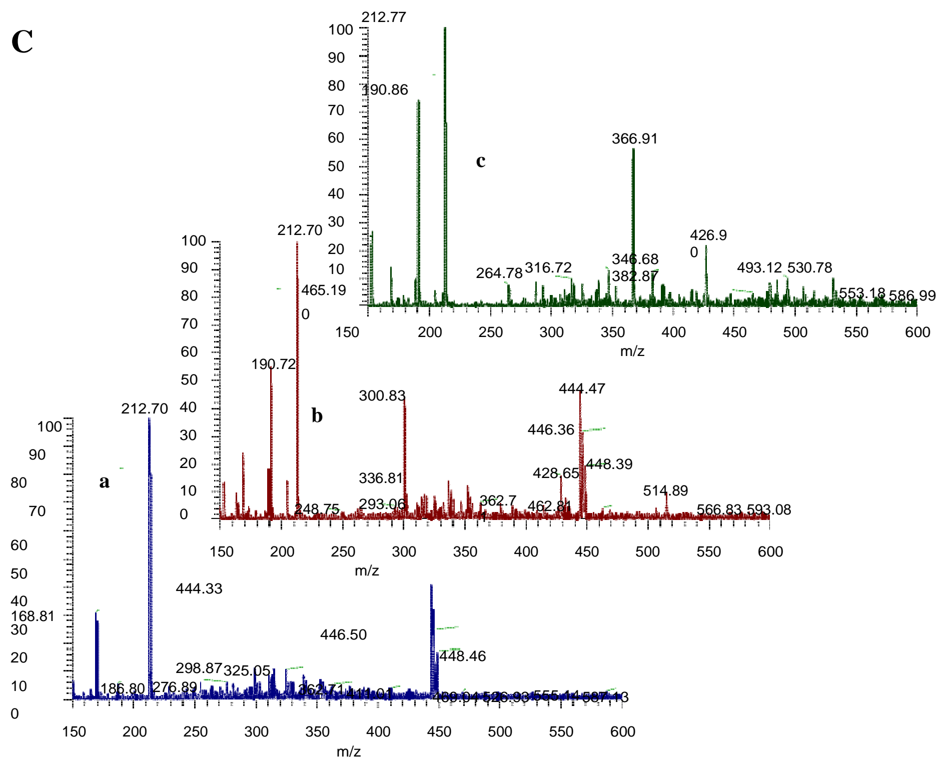


Fig. 2 (continued)

deprotonated quinic acid (*m/z* 191), which was consistent with Sun et al. (2007). Blueberry water extract (Table 2; Fig. 2c) demonstrated a peak at 404.85 (piceatannol 3-*O*-glucoside), 346.68, and 190.93 as a result of destroying 5-heptadecylresorcinol. Ethyl acetate extracts of berries showed similar spectral peaks. Gooseberry (Table 1; Fig. 2—Ba) and cranberry (Table 1; Fig. 2—Bb) were similar in molecular ions but differ in the percentage in MS. Blueberry ethyl acetate extract (Table 2; Fig. 2—Bc) and water extract (Table 2; Fig. 2—Ac) were similar. In the diethyl ether extracts (Table 2; Fig. 2c) of all berries, the main peak was of *m/z* 212.6. The spectra of blueberry differ from gooseberry and cranberry with one peak at *m/z* 366.9. In gooseberry and cranberry extracts, one common peak appeared at *m/z* 444.4, but gooseberry extract is characterized by the peak of gallic acid and in cranberry only quercetin is found.

The recorded spectra were in the same scale (in the range between 100 and 600*m/z*) for comparison. We choose negative mode as the MS method because in many publications it was described that this mode is the best for analysis of low molecular weight phenolic compounds [29, 38–40]. All of the peaks were identified and the recorded MS spectra can be used as a fingerprint for characterization of different berry extracts based on the percentage of the main peaks. Our obtained results by MS are similar to Zuo et al. [39], where 15 benzoic and phenolic acids (benzoic, *o*-hydroxybenzoic, cinnamic, *m*-hydroxybenzoic, *p*-hydroxybenzoic, *p*-hydroxyphenyl acetic, phthalic, 2,3-dihydroxybenzoic, vanillic, *o*-hydroxycinnamic, 2,4-dihydroxybenzoic, *p*-coumaric, ferulic, caffeic, and sinapic acid) were identified in cranberry fruit. The most abundant is benzoic and then *p*-coumaric and sinapic acids. The phenolic

constituents in the berries were identified as chlorogenic acid, *p*-coumaric acid, hyperoside,

quercetin-3-*O*-glucoside, isoorientin, isovitexin, orientin, and vitexin [38]. The AA of blueberry in water extracts (Table 3) as determined by CUPRAC, DPPH, and β-carotene assays (131.09±12.9.3 μM TE/g DW, 108.09±7.2 μM TE/g DW, and 80.11±8.9 %, respectively) in all of the extracts used is significantly higher than that recorded for other berries studied (P<0.05). The AA of gooseberry is lower by about nine times than in blueberries and four times than in cranberries. As was calculated, a very good correlation was found between the antioxidant activity and the contents of total polyphenols in water extracts. All groups of data (Tables 1 and 3) were tested for character of their distribution and homogeneity of variance at 0.95 confidence level. The Shapiro–Wilk normality test showed that all the data in groups are normally distributed, with the exception of flavanols in gooseberry water and ethyl acetate extracts with no quantified content. Levene's F test, which is widely accepted as the most powerful homogeneity of variance test, indicated extract types which have no the same variance tested at 0.95 confidence level. Table 4 presents significant differences (with P values <0.05) between bioactive compounds contents and antioxidant activities in different extracts of berries found by multiple comparisons using the method of Student–Newman–Keuls. The method denotes significantly different pairs, and the group in the first position means that it is higher in the contents of bioactive substances. For example, the case of polyphenols in line G/W-G/D means a statistically different content of polyphenols between gooseberry water and diethyl ether extracts. Water extract is higher in the content of polyphenols of about 10.2 mg GAE/g DW. From Table 4, it is evident that in majority of the cases, water extraction yields the highest content of bioactive compounds and antioxidant activities.

The antioxidant activity of different extracts was evaluated by DPPH free radical scaveng- ing activity, taking total phenolic content as an index [41]. Our obtained results correspond with the data of Kusznierewicz et al. [17], where the DPPH antioxidant activity varied from 93 to 166 mol TE/g DW. The obtained phenolic compounds and DPPH values (Tables 1 and 2) were as well in the range of those reported by Li et al. [42] of four berry fruits (strawberry, Saskatoon berry, raspberry, and wild blueberry), chokecherry, and seabuck thorn ranging from

Table 3 Antioxidant activities in water, ethyl acetate, and diethyl ether extracts of gooseberries (*P. peruviana*), cranberries (*V. macrocarpon*), and blueberries (*V. corymbosum*) per gram dry weight

Extracts	Indices		
	DPPH, μM TE/g DW	CUPRAC, μM TE/g DW	β-carotene, %
GOOSEB, H_2O	8.39±0.9	11.25±1.1	11.40±0.9
CRAN, H_2O	46.58±4.5	49.38±4.4	36.71±3.8
BLUEB, H_2O	108.09±7.2	131.09±9.6 0.88±0.1	80.10±6.6
GOOSEB, EtOAc	0.35±0.1		0.54±0.1
CRAN, EtOAc	3.02±0.4	9.20±1.1	6.09±0.6
BLUEB, EtOAc	8.83±4.4	12.40±1.1	8.13±0.9
GOOSEB, DETETHR	0.16±0.01	0.24±0.01	0.20±0.01
CRAN, DETETHR	3.42±0.4	5.77±0.6	3.48±0.3
BLUEB, DETETHR	10.97±0.9	14.87±1.1	6.79±0.7

Values are means ± SD of five measurements. All statistical data are shown in Table 4

DW dry weight, *DPPH* 2,2-diphenyl-*1*-picrylhydrazyl, *CUPRAC* cupric reducing antioxidant capacity, β-*carotene* β-carotene linoleate assay, *GOOSEB* gooseberries (*P. peruviana*), *CRAN* cranberries (*V. macrocarpon*), *BLUEB* blueberries (*V. corymbosum*), *EtOAc* ethyl acetate, *DETETHR* diethyl ether

Table 4 Statistically significant differences between the content of bioactive compounds in different extracts of berries by Student–Newman–Keuls multiple comparisons

Comparison between berries extracts	Difference	Standard error	*q* stat	Table *q*	Probability, $P<0.05$
Polyphenols					
G/W–G/D	10.2053	0.7071	14.4325	3.6332	0.0000
G/E–G/D	8.6337	0.7071	12.2099	2.7718	0.0000
B/W–B/D	4.3603	0.7071	6.1665	3.6332	0.0001
B/W–B/E	3.8084	0.7071	5.3860	3.3145	0.0004
Flavonoids					
G/W–G/E	2.7948	0.7071	3.9525	3.6332	0.0267
C/W–C/D	7.0963	0.7071	10.0357	4.0301	0.0000
C/E–C/D	4.3453	0.7071	6.1452	3.8577	0.0001
B/W–B/E	4.1482	0.7071	5.8665	3.8577	0.0003
B/W–B/D	4.1482	0.7071	5.8665	3.6332	0.0002
C/W–C/E	2.7510	0.7071	3.8905	2.7718	0.0059
Flavanols					
G/W–G/D	3.2040	0.7071	4.5311	3.3145	0.0039
G/E–G/D	3.2040	0.7071	4.5311	2.7718	0.0014
C/W–C/D	6.3189	0.7071	8.9363	3.8577	0.0000
C/E–C/D	3.9136	0.7071	5.5347	3.3145	0.0003
B/W–B/D	4.6555	0.7071	6.5839	3.8577	0.0000
C/W–C/E	2.4053	0.7071	3.4016	3.3145	0.0427
B/W–B/E	2.7159	0.7071	3.8409	3.3145	0.0181
DPPH					
G/W–G/D	12.0877	0.7071	17.0946	4.0301	0.0000
G/E–G/D	7.8126	0.7071	11.0486	2.7718	0.0000
G/W–G/E	4.2751	0.7071	6.0460	3.8577	0.0002
C/W–C/E	4.3824	0.7071	6.1976	4.0301	0.0002
C/W–C/D	4.3289	0.7071	6.1219	3.8577	0.0001
B/W–B/D	4.2085	0.7071	5.9517	3.8577	0.0002
B/W–B/E	2.8095	0.7071	3.9733	3.3145	0.0138
CUPRAC					
G/W–G/D	9.7648	0.7071	13.8095	4.0301	0.0000
G/E–G/D	4.4785	0.7071	6.3335	2.7718	0.0000
G/W–G/E	5.2863	0.7071	7.4760	3.8577	0.0000
C/W–C/D	4.8131	0.7071	6.8068	4.0301	0.0000
B/W–B/E	4.3484	0.7071	6.1495	4.0301	0.0002
B/W–B/D	4.1359	0.7071	5.8490	3.8577	0.0003
C/W–C/E	2.9609	0.7071	4.1874	2.7718	0.0031
β-CAROTENE					
G/W–G/D	8.5379	0.7071	12.0744	4.0301	0.0000
G/E–G/D	3.8783	0.7071	5.4847	2.7718	0.0001
G/W–G/E	4.6596	0.7071	6.5897	3.8577	0.0000
C/W–C/D	5.2270	0.7071	7.3921	4.0301	0.0000

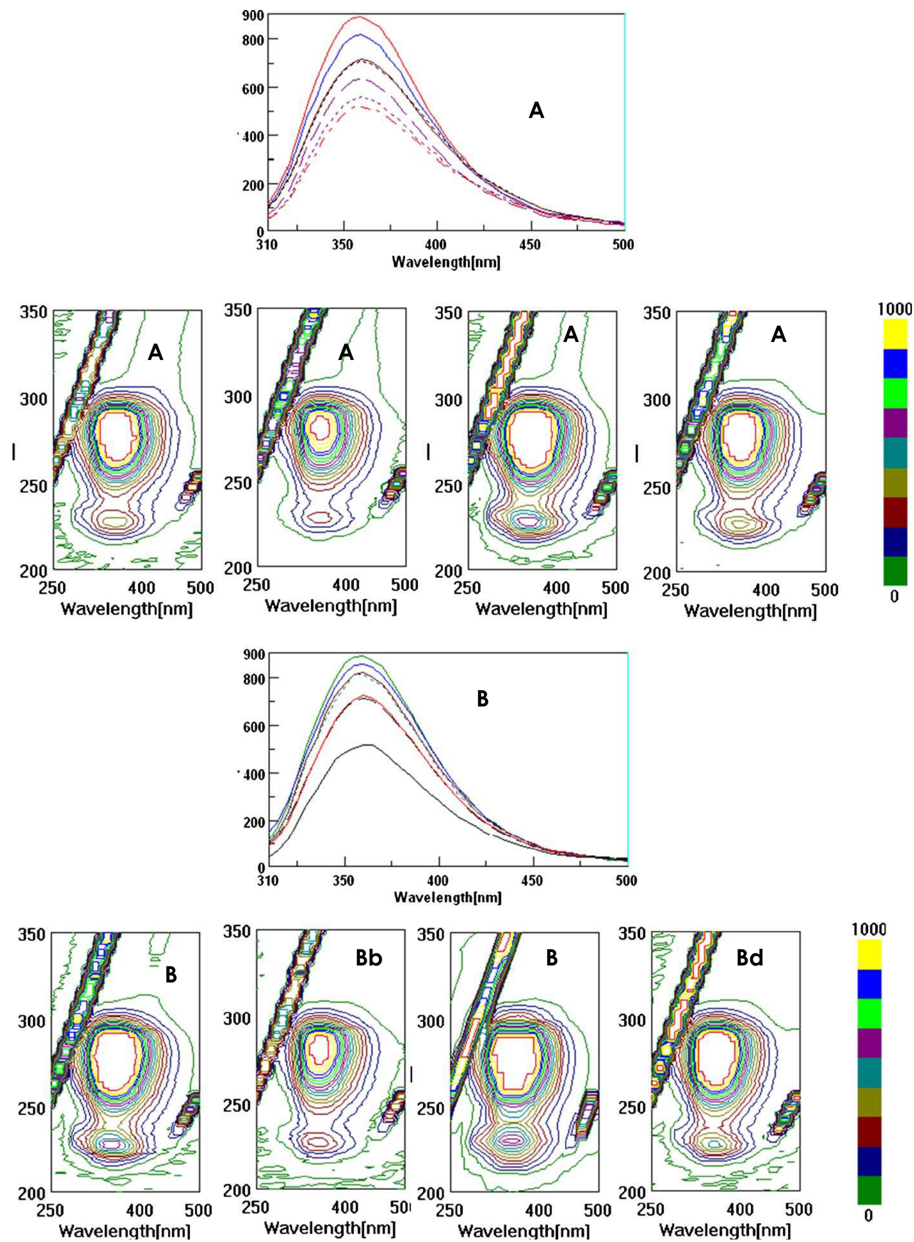
Table 4 (continued)

Comparison between berries extracts	Difference	Standard error	q stat	Table q	Probability, $P<0.05$
C/W–C/E	3.6094	0.7071	5.1045	3.8577	0.0028
B/W–B/D	4.0614	0.7071	5.7437	3.8577	0.0005

B berries, *G* gooseberries, *C* cranberries, *B* blueberries, *W* water, *E* ethyl acetate, *D* diethyl ether

22.83 to 131.88 g/kg and DPPH ranging from 29.97 to 78.86 %. The bioactivity of blueberry is significantly higher than the bioactivity of other berries; however, this index in the gooseberry is comparable with the studied samples. According to the results of Table 4, the antioxidant activities of extracts, partitions, and fractions were strongly correlated with the highest polyphenol contents. Correlation between polyphenols and antioxidant properties exactly corresponded with our results: the highest phenolic content was found in walnut, which revealed the best antioxidant properties [43]. This corresponds with Seeram [8], who discussed also that phytonutrients ranged from fat-soluble/lipophilic to water-soluble/hydrophilic compounds. Our results about the high antioxidant activity of berries (Table 3) are in line with Elberry et al. [11], showing a high antioxidant activity of cranberry extract. Pronounced antioxidant and radical scavenging properties of cranberry was shown by Wojnicz et al. [12]. Ethanol-soluble acidic components were used in order to determine the bioactivity of natural novel sources against oxidation [44]. Our results are in accordance with You et al. [45], where four Rabbiteye blueberry cultivars grown organically and conventionally were compared by their total phenolic content and antioxidant values by DPPH and CUPRAC. Our studies are not in full correspondence with others [15] based on the different extraction systems. In our case, the most active was the water fraction of *P. peruviana* (PP) in comparison with ethyl acetate and diethyl ether. As was reported by Wu et al. [15], supercritical carbon dioxide SCEPP-5 PP extracts in comparison with hot water and ethanol possessed the highest total flavonoid (226.19 mg/g) and phenol (100.82 mg/g) contents. Our results connected with other reports [41, 46], where the methanol extract of leaves from some plants was more potent against *Aspergillus fumigatus* and *Candida tropicana.* The lowest MIC values obtained for LM, LA, and LH were 78, 156, and 625 μg/mL against *A. fumigatus*, *C. tropicana*, and

Fig. 3 Two-dimensional fluorescence (2D-FL) and three (3D-FL) spectra illustrate the interaction between HSA, quercetin, aqueous (positions *Aa*, *Ab*, *Ac*, and *Ad*), and ethyl acetate (positions *Ba*, *Bb*, *Bc*, and *Bd*) extracts of studied berries. a Change in the fluorescence intensity as a result of binding affinity with water extracts: HSA [*first line* from the *top* with FI of 890.21], HSA + WGOOSEB (*second line* from the *top* with FI=817.50), HSA + WCRAN (*third line*, FI=717.39), HSA + WBLUEB (*fourth line*, FI=709.75), HSA + WGOOSEB + QUE (*fifth line*, FI=635.24), HSA + WCRAN + QUE (*sixth line*, FI=560.83), and HSA + WBLUEB + QUE (seventh line, FI=518.96). *Aa*–*Ad* cross maps from the 3D-FL spectrum of HSA + WBLUEB, HSA + WBLUEB + QUE, HSA + WGOOSEB, and HSA + WGOOSEB + QUE. b Change in the fluorescence intensity as a result of binding affinity of HSA with ethyl acetate extracts: HSA [*first line* from the *top* with FI of 890.21], HSA + EtOAcGOOSEB (*second line*, FI= 834.70), HSA + EtOAcCRAN (*third line*, FI= 821.65), HSA + EtOAcBLUEB (*fourth line*, FI=811.70), HSA + EtOAcGOOSEB + QUE (*fifth line*, FI=724.76), HSA + EtOAcCRAN + QUE (*sixth line*, FI=713.41), and HSA + EtOAcBLUEB + QUE (*seventh line*, FI=618.96). *Ba*–*Bd* cross maps from the 3D-FL spectrum of HSA + EtOAcBLUEB, HSA + EtOAcBLUEB + QUE, HSA + EtOAcGOOSEB, and HSA + EtOAcGOOSEB + QUE. In all reactions, the following conditions were used: HSA (2.0×10^{-6} mol/L), quercetin (1.7×10^{-6} mol/L), and water and EtOAc extracts in concentration of 25 and 50 μg/ml, respectively. Binding was during 1 h at 25 °C. Fluorescence intensities are on *y*-axis and emission wavelengths are on *x*-axis. *HSA* human serum albumin, *QUE* quercetin, *EtOAc* ethyl acetate, *WGOOSEB* water extracts of gooseberry, *WCRAN* water extracts of cranberry, *WBLUEB* water extracts of blueberry, *EtOAcGOOSEB* ethyl acetate extracts of gooseberry, *EtOAcCRAN* ethyl acetate extracts of cranberry, *EtOAcBLUEB* ethyl acetate extracts of blueberry



C. albicans, respectively [41]. Our results correspond as well with Suwalsky et al. [19], who showed a new kind of Chilean berries, and the polyphenol aqueous extracts of leaves and whole fruit were responsible for the antioxidant properties when the extracts were induced to interact with human red cells. The results of the CUPRAC test showed that cranberry juice had the highest level of antioxidant reactivity, blueberry juice had an intermediate activity, and orange juice had the lowest. It was determined, however, that contrary to the hypothesis,

orange juice was significantly more potent in protecting the bladder against ischemia/reperfusion damage than either blueberry or cranberry juice. Thus, it is concluded that chemical tests for TAA do not necessarily correlate with their physiological activity [2]. The obtained antioxidant activity by FRAP of blueberry and cranberry extracts was similar to other studies. Probably, a complex spectrum of anthocyanins was the major contributor to the antioxidant activity [47].

Fluorometry Spectra Studies

The binding properties of the berry samples in comparison with the pure flavonoids such as quercetin are shown in 3D- FL spectra, which illustrated the elliptical shape of the cross map. The results showed that the 3D- FL cross maps of berries differed. One of the main peaks for HSA was found at λex/em of 220/360 nm. The second main peak appeared for these samples at λex/em of 280/350 nm (Fig. 3a, b). The interaction of HSA and the water and ethyl acetate extracts of berries (Fig. 3—Aa, Ac, Ba, and Bc), HSA, water, and ethyl acetate extracts, and quercetin (Fig. 3—Ab, Ad, Bb, and Bd) showed a slight change in the position of the main peak at the wavelength of 360 nm and a decrease in fluorescence intensity (FI). The following changes appeared when the water extracts of berries were added to HSA [initially the main peak at emission 360 nm and FI of 890.21] (Figs. 3a, b and 4a, b; the upper line is HSA). The reaction with the berry extracts and quercetin decreased the FI of HSA (Fig. 3a, b; middle and low lines). The following decrease in the FI (%) occurred during the interaction of water extracts with HSA: HSA+WGOOSEB=8, HSA+WCRAN=19.4, and HSA+WBLUEB= 37. The decrease in the FI with ethyl acetate extracts was lower than with water extract: HSA+EtOAcGOOSEB=6.0, HSA+EtOAcCRAN=7.7, and HSA+EtOAcBLUEB=8.2. The diethyl ether extracts did not show any binding properties with HSA. These results are in direct relationship with the antioxidant properties of the extracts. The synergism of bioactive compounds is shown when quercetin was added to the mixture of HSA and extracts of berries. The decrease in the FI of HSA with WGOOSEB, WCRAN, and WBLUEB was 28.6, 37.0, and 41.7, respectively (fifth, sixth, and seventh lines (Fig. 3a)). Therefore, the participation of quercetin in synergism was 20.6, 17.6, and 21.4 for WGOOSEB, WCRAN, and WBLUEB, respectively. With ethyl acetate extracts, the participation of quercetin was 13.9, 10.9, and 17.6 for GOOSEB, CRAN, and BLUEB, respectively (Fig. 3b).

The concentrations of water extracts of berries in the interaction with HSA are 3.01971, 5.12232, and 5.23493×10^{-8} QUE for GOOSEB, CRAN, and BLUEB, respectively. Ethyl acetate extracts showed lower concentrations at 2.5751, 2.90949, and 3.16139×10^{-8} for GOOSEB, CRAN, and BLUEB, respectively. Our very recent results showed that the fluorescence is significantly quenched because the conformation of the HSA changes in the presence of pure flavonoids and berry extracts. This interaction between quercetin and HSA was investigated using tryptophan fluorescence quenching. Our result is in agreement with others that quercetin, as an aglycon, is more hydrophobic and demonstrates strong affinity toward HSA. Other results [20, 21] differ from those reported by us, probably because of the variety of antioxidant abilities of pure flavonoids and different ranges of fluorometry scanning ranges used in a similar study. The biological relevance of quercetin interaction in human organism is important from the point of view that this molecule of polyphenolic type extensively binds to HSA, the most abundant carrier protein in the blood. Our in vitro results of interaction of HSA and quercetin can be compared with other reports in vivo, showing the protective effects of quercetin on hepatic injury induced by different chemical reactions. Our results on BSA binding with other types of berry correspond with our present results with HSA and investigated berries. Results on water extracts of blueberries were similar to these samples

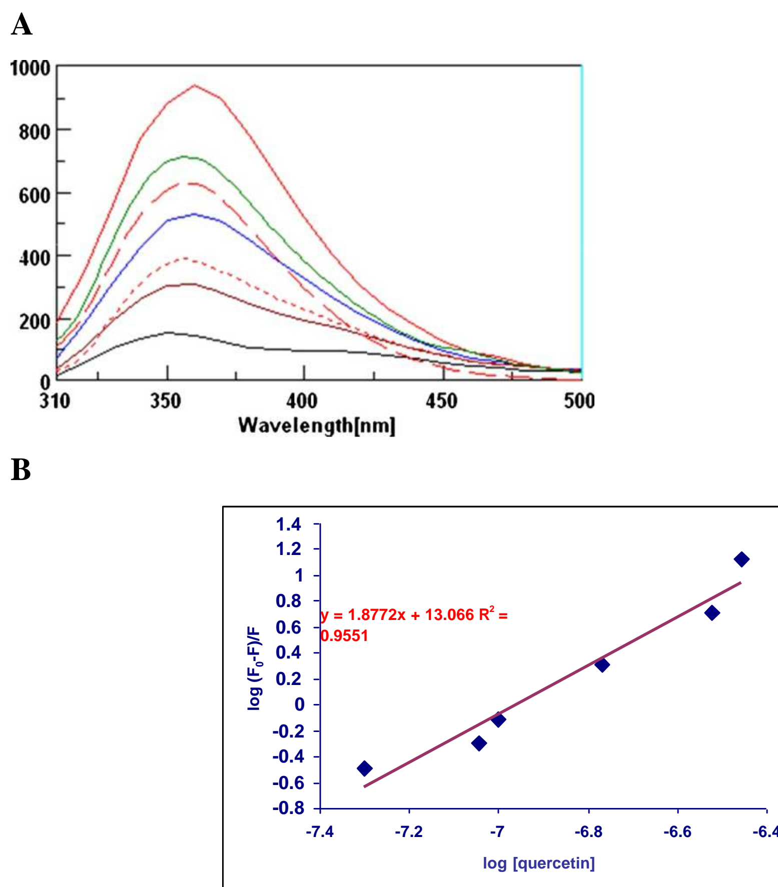


Fig. 4 a Fluorescence spectra of aqueous solutions of HSA (2.0×10^{-6} mol/L) in the presence of different concentrations of quercetin: 0, 0.17, 0.30, 1.0, and 1.7×10^{-6}) mol/L at pH 7.4 at excitation wavelength of 290 nm. b Linear plot for log $(F_0-F)/F$ vs log [quercetin], where F_0 and F represent the fluorescence intensity of HSA in the absence and in the presence of polyphenols, respectively

[48, 49]. Strong binding properties have been confirmed for the compounds containing high bioactivity. The strong binding properties of phenolics show that they may be effective in the prevention of atherosclerosis under physiological conditions. Quercetin can suppress HSA. These results demonstrate that quercetin and other phenolic compounds can effectively protect from atherosclerosis under physiologically relevant conditions, providing insight into the mechanism of action of bioactive phenolics. Our explanation of the binding affinity of berry polyphenols is similar to the description of Xiao et al. [20] and Xiao and Kai [21] that one or more hydroxyl groups in the B-ring of flavonoids enhanced the binding affinities to proteins. Much of the bioactivities of citrus flavanones significantly appear to impact blood and microvascular endothelial cells [50]; therefore, it was essential to investigate the interaction between berry polyphenols and serum albumin. The binding constants ranked in the following order: quercetin>rutin>calycosin>calycosin-7-*O*-(sup)-*D*-glucoside [formononetin-7-*O*-(sup)-*D*-glucoside [51]. 3D fluorescence can be used as an additional tool for the characterization of the polyphenol extracts of berry cultivars and their binding properties.

Conclusion

There are many reports on the antioxidant properties of berries; however, there is little information about the binding properties of blueberries and cranberries and even less information about gooseberries. The gooseberry, in comparison with cranberries and blueberries, showed a lower amount of bioactive compounds. Therefore, some of the methods used in this work such as fluorescence were done for the first time. Some of the active compounds may have synergistic interactions with other compounds as was shown when quercetin was added to the reaction. This work demonstrated relatively high antioxidant and binding properties of the investigated berries, especially in water extracts. The possibility of benefit of the consumption of these berries for everyday human health can be suggested.

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Conflict of Interest The authors declare that there is no conflict of interest.

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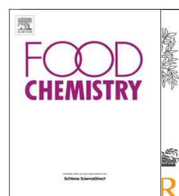

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Bioactive compounds and the antioxidant capacity in new kiwi fruit cultivars

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The aim of this investigation was to find the best among seven different kiwi fruit cultivars ('Hayward', 'Daheung', 'Haenam', 'Bidan', 'Hort16A', 'Hwamei' and 'SKK12') for human consumption and to classify them as groups. Therefore, the contents of bioactive compounds and the level of antioxidant capacities of these cultivars were determined in four different extracts and compared. It was found that the contents of the bioactive compounds and the level of antioxidant capacities in different extracts differ significantly ($P < 0.05$). Bioactive compounds and the antioxidant capacities were significantly higher in 'Bidan' and 'SKK12' cultivars than in other studied samples. The ethanol and water extracts of these cultivars exhibited high binding properties with human serum albumin (HSA) in comparison with catechin. In conclusion, based on fluorescence profiles the seven new kiwi fruit cultivars can be classified for three groups: 'Hayward' (including 'Daheung', 'Haenam', Hwamei' and 'SKK12'), 'Bidan' and 'Hort16A'. In MS – profiles some differences in the peaks were found between the cultivar groups. All studied fruits could be a valuable addition to known disease preventing diets.

Introduction

Nowadays some authors recommend consumption of fruits with high bioactivity (Proteggente et al., 2002; Sun, Chu, Wu, & Liu, 2002), because only such fruits are effective in prevention and treatment of various diseases (Lansky, & Newman, 2007; Larson, Neumark-Sztainer, Hannan, & Story, 2007; Lindeberg et al., 2007; Duttaroy & Jørgensen, 2004). Most of the used fruits have many cultivars (Fukuda, Suezawa, & Katagiri, 2007; Toledo et al., 2008; Wall et al., 2008). It was shown that even cultivars grown in the same geographic and climatic conditions differ significantly (Ercisli, Ozdemir, Sengul, Orhan, & Gungor, 2007; Toledo et al., 2008). So, Toledo et al. (2008) studied the bioactivity of durian cultivars such as Mon Thong, Chani, Kan Yao, Pung Manee and Kradum at the same stage of ripening from the same geographic region grown in the same climatic conditions in order to find the best among them for human consumption. It was concluded that among the studied durian cultivar Mon Thong is preferable (Haruenkit et al., 2010). Widely consumed kiwi fruit has many cultivars (Ercisli et al., 2007). Which of them is preferable for human consumption? In order to answer this question it was decided to investigate seven kiwi fruit cultivars ('Hayward', 'Daheung', 'Haenam', 'Bidan', 'Hort16A', 'Hwamei' and 'SKK12') and to divide them to groups. The content of the bioactive compounds and the level of antioxidant capacity (AC) were determined and compared. In order to receive reliable data the AC was determined by four complementary assays: ABTS, DPPH, FRAP and CUPRAC and the mass-spectra profile. Human serum albumin is the drug carrier's protein and serves to greatly amplify the capacity of plasma for transporting drugs. It is interesting to investigate *in vitro* how this protein interacts with polyphenols extracted from kiwi fruit samples in order to get useful information of the properties of polyphenol–protein complex. Therefore the functional properties

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of new kiwi fruit cultivars were studied by the interaction of ethanol and water polyphenol extracts with a small protein such as HSA, using 3D-FL.

As far as we know not results of such investigations were published.

- Material and methods

- *Chemicals*

Trolox (6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid); 2,2^0 -azobis-2-methyl-propanimidamide; 1,1-diphenyl-2-picrylhydrazyl (DPPH), $FeCl_3 \times 6H_2O$; Folin–Ciocalteu reagent (FCR); *Tris, tris(hydroxymethy1)aminomethane;* lanthanum (III) chloride heptahydrate; $CuCl_2 \times 2H_2O$; and 2,9-dimethyl-1,10-phenanthroline (neocuproine), potassium persulfate, quercetin, human serum albumin, were obtained from Sigma Chemical Co., St. Louis, MO, USA. 2, 4, 6-tripyridyl-s-triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland. All reagents were of analytical grade. Deionised and distilled water were used throughout.

- *Samples*

Kiwi fruits of seven cultivars were harvested at the optimal stage in orchard, located in Haenam county (longitude 126° 15^{00} and latitude 34° 18^{00}), Jeonnam province, Korea, 2012. All cultivars, except 'Hort 16A', are were bred in Korea and classified as 'Hort'. 'Hort 16A' is a New Zealand gold kiwi fruit and was purchased in 2012 from farmer, located in Jeju Island. 'Hwaemi' and 'SKK-12' are green kiwi fruit cultivars of 100 g size as 'Hayward'. 'Bidan' has a smaller size of 20 g and its skin is white (flesh is green). The peeled fruits were weighed, chopped and homogenised under liquid nitrogen in a high-speed blender (Hamilton Beach Silex professional model) for 1 min. A weighed portion (50–100 g) was then lyophilized for 48 h (Virtis model 10–324), and the dry weight was determined. The samples were ground to pass through a 0.5 mm sieve and stored at -20 °C until the bioactive substances were analysed.

- *Determination of bioactive compounds and antioxidant capacity*

The lyophilized samples of kiwi fruit cultivars were extracted with ethanol, water, acetone and hexane at room temperature. The extracts were filtered in a Buchner funnel. After removal of the solvents in a rotary evaporator at a temperature below 40 °C, and the aqueous solution was freeze-dried. The polyphenols were determined by Folin–Ciocalteu method with measurement at 750 nm with spectrophotometer (Hewlett–Packard, model 8452A, Rockvile, USA). The results were expressed as mg of gallic acid equivalents (GAE) per g DW (Singleton, Orthofer, & Lamuela-Raventos, 1999). The extracts of condensed tannins (procyanidins) with 4% methanol vanillin solution were measured at 500 nm. Flavonoids, extracted with 5% $NaNO_2$, 10% $AlCl_3 \times 6H_2O$ and 1 M NaOH, were measured at 510 nm (Bener, Özyürek, Güçlü, & Apak, 2010). The total flavanols amount was estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method, and then the absorbance at 640 nm was read. To ensure the presence of flavanols on the nuclei, subsequent staining with the DMACA reagent resulted in an intense blue coloration in plant extract (Feucht & Polster, 2001). As it was mentioned previously, (+)-catechin served as a standard for flavonoids and flavanols, and the results were expressed as catechin equivalents (CE).

The AC was determined by the following assays:

- 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) method for the screening of antioxidant capacity is reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants. The influences of both the concentra- tion of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant capacity. ABTS radical cation was generated by the interaction of ABTS (7 mM/L) and $K_2S_2O_8$ (2.45 mM/L). This solution was diluted with metha- nol until the absorbance in the samples reached 0.7 at 734 nm (Re et al., 1999).
- Cupric reducing antioxidant capacity (CUPRAC): This assay is based on utilising the copper (II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidising agent. To the mixture of 1 ml of copper (II)-neocuproine and NH_4Ac buffer solution, acidified and non acidified methanol extracts of fruits (or standard) solution (x, in ml) and H_2O [(1.1-x) ml] were added to make the final volume of 4.1 ml. The absorbance at 450 nm was recorded against a reagent blank (Apak, Guclu, Ozyurek, & Karademir, 2004).
- Scavenging free radical potentials were tested in solution of 1, 1-Diphenyl-2-picrylhydrazyl (DPPH). In its radical form, DPPH has an absorption band at 515 nm which disappears upon reduction by an antiradical compounds. DPPH solution (3.9 mL, 25 mg/L) in methanol was mixed with the samples extracts (0.1 mL), then the reaction progress was monitored at 515 nm until the absorbance was stable (Brand-WIlliams, Cuvelier, & Berset, 1995).
- Ferric-reducing/antioxidant power (FRAP) assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripiridyltriazine [Fe (III)-TPTZ] to a ferrous form [(Fe (II)]. FRAP reagent (2.5 mL of a 10 mmol ferric-tripiridyltriazine solution in 40 mmol HCl plus 2.5 mL of 20 mmol $FeCl_3xH_2O$ and 25 mL of 0.3 mol/L acetate buffer,

pH 3.6) of 900]L was mixed with 90]L of distilled water and 30]L of kiwi fruit extract samples as the appropriate reagent blank. The absorbance was measured at 595 nm (Benzie & Strain, 1996).

- *Fluorometric measurements*

Fluorometric measurements were used for the evaluation of binding properties of kiwi fruit extracts to human serum albumin. Two dimensional (2D-FL) and three dimensional (3D-FL) fluorescence measurements for all kiwi fruit extracts at a concentration of 0.01 mg/mL were recorded on a model FP-6500, Jasco spectrofluorometer, serial N261332, Japan, equipped with 1.0 cm quartz cells and a thermostat bath. The 2D-FL was taken at emission wavelengths from 310 to 500 nm; and at excitation of 295 nm. The 3D-FL spectra were collected with subsequent scanning emission spectra from 250 to 500 nm at 1.0 nm increments by varying the excitation wavelength from 200 to 350 nm at 10 nm increments. Catechin was used as a standard. All solutions for protein interaction were prepared in 0.05 mol/l Tris–HCl buffer (pH 7.4), containing 0.1 mol/l NaCl. The final concentration of HSA was 2.0×10^{-6} mol/l. The HSA was mixed with quercetin in the proportions of HSA:extract = 1:1.

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- *MS analysis*

In order to compare the extracted phenolics in additional to the used solvents 50% methanol in water acidified with 1% formic acid; and 50% methanol in water were used. Different extractions were carried out in order to achieve the better phenols recovery using variable ratio of water and methanol, with and without formic acid in mass-spectra profiles (Fracassetti, Costa, Moulay, & Tomás-Barberán, 2013). These extracts were submitted to MS

analysis for determination of bioactive compounds (Sanz et al., 2010). A mass spectrometer, a TSQ Quantum Access Max (Thermo Fisher Scientific, Basel, Switzerland) was used. Analytes were ionised by electrospray ionoization (ESI) in negative mode. Vaporizer temperature was kept at 100 °C. All samples were done by direct infusion in the mass spectrometer by use ESI source at negative ion mode, full scan analysis, range of 100–900 m/z. For optimisation of the acquisition parameters and for identity confirmation only a part of standards was employed, not for all compounds that were found in the investigated samples. Settings for the ion source were as follows: spray voltage 3000 V, sheath gas pressure 35 AU; ion sweep gas pressure 0 AU; auxiliary gas pressure at 30 AU; capillary temperature at 200 °C, skimmer offset 0 V (Gómez-Romero et al., 2011; Mikulic-Petkovsek, Slatnar, Stampar, & Veberic, 2012).

- *Statistical analyses*

To verify the statistical significance, mean ± SD of five independent measurements were calculated. Differences between groups were tested by two ways ANOVA. In the assessment of the antioxidant capacity, Spearman correlation coefficients (R) were used. Linear regressions were also calculated. P-values of <0.05 were considered significant.

· Results and discussion

- *Polyphenols, flavonoids, flavanols and tannins*

The combination of determination of bioactive compounds as total phenols, total flavonoids, total flavanols and tannins, determined spectroscopically, and with antioxidant assays, fluorescence and mass spectra can be used in comparison and fingerprinting analysis of new kiwi fruit cultivars. These methods can be used for rapid distinguishing of the cultivars.

The results of the determination of the contents of these bioac- tive compounds in all seven studied kiwi fruits cultivars are shown in the Table 1. As can be seen, the contents of polyphenols in ethanol and water extracts were significantly higher than in acetone and hexane extracts (P in all cases < 0.05). The contents of flavonoids in ethanol extract were significantly higher in 'Haenam' and 'Bidan', in water extracts – in 'SKK12' and 'Hwamei', in acetone and hexane extracts – in 'Bidan' (P in all cases < 0.05). The contents of flavanols in ethanol and water extracts were significantly higher in 'Haenam', and 'Bidan', in acetone and hexane extracts – in 'Haenam' (P in all cases < 0.05). The contents of tannins in ethanol extracts were significantly higher in 'SKK12', in water and acetone extracts – in 'Bidan', and in hexane extracts – in 'SKK12' (P in all cases < 0.05). As can be seen, the contents of the bioactive compounds extracted by different solvents differ significantly: the content of the main bioactive compound – polyphenols was significantly higher in 'SKK12', 'Hwamei' and 'Bidan' (P < 0.05).

- *Antioxidant capacity*

The results of the determination of the level of antioxidant capacity of seven studied kiwi fruit cultivars are shown in the Table 2. As can be seen: (a) according to all assays the significantly highest level of AC in all extracts was in 'SKK12', following by 'Hwamei' and 'Bidan' (P < 0.05). ABTS and CUPRAC are two electron transfer assays and therefore the obtained results are similar. As can be seen, according to all four used assays, the significantly highest level of antioxidant capacity was registered in 'Bidan', 'SKK12' and 'Hwamei' cultivars (P < 0.05). As was shown above, these cultivars have also the highest content of polyphenols among studied cultivars (Table 1).

- *Fluorometric data*

The 3D-FL of kiwi fruit cultivars ethanol extracts differ by the wavelengths of the peaks and their fluorescence intensity (FI), and could be classified according to the fluorescence results to three groups 'Hayward'(including 'Daheung', 'Haenam', Hwamei' and 'SKK12'), 'Bidan' and 'Hort 16A'. The following common peaks appeared in three groups: at ƙex/em of 290/220, 400/230 and 600/210 nm. 'Hort 16A' showed one big peak at 400/300 nm, which was not found in any of cultivars. 'Hwamei', which is similar to 'Hayward' showed one peak at 300/280, characteristic only for this cultivar. At ƙex/em of 700/400 nm the biggest prominent peak was in 'Bidan' cultivar, decreasing for 'Hayward', 'Hwamei' and' Hort 16A' (Fig. 1C, B, D and A, respectively). The binding properties of the kiwi fruit samples in comparison with the pure flavonoids such as catechin are shown in two-dimensional fluorescence spectra (2D-FL). One of the main peaks for HSA was found at ƙex/em of 220/357 nm (Fig. 1E). The interaction of HSA and the ethanol extracts of kiwi fruit cultivars (Fig. 1E) showed slight change in the position of the main peak at the wavelength of 357 nm and the decrease in the fluorescence intensity (FI). The following changes appeared when the ethanol extracts of kiwi fruit were added to HSA [initially the main peak at emission 357 nm and FI of 961.00 (Fig. 1E, the upper line is HSA). The reaction with the kiwi fruit extracts and catechin decreased the FI of HSA (Fig. 1E, the lowest line). The following decrease in the FI (%) occurred during the interaction of ethanol extracts with HSA: HSA + 'Hayward' = 3.86; HSA + 'Haenam' = 6.71; HSA + 'Hort 16A' = 7.63; HSA + 'Bidan' = 10.18; HSA + 'Bidan' = 12.03; HSA + 'Hwamei' = 15.05; HSA + 'SKK 12' = 11.65; HSA + catechin = 15.41. The water extracts showed the results of the decrease (%) of HSA intensity (Fig. 1F): HSA + 'Hayward' = 2.03; HSA+'Hort 16A' = 10.79; HSA + 'Bidan' = 15.47; HSA + catechin = 15.89; HSA + 'Hwamei' = 18.76; HSA + 'SKK 12' = 21.24. These data were slightly higher than with ethanol extracts and such strong binding properties of water extracts are proportional to their amount of polyphenols (Table 1). These results were in direct relationship with the antioxidant capacities of the extracts (Table 2). The synergism of bioactive compounds is shown when to the mixture of HSA and extracts of kiwi fruit catechin was added. Our very recent results showed that the fluorescence is significantly quenched, because of the conformation of proteins, phenolic acids and flavonoids (Namiesnik et al., 2013). This interaction was investigated using tryptophan fluorescence quenching. Our result is in agreement with others that quercetin, as an aglycon, is more hydrophobic and demonstrates strong affinity toward HSA. Other results (Xiao, Chen, Cao, Chen, & Yang, 2011) differ from the reported by us, probably because of the variety of antioxidant abilities of pure flavonoids and different ranges of fluorometry scanning ranges used in a similar study. The strong binding properties of phenolics show that they may be effective in prevention of atherosclerosis under physiological conditions. Quercetin can suppress HSA. Much of the bioactivities of citrus flavanones significantly appear to impact blood and microvascular endothelial cells, therefore it was essential to investigate the interaction between kiwi fruit polyphenols and serum albumin. The binding constants ranked in the following order querce- tin > rutin > calycosin > calycosin-7-O-(sup)-D-glucoside [formononetin-7-O-(sup)-D-glucoside (Liu et al., 2010). 3-D fluorescence can be used as an additional tool for the characterisation of the polyphenol extracts of kiwi fruit cultivars and their binding properties.

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- *MS spectra*

The ESI-MS in negative ion mode of studied extracts slightly dif- fer between cultivars. As it was shown previously the cultivars were

Table 1
Bioactive compounds of seven kiwi fruit cultivars in ethanol (Et), water (W), acetone (Ac) and hexane (He) extracts.[1,2,3]

	POL (mg GAE/g)	FLAVON (mg CE/g)	FLAV (lg CE/g)	TAN (mg CE/g)
HaywardEt	4.48 ± 0.44^{a}	1.22 ± 0.12^{a}	37.84 ± 3.67^{de}	2.84 ± 0.26^{c}
Daheung Et	4.18 ± 0.40^{a}	0.99 ± 0.11^{a}	5.82 ± 0.56^{a}	1.63 ± 0.16^{a}
HaenamEt	6.82 ± 0.55^{b}	4.25 ± 0.41^{c}	42.96 ± 0.45^{e}	2.85 ± 0.21^{c}
BidanEt	11.45 ± 1.12^{c}	4.32 ± 0.38^{c}	15.80 ± 1.51^{c}	2.48 ± 0.23^{b}
Hort16AEt	10.23 ± 1.07^{c}	1.23 ± 0.09^{a}	31.88 ± 3.21^{d}	2.88 ± 0.28^{c}
SKK12Et	14.48 ± 1.46^{d}	2.39 ± 0.21^{b}	10.53 ± 1.07^{b}	3.01 ± 0.28^{c}
HwameiEt	13.11 ± 1.29^{cd}	2.23 ± 0.21^{b}	9.46 ± 0.98^{b}	2.81 ± 0.27^{c}
HaywardW	5.30 ± 0.45^{a}	0.57 ± 0.12^{a}	16.35 ± 1.65^{b}	1.17 ± 0.14^{a}
DaheungW	5.50 ± 0.54^{a}	0.55 ± 0.06^{a}	7.90 ± 0.78^{a}	1.57 ± 0.14^{b}
HaenamW	7.69 ± 0.69^{b}	0.70 ± 0.09^{b}	8.87 ± 0.88^{a}	1.17 ± 0.11^{a}
BidanW	13.97 ± 1.32^{d}	1.00 ± 0.11^{b}	39.92 ± 3.83^{d}	3.04 ± 033^{d}
Hort16AW	11.08 ± 1.14^{c}	1.37 ± 0.13^{c}	8.59 ± 0.81^{a}	2.37 ± 2.24^{c}
SKK12 W	16.34 ± 1.11^{e}	1.75 ± 0.07^{d}	19.68 ± 1.94^{c}	1.60 ± 0.03^{b}
HwameiW	14.23 ± 1.39^{d}	1.62 ± 0.11^{d}	14.47 ± 1.44^{ab}	2.50 ± 0.15^{c}
HaywardAc	1.15 ± 0.05^{a}	0.61 ± 0.07^{b}	18.91 ± 1.87^{e}	1.42 ± 0.18^{c}
DaheungAc	0.84 ± 0.07^{a}	0.48 ± 0.06^{a}	2.98 ± 0.27^{a}	0.82 ± 0.09^{a}
HaenamAc	1.82 ± 0.04^{b}	2.11 ± 0.24^{d}	21.43 ± 2.32^{f}	1.43 ± 0.16^{c}
BidanAc	3.39 ± 0.33^{d}	2.17 ± 0.22^{d}	7.84 ± 0.78^{c}	1.25 ± 0.13^{b}
Hort16AAc	2.74 ± 0.21^{c}	0.62 ± 0.08^{b}	15.91 ± 1.58^{d}	1.44 ± 0.15^{c}
SKK12Ac	5.11 ± 0.52^{e}	1.21 ± 0.23^{c}	5.24 ± 0.51^{b}	1.51 ± 0.16^{c}
HwameiAc	4.85 ± 0.48^{e}	1.12 ± 0.12^{c}	4.71 ± 0.47^{b}	1.45 ± 0.14^{c}
HaywardHe	0.49 ± 0.03^{a}	0.42 ± 0.07^{a}	12.63 ± 1.32^{d}	0.95 ± 0.9^{b}
DaheungHe	0.31 ± 0.04^{a}	0.32 ± 0.03^{a}	1.97 ± 0.19^{a}	0.55 ± 1.2^{a}
HaenamHe	1.15 ± 0.13^{b}	1.43 ± 0.16^{c}	14.31 ± 1.34^{e}	0.95 ± 0.7^{b}
BidanHe	2.07 ± 0.25^{c}	1.45 ± 0.14^{c}	5.26 ± 0.52^{c}	0.83 ± 0.6^{b}
Hort16AHe	1.67 ± 0.14^{bc}	0.41 ± 0.04^{a}	10.63 ± 1.13^{cd}	0.96 ± 0.5^{b}
SKK12He	3.42 ± 0.33^{d}	0.81 ± 0.08^{b}	3.49 ± 0.32^{b}	1.03 ± 0.09^{b}
HwameiHe	3.04 ± 0.33^{d}	0.75 ± 0.07^{b}	3.14 ± 0.31^{b}	0.97 ± 0.09^{b}

POL, polyphenols; FLAVON, flavonoids; FLAV, flavanols; TAN, tannins; CE, catechin equivalent; GAE, gallic acid equivalent; HaywardEt, DaheungEt, HaenamEt, HwameiEt, Hort16AEt, SKK12Et and BidanEt, kiwi fruit cultivars extracted with 100% ethanol; HaywardW, DaheungW, HaenamW, HwameiW, Hort16AW, SKK12W and BidanW, kiwi fruit cultivars extracted with water; HaywardAc, DaheungAc, HaenamAc, HwameiAc, Hort16AAc, SKK12Ac and BidanAs, kiwi fruit cultivars extracted with acetone; HaywardHe, DaheungHe, HaenamHe, HwameiHe, Hort16He, SKK12He and BidanHe, kiwi fruit cultivars extracted with hexane.

[1] Values are means ± SD of 5 measurements.

[2] Values in columns for every bioactive compound with the same solvent bearing different superscript letters are significantly different ($P < 0.05$).

[3] Per g dry weight.

Table 2
The antioxidant capacities of seven kiwi fruit cultivars (lmolTE/g DW) in ethanol[A], water[B], acetone[C], and hexane[D] extracts.[1,2,3]

	Hayward	Daheung	Haenam	Bidan	Hort 16A	SKK12	Hwamei
ABTS[A]	18.21 ± 1.65^{a}	17.42 ± 1.65^{a}	22.43 ± 2.18^{a}	34.25 ± 3.23^{c}	31.15 ± 3.11^{b}	37.18 ± 3.65^{c}	33.25 ± 3.31^{b}
ABTS[B]	20.41 ± 2.11^{a}	22.40 ± 2.23^{a}	26.18 ± 2.43^{a}	39.16 ± 3.87^{c}	34.12 ± 3.41^{b}	42.14 ± 4.32^{d}	39.35 ± 3.87^{c}
ABTS[C]	4.82 ± 0.45^{a}	4.05 ± 0.42^{a}	5.42 ± 0.52^{a}	12.41 ± 1.24^{ab}	11.12 ± 1.11^{ab}	14.15 ± 1.43^{b}	13.16 ± 1.31^{b}
ABTS[D]	1.61 ± 0.15^{a}	1.42 ± 0.14^{a}	1.83 ± 0.18^{a}	4.23 ± 0.41^{b}	4.11 ± 0.41^{b}	4.83 ± 0.48^{b}	4.52 ± 0.45^{b}
CUPRAC[A]	20.18 ± 2.04^{a}	19.44 ± 1.87^{a}	24.12 ± 2.32^{ab}	35.42 ± 3.23^{bc}	32.14 ± 2.16^{b}	38.15 ± 3.87^{c}	34.18 ± 3.21^{bc}
CUPRAC[B]	21.14 ± 2.11^{a}	23.40 ± 1.87^{a}	27.41 ± 2.12^{b}	40.18 ± 3.23^{d}	35.61 ± 2.76^{c}	43.27 ± 3.23^{d}	40.91 ± 3.45^{d}
CUPRAC[C]	4.01 ± 0.32^{a}	4.94 ± 0.27^{a}	6.12 ± 0.54^{ab}	13.13 ± 1.21^{c}	12.43 ± 0.85^{b}	15.25 ± 1.32^{d}	14.21 ± 1.34^{c}
CUPRAC[D]	1.51 ± 0.13^{a}	1.38 ± 0.11^{a}	1.73 ± 0.14^{ab}	4.11 ± 0.41^{bc}	3.85 ± 0.34^{b}	4.63 ± 0.43^{c}	4.41 ± 0.27^{bc}
FRAP[A]	6.12 ± 0.56^{a}	5.42 ± 0.54^{a}	10.21 ± 1.01^{ab}	18.44 ± 1.76^{c}	11.25 ± 1.12^{b}	21.15 ± 1.71^{c}	20.14 ± 1.98^{c}
FRAP[B]	7.12 ± 0.65^{a}	7.88 ± 0.67^{a}	11.33 ± 1.08^{ab}	21.32 ± 1.78^{c}	13.12 ± 1.31^{b}	24.55 ± 2.18^{c}	23.11 ± 2.11^{c}
FRAP[C]	1.58 ± 0.15^{a}	1.15 ± 0.09^{a}	2.43 ± 0.18^{ab}	4.75 ± 0.28^{c}	3.81 ± 0.32^{b}	5.36 ± 0.43^{c}	5.05 ± 0.41^{c}
FRAP[D]	0.53 ± 0.04^{a}	0.48 ± 0.03^{a}	0.81 ± 0.07^{ac}	1.65 ± 0.09^{c}	1.31 ± 0.12^{b}	1.98 ± 0.11^{d}	1.79 ± 0.14^{d}
DPPH[A]	6.95 ± 0.54^{a}	5.80 ± 0.45^{a}	7.65 ± 0.45^{ab}	14.41 ± 1.34^{c}	11.18 ± 1.13^{b}	17.23 ± 1.43^{d}	15.42 ± 1.28^{c}
DPPH[B]	6.08 ± 0.56^{a}	6.90 ± 0.43^{a}	9.14 ± 0.41^{ab}	17.15 ± 1.54^{c}	13.24 ± 1.43^{b}	18.42 ± 1.67^{d}	17.85 ± 1.87^{c}
DPPH[C]	1.75 ± 0.17^{a}	1.41 ± 0.12^{a}	2.18 ± 0.15^{ab}	4.15 ± 0.32^{c}	3.18 ± 0.23^{b}	4.87 ± 0.28^{c}	4.37 ± 0.32^{c}
DPPH[D]	0.65 ± 0.07^{a}	0.52 ± 0.05^{a}	0.74 ± 0.08^{ab}	1.48 ± 0.12^{c}	1.21 ± 0.09^{b}	2.03 ± 0.04^{d}	1.74 ± 0.06^{c}

[1]Values are means ± SD of 5 measurements; [2]Values in columns for kiwi fruits with the same solvent bearing different superscript letters are significantly different ($P < 0.05$); [3]per g dry weight. Cupric reducing antioxidant capacity (CUPRAC), 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) Ferric-reducing/antioxidant power (FRAP).

[A,B] Extracted at room temperature in concentration of 25 mg lyophilized sample in 1 mL ethanol, 1 mL water, respectively.

[C] Extracted at room temperature in concentration of 40 mg lyophilized sample in 1 ml acetone.

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[D] Hexane.

[1] Values are means ± SD of 5 measurements.

[2] Values in rows with different superscript letters are significantly different ($P < 0.05$).

[3] Per g dry weight.

classified according to fluorometric measurements to three groups: 'Hayward' (including 'Daheung', 'Haenam', Hwamei' and 'SKK12'), 'Bidan' and 'Hort 16A'. There were done all the spectra analyses, but only these groups are presented in Fig. 2 and Table 3. In all cultivars the main peak was at m/z 190.97 (100%) corresponded to quinic acid (Table 3, Fig. 2), but small peaks differ from one group to another (Table 3). 'Hwamei' slightly differ in methanol extracts from the other four cultivars which belong to the 'Hayward' group (Table 3, Fig. 2B). MeOH/water/50/50 showed as well differences in these 3 groups (Table 3, Figs. 2B, F, J). 'Bidan' contained also the main peak with m/z 191(100%) with average peaks different from the first group such as 308.95 and 366.91 (Table 3). MeOH/water/

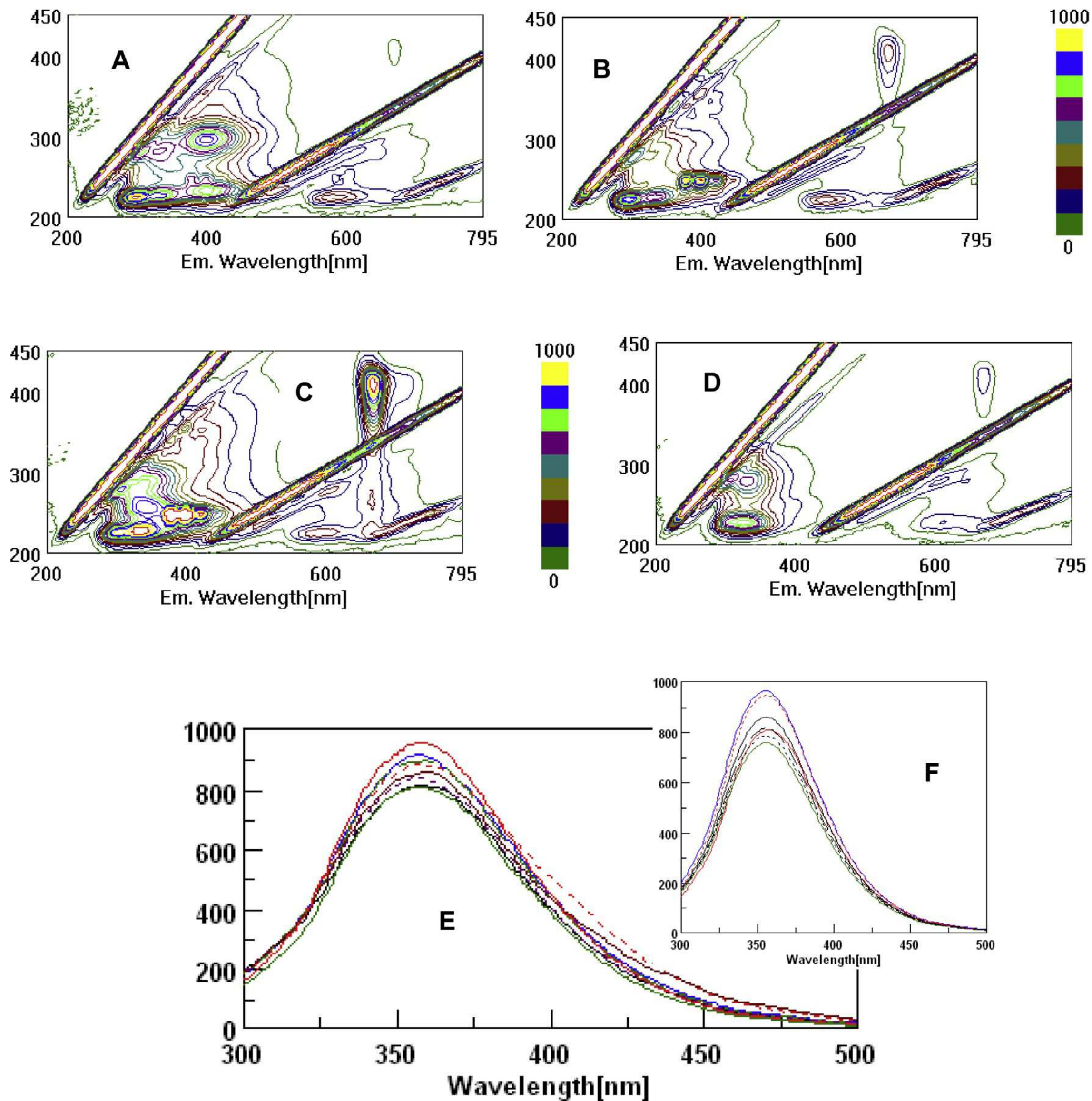

Fig. 1. Contour maps of three dimensional fluorescence (3D-FL) spectra of ethanol extracts of A, B, C, D, 'Hort16A' 'Hayward'; 'Bidan', D, and 'Hwamei'. 2D-FL spectrum illustrate the interaction between humam serum albumin (HSA), catechin, ethanol (E) and water (insert F) extracts of kiwi fruit cultivars. The change in the fluorescence intensity as a result of binding affinity with kiwi fruit extracts: E, HSA [first line from the top with fluorescence intensity (FI) of 961.00]; HSA + 'Hayward' (second line from the top with FI = 923.94), HSA + 'Haenam' (third line, FI = 896.54), HSA + 'Hort16A' (fourth line, FI = 887.66), HSA + 'Bidan' (fifth line, FI = 863.18), HSA + 'Hwamei' (sixth line, FI = 845.40), HSA + 'SKK12' (seventh line, FI = 816.41), HSA + catechin (eighth line, FI = 812.90). Insert F, HSA [first line from the top with fluorescence intensity (FI) of 967.64]; HSA + 'Hayward' (second line from the top with FI = 948.00), HSA + 'Hort16A' (third line, FI = 863.23), HSA + 'Bidan' (fourth line, FI = 817.90), HSA + catechin (fifth line, FI = 813.85), HSA + 'Hwamei' (sixth line, FI = 786.39), HSA + 'SKK12' (seventh line, FI = 762.12). In all reactions were used the following conditions: HSA (2.0×10^{-6} mol/L); catechin (1.7×10^{-6} mol/L); ethanol extracts in concentration of 50]g/mL. The binding was during 1 h at 25 °C. Fluorescence intensities are on *y*-axis and emission wavelengths – on *x*-axis.

formic acid/50%/49%/1% extracts were different and contained different peaks mostly in 'Hayward' and 'Bidan' groups of *m*/*z* 370.97 and 225.02, respectively (Table 3, Figs. G, K). Acetone fractions of the groups showed one main peak of m/z 191 with a number of small peaks with different masses (Table 3, Figs. 2D, H, L, P). As can be seen all kiwi fruit ethanol extracts characterised by chlorogenic acid of the [M-H] – deprotonated molecule (*m*/*z* 353) and the ion corresponding to the deprotonated quinic acid (*m*/*z* 191), which was consistent with Sun, Liang, Bin, Li, and Duan (2007). The recorded spectra were in the same scale (in the range between 100 and 600 *m*/*z*) for comparison. We choose negative mode for the MS method because in many publications was described that this mode is the best for analysis of low- molecular phenolic compounds (Gómez-Romero et al., 2011; Sun et al., 2007). The main peaks were identified and the recorded MS spectra can be used as a fingerprint for characterisation of different kiwi fruit cultivars, based on the percentage of the main peaks. The most abundant is chlorogenic acid. This is in agreement with Mittelstadt, Negron, Schofield, Marsh, and Parker (2013), who showed that one of the novel aspects of kiwifruit is the presence of a high level of quinic acid which contributes to the flavour of the fruit. Quinic acid metabolism intersects with the shikimate pathway, which is responsible for the de novo biosynthesis of primary and secondary aromatic metabolites. Our results are in accordance with Clifford (2000), Fiorentino et al. (2009) and Sârbu et al. (2012), where fingerprinting of kiwi fruit was suggested. Palafox-Carlos et al. (2012) showed the interactions of four major phenolic compounds (chlorogenic, gallic, protocatechuic and vanillic acid) found in 'Ataulfo' mango pulp. Significant synergism was found in the majority of the all combinations, as well as the combination of the four phenolics. Cultivars

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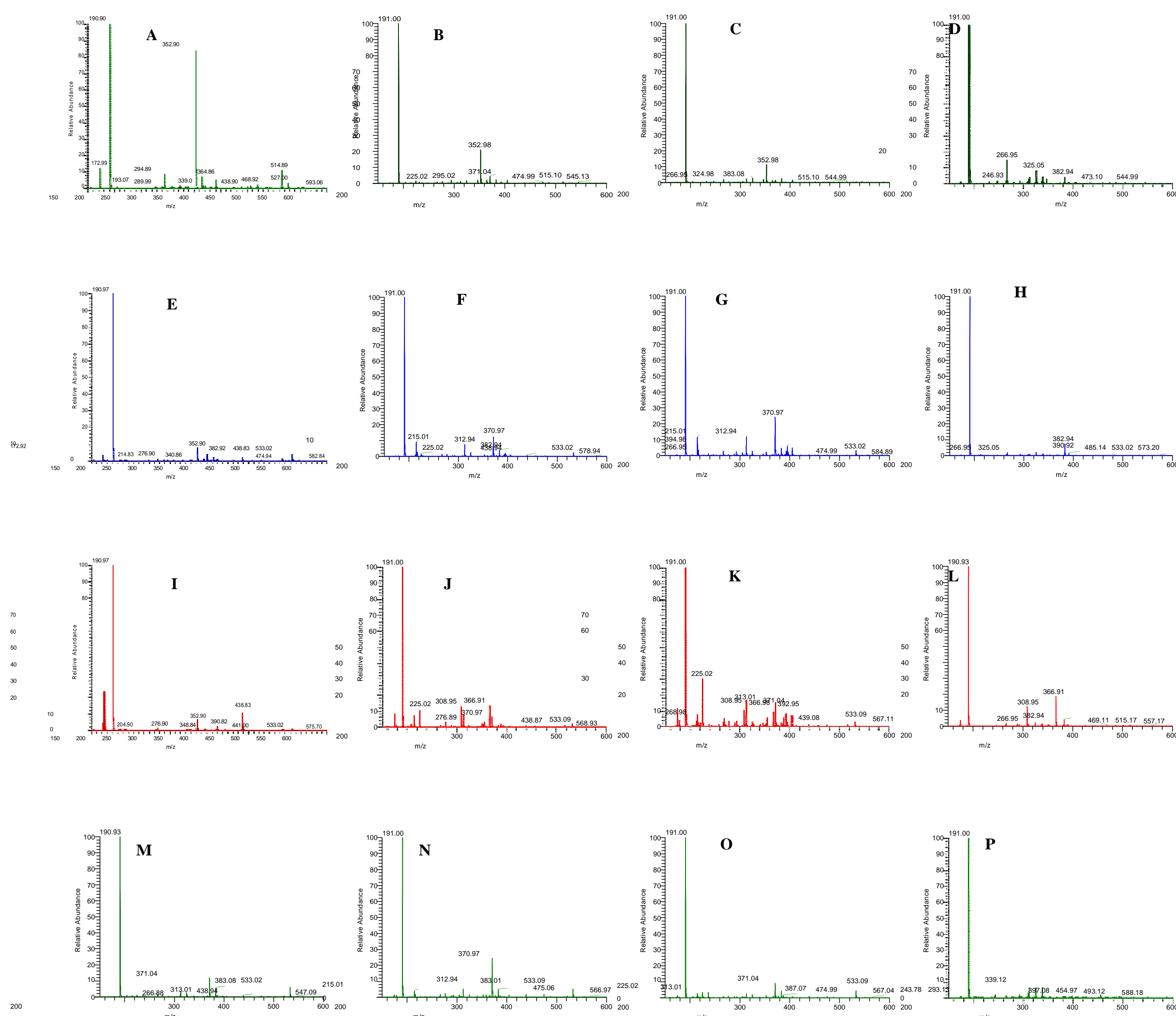

Fig. 2. ESI-MS spectra in negative ion mode of kiwi fruit cultivar groups extracts. A, B, C, D, EtOH, MeOH/water, MeOH/water/acid, acetone of 'Hort 16A'; E, F, G, H, EtOH, MeOH/water, MeOH/water/acid, acetone of 'Hayward'; I, J, K, L, EtOH, MeOH/water, MeOH/water/acid, acetone of 'Bidan'; M, N, O, P, EtOH, MeOH/water, MeOH/water/acid, acetone of 'Hwamei'.

of fruits and vegetables even grown in the same geographic and climatic conditions could different significantly and therefore, it must be taken into consideration (Koh, Wimalasiri, Chassy, & Mitchell, 2009; Toledo et al., 2008). Manolopoulou and Papadopoulou (1998), described such differences in kiwi fruit cultivars. However, in their study were investigated mainly respiratory and physicochemical changes of four kiwi fruit cultivars during cool-storage. Manolopoulou and Papadopoulou (1998) investigated only four cultivars: Allison, Bruno, Hayward and Monty harvested at the proper stage of maturity. They investigated respiration rates, production of ethylene, shelf-life. Among bioactive compounds only ascorbic acid content was measured. No changes in antioxidant activity were described. Therefore, it was decided to study seven well known kiwi fruit cultivars, determine and compare contents of main bioactive compounds and the level of the antioxidant capacity in order to find the best for human consumption. It must be underlined once again that these fruits were at the same stage of ripening and grown in the same geographic and climatic conditions. Therefore, no doubt, the determined data must be reliable. The results of present investigation show that all kiwi fruit cultivars contain high quantities of bioactive compounds. Also our previous data (Park et al., 2008) and of others (Amodio, Colelli, Hasey, & Kader, 2007; Jeong, Lee, Bae, & Choi, 2007; Tavarini, Degl'Innocenti, Remorini, Massai, & Guidi, 2008) are in agreement with our present results. However, the results are different for different cultivars (Castaldo, Lo Voi, Trifiro, & Gherardi, 1992; Du, Li, Ma, & Liang, 2009; Samadi-Maybodi, & Shariat, 2003). So, the contents of the main bioactive compound – polyphenols was significantly higher in 'SKK12', 'Bidan' and 'Hwamei' ($P < 0.05$). The obtained results depend on the year of collection and the extraction procedure, therefore our recent published results differ from the presently reported (Park et al., 2011). Also the significant highest level of antioxidant capacity and binding abilities were registered in the same cultivars: 'SKK12', 'Bidan' and 'Hwamei' ($P < 0.05$).

· Conclusions

Seven relatively new cultivars were divided to three groups mostly based on fluorometric measurements and supported by MS-spectra. The contents of bioactive compounds, antioxidant

Table 3
Mass spectral data (molecular ion and the major fragment ions of polyphenols extracted from kiwi fruit).

Extracts		[M–H–] and fragmentation in ESI, (% in MS)	Compound
Ethanol	Hort 16A	190.90(100)	Quinic acid
		352.9(85)	Caffeoylquinic acid
	Hayward	191.0(100)	Quinic acid
		352.9(10)	Caffeoylquinic acid
	Bidan	190.97(100)	Quinic acid
		352.9(10)	Caffeoylquinic acid
		438.83(15)	n-Triacontanol
	Hwamei	191.0(100)	Quinic acid
		371.04(12)	Sinensetin or tangeretin
Methanol:water (50:50)	Hort16A	191.0(100)	Quinic acid
		352.98(22)	Caffeoylquinic acid
	Hayward	191.0(100)	Quinic acid
		312.94(8)	Cirsimaritin
		370.97(11)	Sinensetin or tangeretin
	Bidan	191.0(100)	Quinic acid
		308.95(14)	Cinnamoyl glucose
		366.91(14)	Feruloylquinic acid
	Hwamei	191.0(100)	Quinic acid
		370.97(24)	Sinensetin or tangeretin
Methanol:water:acid	Hort16A	191.0(100)	Quinic acid
		352.98(12)	Caffeoylquinic acid
	Hayward	191.0(100)	Quinic acid
		215.01(14)	Bergapten
		312.94(14)	Cirsimaritin
		370.97(25)	Sinensetin or tangeretin
	Bidan	191.0(100)	Quinic acid
		225.02(30)	Uknown
		308.95(12)	Cinnamoyl glucose
		313.01(16)	Cirsimaritin
		371.04(15)	Sinensetin or tangeretin
	Hwamei	191.0(100)	Quinic acid
		371.04(10)	Sinensetin or tangeretin
Acetone	Hort16A	191.0(100)	Quinic acid
		266.95(15)	Genistin
	Hayward	191.0(100)	Quinic acid
		382.94(10)	Uknown
	Bidan	190.93(100)	Quinic acid
		308.95(15)	Cinnamoyl glucose
		366.91(18)	Feruloyquinic acid
	Hwamei	191.0(100)	Quinic acid
		339.12(8)	Phenylnaringenin

capacity and binding properties are significantly higher in SKK12', 'Bidan' and 'Hwamei' cultivars. The SKK12', 'Bidan' and 'Hwamei' and to less degree other four studied cultivars could be a valuable addition to known disease preventing diets.

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Original article

Comparative assessment of two extraction procedures for determination of bioactive compounds in some berries used for daily food consumption

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Summary Two extractions with methanol and water were used to determine the antioxidant and binding properties of some berries as a supplement to food. Fluorometry, FTIR spectra and radical scavenging assays were used for characterisation of bioactive compounds (polyphenols, flavonoids, flavanols and tannins) and the levels of their antioxidant activities (AAs). The contents of bioactive compounds and AAs in water and methanol polyphenol extracts in gooseberries, blueberries and cranberries differed, but not always signifi- cantly. Water extracts of gooseberries showed the lowest amounts of polyphenols (mg GAE g^{-1}),
6.24 ± 0.6, and flavonoids (mg CE g^{-1}), 0.29 ± 0.01, and AAs (lMTE g^{-1}) determined by DPPH,
FRAP, ABTS and CUPRAC assays such as 6.05 ± 0.6, 8.07 ± 0.9, 18.70 ± 1.8 and 13.44 ± 1.2, respec- tively, in comparison with blueberries and cranberries. Polyphenol content highly correlated with antioxi- dant activity (R^2 from 0.94 to 0.81). The quenching properties of berries were studied by the interaction of water and methanol polyphenol extracts with HSA by 3D fluorescence. In conclusion, the bioactivity of gooseberries was lower than in blueberries and cranberries. Gooseberries can be used as a new source for food consumption and supplementation based on their antioxidant and binding properties. 3D fluores- cence spectroscopy and FTIR spectroscopy can be applied as additional analytical tools for rapid estima- tion of the quality of different food products.

Keywords Antioxidant activity, berries, bioactive compounds, food consumption.

Introduction

Consumption of berries has become popular among health-conscious consumers due to the high levels of valuable antioxidants, such as phenolics, which include flavonoids, tannins, flavanols and phenolic acids (Wolfe *et al*., 2008 Battino *et al.*, 2009; El Gharras, 2009; Paredes-Lopez *et al.*, 2010; You *et al.*, 2011; Kang *et al.*, 2012). Recent studies *in vitro* and *in vivo* have improved the scientific understanding of how ber- ries promote human health and prevent chronic ill- nesses such as some cancers, heart and neurodegenerative diseases (Seeram, 2010). Adminis- tration of a freeze-dried powder of mulberry (*Morus alba* L.) fruit to rats on a high-fat diet resulted in a significant decline in levels of serum and liver triglycer- ide, total cholesterol and serum low- density lipoprotein cholesterol, and a decrease in the atherogenic index (Yang *et al.*, 2010). Oxidative stress and hypogonadism are linked to the increased inci- dence of cardiovascular disease (Deyhim *et al.*, 2007). Cranberry was investigated as a chemotherapeutic agent (Elberry *et al.*, 2010). The effect of particle size,

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use of infrared radiation and type of freeze-drying (vacuum or atmospheric) on some nutritional proper- ties of blueberries was investigated (Reyes *et al.*, 2011). The purpose of some studies was to investigate and compare the composition, stability and antioxidant properties of berry extracts from selected cultivars using some extraction methods (Chanda & Kaneria, 2012; Khoo *et al.*, 2012). *Physalis peruviana*, com- monly known as cape gooseberry, is an Andean Solan- aceae fruit with high nutritional value and interesting medicinal properties. *Physalis peruviana* has been used in folk medicine for its medicinal properties including anticancer, antimycobacterial, antipyretic, diuretic, immunomodulatory and anti-inflammatory properties (Franco *et al.*, 2007). Three species of Physalis fruit (*Physalis ixocarpa* Brot, *Physalis pruinosa* L. and *Phy- salis peruviana* L.) from Colombia, Egypt, Uganda and Madagascar were analysed by multivariate analysis (El Sheikha *et al.*, 2012). In our recent research, the meth- anol extracts from different berries were investigated and compared (Arancibia-Avila *et al.*, 2011). Bioactive compounds (polyphenols, flavonoids, flavanols, tan- nins, anthocyanins and ascorbic acid) and the level of antioxidant activity (AA) estimated by ABTS, DPPH, FRAP and CUPRAC assays of water, acetone and hexane extracts of Chilean 'Murtilla' and 'Myrteola' berries, Chilean and Polish blueberries, Chilean rasp- berries and Polish black chokeberry were determined and compared (Arancibia-Avila *et al.*, 2012). We were interested to investigate water and methanol extracts of relatively less known gooseberry and to compare its composition with the widely consumed berries. The water extracts of berries are important from the point of view of tea consumption. To meet this aim, the con- tents of bioactive compounds (polyphenols, flavonoids, flavanols and tannins) and the level of antioxidant activities (AAs) were determined and compared. To receive reliable data, the AA was determined by four assays: CUPRAC, ABTS, DPPH and FRAP (Brand-Williams *et al.*, 1995; Benzie & Strain, 1996; Re *et al.*, 1999; Apak *et al.*, 2004). To determine the fluorescence properties of the extracted bioactive com- pounds, *in vitro* studies were performed by interaction of proteins with flavonoids. Human serum albumin is the drug carrier protein and serves to greatly amplify the capacity of plasma to transport drugs. It was inter- esting to investigate *in vitro* how this protein interacts with flavonoids extracted from berry samples in order to obtain useful information about the properties of flavonoid–protein complex. Therefore, the functional properties of a new kind of berry were studied by the interaction of water and methanol polyphenol extracts with a small protein such as HSA (Zhang *et al.*, 2009). Therefore, the aim of this research was to evaluate the bioactivity of gooseberries in comparison with more consumed ones such as blueberries and cranberries.

FTIR spectra and fluorescence spectroscopy were used for characterisation of the phytochemicals in berries, extracted with water and methanol. As far as we know, no results of such investigations were published.

Material and methods

Reagents

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-azino-bis (3-ethylbenzthiazoline-6-sul- phonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent (FCR), $CuCl_2$ 9 $2H_2O$, 2,9-dimethyl-1,10-phenanthroline (neocuproine), lanthanum (III) chloride heptahydrate and $FeCl_3$ 9 $6H_2O$ were purchased from Sigma Chemical Co., St Louis, MO, USA. 2, 4, 6-Tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland. All reagents used were of analytical grade. Deionised and distilled water was used throughout the experiment.

Samples

Cape gooseberries (*Physalis peruviana*), blueberries (*Vaccinium corymbosum*) and cranberries (*Vaccinium macrocarpon*) were investigated. The fruits were har- vested at their mature stage. All berries were pur- chased at the local market in Gdansk and Warsaw, Poland. For the investigation, five replicates of five berries each were used. Their edible parts were sepa- rated manually without using steel knives. The sepa- rated berries were weighed, chopped and homogenised under liquid nitrogen in a high-speed blender (Hamil- ton Beach Silex professional model) for 1 min. A weighed portion (50–100 g) was then lyophilised for 48 h (Virtis model 10–324), and the dry weight was determined. The samples were ground to pass through a 0.5-mm sieve and stored at -20 °C until the bioac- tive substances were analysed.

Determination of bioactive compounds and antioxidant activity

The contents of polyphenols, tannins, flavonoids and flavanols in the extracts of the studied berries were determined as previously described (Gorinstein *et al.*, 2009, 2010). The lyophilised samples of berries (1 g) were extracted with 100 mL of methanol and water (1:1) at room temperature and in darkness for 24 h. The extracts were filtered in a Buchner funnel. The polyphenols were determined by the of Folin–Ciocal- teu method with measurement at 750 nm using spec- trophotometer (model 8452A; Hewlett-Packard, Rockville, MD, USA). The results were expressed as mg of gallic acid equivalents (GAE) per g DW (Single- ton *et al.*, 1999). Condensed tannins (procyanidins)

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were extracted with 4% methanol vanillin solution, and the extracts were measured at 500 nm. Flavo- noids, extracted with 5% $NaNO_2$, 10% $AlCl_3$ 9 $6H_2O$ and 1 M NaOH, were measured at 510 nm. The amount of total flavanols was estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method, and then the absorbance was read at 640 nm (Feucht & Polster, 2001). (+)-Catechin served as a standard for spectrophotometric determination of flavonoids, flavanols and tannins, and the results were expressed as catechin equivalents (CEs).

The antioxidant activity was determined by four assays

- 2, 2-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt ($ABTS^{•+}$) radical cation was prepared by the addition of ABTS (7 mM) and $K_2S_2O_8$ (2.45 mM). This solution was diluted with methanol until the absorbance of the samples reached 0.7 at 734 nm (Re *et al.*, 1999).
- Ferric-reducing antioxidant power (FRAP) reagent (2.5 mL of a 10 mmol ferric-tripyridyltriazine solution in 40 mmol HCl plus 2.5 mL of 20 mmol $FeCl_3$ 9 H_2O and 25 mL of 0.3 M acetate buffer, pH 3.6) (900 lL) was mixed with 90 lL of distilled water and 30 lL of berry samples as the appropriate reagent blank. The absorbance was measured at 595 nm (Ben- zie & Strain, 1996).
- Cupric reducing antioxidant capacity (CUPRAC) was determined based on the utilisation of the copper (II)–neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidising agent. The absorbance at 450 nm was recorded against a reagent blank (Apak *et al.*, 2004).
- 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) has an absorption band at 515 nm, which disappears upon reduction by an antiradical compound. DPPH solution (3.9 mL, 25 mg L^{-1}) in methanol was mixed with the sample extracts (0.1 mL) and then the reaction progress was monitored at 515 nm until the absorbance was stable (Brand-Williams *et al.*, 1995).

Fluorometry and fourier transform infrared (FT-IR) spectra studies

Two-dimensional (2D FL) fluorescence spectra measurements for all berry extracts at a concentration of 0.01 mg mL^{-1} were recorded on a model FP-6500, Jasco spectrofluorometer, serial N261332, Japan, equipped with 1.0-cm quartz cells and a thermostat bath. The 2D FL spectroscopy measurement was taken at emission wavelengths from 310 to 500 nm and at excitation of 295 nm (Arancibia-Avila *et al.*, 2011, 2012). Quercetin was used as a standard. All solutions for protein interaction were prepared in 0.05 M Tris-HCl buffer (pH 7.4), containing 0.1 M NaCl. The final concentration of HSA was 2.0 9 10^{-6} M. The HSA was mixed with quercetin in the proportions of HSA–extract = 1:1 (Wulf *et al.*, 2005; Xiao *et al.*, 2011a,b).

The presence of polyphenols in the investigated berry samples was studied by Fourier transform infra- red (FT-IR) spectroscopy. A Nicolet iS 10 FT-IR Spectrometer (Thermo Scientific Instruments LLC, Madison, WI, USA), with the smart iTR™ ATR (Attenuated Total Reflectance) accessory, was used to record IR spectra (Sinelli *et al.*, 2008).

Statistical analyses

To verify the statistical significance, mean ± SD of five independent measurements was calculated. Differences between groups were tested by one-way ANOVA. In the assessment of the antioxidant activity, Spearman's correlation coefficients (*R*) were used. Linear regressions were also calculated. *P*-values of <0.05 were considered significant.

Results

Bioactive compounds

The results of the determination of the contents of the bioactive compounds in all studied samples are summarised in Table 1. Water and methanol extracts of gooseberries showed lower amounts of polyphenols, flavonoids, flavanols and tannins (6.24–3.77 mg GAE g^{-1}; 0.29–0.45 mg CE g^{-1}; 6–8 lg CE g^{-1}; and 1.01–1.24 mg CE g^{-1}, respectively, Table 1) than blueberries and cranberries.

Antioxidant activity

As can be seen from Table 2, the AA (lM TE g^{-1}) for gooseberries by DPPH, FRAP, ABTS and CU- PRAC assays was 6.05–4.61; 8.07–7.61; 18.70–19.13; and 13.44–12.71, respectively. The antioxidant activ- ity of blueberries was higher than that of gooseber- ries and cranberries. As was calculated, a very good correlation was found between the antioxidant activity and the contents of total polyphenols in water and methanol extracts. The correlation between the antioxidant activity and polyphenols was between 0.87 and 0.78.

Fluorometry spectra studies and FTIR spectra

The quenching properties of the berry samples are shown in two-dimensional fluorescence spectra (2D FL) and also their comparison with quercetin (Q). One of the main peaks for HSA was found at kex/em of 220/360 nm. The second main peak appeared for

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Table 1 Bioactive compounds in water (H_2O) and methanol (MeOH) polyphenol extracts of gooseberries (GOOSEB, *Physalis peruviana*), cranberries (CRAN, *Vaccinium macrocarpon*) and blueberries (BLUEB, *Vaccinium corymbosum*)*,†,‡

	Indices, g^{-1} DW			
Extracts of berries	POLYPHEN, mg GAE	FLAVON, mg CE	FLAVAN, lg CE	TANNINS, mg CE
GOOSEB, H_2O	6.24 ± 0.6^c	0.29 ± 0.01^c	6 ± 0.8^d	1.01 ± 0.2^c
CRAN, H_2O	15.32 ± 2.5^b	3.06 ± 0.4^b	249 ± 14.5^c	2.30 ± 0.7^c
BLUEB, H_2O	57.47 ± 4.2^a	6.68 ± 0.6^a	1762 ± 25.6^b	5.00 ± 0.6^b
GOOSEB, MeOH	3.77 ± 0.1^c	0.45 ± 0.01^c	8 ± 1.1^d	1.24 ± 0.1^c
CRAN, MeOH	20.25 ± 0.4^b	2.20 ± 0.1^b	393 ± 20.3^c	1.76 ± 0.1^c
BLUEB, MeOH	57.96 ± 0.4^a	6.68 ± 0.7^a	3210 ± 40.4^a	24.80 ± 2.5^a

POLYPHEN, polyphenols; CE, catechin equivalent; GAE, gallic acid equivalent; FLAVON, flavonoids; FLAVAN, flavanols; nd, not determined; DPPH, 2,2-diphenyl-1-picrylhydrazyl; CUPRAC, cupric reducing antioxidant capacity; ABTS, 2, 2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammo- nium salt; FRAP, ferric reducing antioxidant power.

*Values are means ± SD of five measurements.

†Values in columns for every bioactive compound bearing different superscript letters are significantly different ($P < 0.05$).

‡Per gram dry weight.

Table 2 Antioxidant activities in water (H_2O) and methanol (MeOH) extracts of gooseberries (GOOSEB, *Physalis peruviana*), cranberries (CRAN, *Vaccinium macrocarpon*) and blueberries (BLUEB, *Vaccinium corymbosum*)*,†,‡

	Indices, lM TE g^{-1} DW			
Extracts of berries	DPPH	FRAP	ABTS	CUPRAC
GOOSEB, H_2O	6.05 ± 0.6^e	8.07 ± 0.9^c	18.70 ± 1.8^d	13.44 ± 1.2^c
CRAN, H_2O	44.23 ± 4.5^c	22.45 ± 2.4^b	64.83 ± 6.5^c	28.45 ± 2.7^b
BLUEB, H_2O	75.09 ± 6.2^b	177.25 ± 14.6^a	254.83 ± 25.6^b	250.95 ± 18.6^a
GOOSEB, MeOH	4.61 ± 0.4^e	7.61 ± 0.9^c	19.13 ± 2.1^d	12.71 ± 1.1^c
CRAN, MeOH	23.25 ± 2.4^d	26.11 ± 2.1^b	68.40 ± 6.3^c	32.67 ± 3.1^b
BLUEB, MeOH	142.03 ± 11.4^a	149.00 ± 11.7^a	265.92 ± 25.4^a	265.76 ± 20.5^a

POLYPHEN, polyphenols; CE, catechin equivalent; GAE, gallic acid equivalent; FLAVON, flavonoids; FLAVAN, flavanols; nd, not determined; DPPH, 2,2-diphenyl-1-picrylhydrazyl; CUPRAC, cupric reducing antioxidant capacity; ABTS, 2, 2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammo- nium salt; FRAP, ferric reducing antioxidant power.

*Values are means ± SD of five measurements.

†Values in columns for every bioactive compound bearing different superscript letters are significantly different ($P < 0.05$).

‡Per gram dry weight.

these samples at k ex/em of 280/350 nm (Fig. 1). Water phenolic extracts showed slightly higher antioxi- dant properties than the methanol ones, but the differ- ences were not always significant in all extracts. The interaction between HSA and the water extracts (WE) of berries, HSA, WE and Q (Fig. 1a), showed slight change in the position of the main peak at the wave- length of 360 nm and the decrease in the relative fluo- rescence intensity (RFI). The following changes appeared when the water extracts of berries were added to HSA [initially the main peak was at emission of 360 nm and FI of 890.21 (Fig. 1a, the upper line is HSA). The reaction of blueberry water extracts (BLUEBWE) with HSA (second line from the top) and with BLUEBWE, HSA and Q (fifth line from the top) decreased the RFI of HSA by 28.1% and 41.7%, respectively. The reaction of cranberry water extracts (CRANBWE) with HSA (third line from the top) and with CRANWE, HSA and Q (sixth line from the top) decreased the RFI of HSA by 13% and 29.9%, respec- tively. The reaction of gooseberry water extracts (GOOSEBWE) with HSA (fourth line from the top) and with GOOSEBWE, HSA and Q (seventh line from the top) decreased the RFI of HSA by 3.9% and 27.8%, respectively. These results showed that the binding properties of gooseberries were 7.2 and 1.5 times and 3.3 and 1.1 times lower than that of blueber- ries and cranberries, respectively. The following changes appeared when the methanol extracts of ber- ries were added to HSA (initially the main peak was at emission of 360 nm and FI of 890.21, Fig. 1b, the upper line is HSA). The reaction of blueberry metha- nol extracts (BLUEBMeOHE) with HSA (second line from the top) and with BLUEBMeOHE, HSA and Q

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(fifth line from the top) decreased the RFI of HSA by 13.9% and 31.3%, respectively. The reaction of cranberry methanol extracts (CRANBMeOHE) with HSA (fourth line from the top) and with CRANBMeOHE, HSA and Q (seventh line from the top) decreased the RFI of HSA by 8.0% and 24.0%, respectively. The reaction of gooseberry methanol extracts (GOOSEBMeOHE) with HSA (third line from the top) and with GOOSEBMeOHE, HSA and Q (sixth line from the top) decreased the RFI of HSA by 1.3% and 18.9%, respectively. The lowest decrease was with GOOSEBMeOHE without quercetin, but the synergism of quercetin with cranberries and gooseberries showed similar results. The water extracts showed higher binding properties to berries than the methanol, and the difference was significant in all berries.

Our most recent results showed that the fluorescence is significantly quenched, because of the conformation of the HSA changes in the presence of quercetin and berry extracts. This interaction between quercetin and HSA was investigated using tryptophan fluorescence quenching. Other results (Xiao *et al.*, 2011a,b; Zhang *et al.*, 2009) differ from that reported by us, probably because of the variety of antioxidant abilities of pure flavonoids and different ranges of fluorometry scanning used in a similar study. Our *in vitro* results of interaction between HSA and quercetin can be compared with other reports (Zhang *et al.*, 2009). There are not too many applications of 3D fluorescence spec- tra; therefore, our present conclusions – that 3D fluorescence can be used as an additional tool for the characterisation of the polyphenol extracts of berries cultivars – correspond with the previous data (Gorinstein *et al.*, 2010) and can be applied to any food analysis.

FTIR spectra of water (a) and methanol (b) extracts of gooseberries, blueberries and cranberries are presented in Fig. 2 (lines from the bottom to the top). The FTIR wave numbers in polyphenol water extracts showed a broad band at 3273 cm^{-1} for gooseberries and blueberries, but for cranberries, there was a shift to 3332 cm^{-1} (phenolic OH band). Other bands were detected at 2342, 2349 and 2345 cm^{-1} for gooseberry, blueberry and cranberry, respectively. At 1642 cm^{-1} (C=O stretching phenyl ring amino acid-1), this band was detected for gooseberry and blueberry and at 1636 cm^{-1} only for cranberry (Fig. 2a). The methanol polyphenol extracts (Fig. 2b) showed similar bands at 3313, 2943 and 2834 cm^{-1} for three berries. At 1652 cm^{-1} (characteristic CO stretching), bands appeared for gooseberry and blueberry and at 1715 cm^{-1} for cranberry. In the range of 1445 cm^{-1}, a band was found for gooseberry. At 1410 cm^{-1}, a band was found for blueberry and at 1391 cm^{-1} (–OH phenolic bending) for cranberry. The common bands at 1115 cm^{-1} (aromatic bending and stretching) and at 821 cm^{-1} were estimated for all berries. The comparison between the berries, their extracts and some standards in the range of common peaks is shown in Tables 3–4. The best matching in the common range of the peaks was in water extracts of the berries between 3200 and 3000 cm^{-1} (Table 3) of 87% with tannic acid, 78% with hesperidin and 64% with gallic acid. Caffeic and tannic acids showed the matching in the range of 2500–2000 cm^{-1} (Table 3) of 40%. In phenolic extracts with methanol, similar matching of the peaks was found in comparison with tannic acid (84%) and hesperidin (70%). Quercetin in the range of 3500–3100 cm^{-1} (Table 4) showed similarity with the same bands of 70%, which was three times higher than that in water phenolic extract. In the range of 3000–1600 cm^{-1} (Table 4), caffeic, gallic, tannic and ferulic acids showed matching with the investigated berries from 30 to 12%. These matching results for the first time show that FTIR spectra can be used for the rapid estimation of extracted bioactive compounds. Quercetin exhibited the highest matching in the investigated fruit extracts in comparison with fisetin, and caffeic and gallic acids in methanol extracts of investigated berries. Difference between the standards and the investigated samples can be explained by the extraction procedures of the main polyphenols.

Discussion

A number of reviewed articles showed that the main bioactive compounds determining the nutritional quality of berries are polyphenols, anthocyanins and flavonoids (Battino *et al.*, 2009; Dai *et al.*, 2009; Bo- wen-Forbes *et al.*, 2010). Seeram (2010) discussed also that phytonutrients ranged from fat-soluble/lipophilic to water-soluble/hydrophilic compounds. The health benefits of blueberries and cranberries have long been recognised, but less is known about gooseberries. It was of great interest to compare gooseberry with blue- berry and to find out whether the bioactivity of goose- berry is on the same level as these berries in order to use it as a novel additional food source. As was declared in the Results, the contents of bioactive com- pounds (polyphenols, flavonoids, flavanols and tan- nins) and AA in water and methanol extracts were the lowest in gooseberries. Our results, connected with the bioactive compounds and AAs, are in correspondence with others, showing that water extracts of blackber- ries contain high amounts of bioactive compounds (Dai *et al.*, 2009). Our results correspond also with the research of Wu *et al.* (2006), where concentrations of total anthocyanins varied considerably from 0.7 to 1480 mg per 100 g FW in gooseberry ('Careless' variety) and chokeberry, respectively. DPPH radical scavenging activity of currant varied from 12.67 to 31.18 mmol TE kg^{-1} (Wojdyo *et al.*, 2013), and it was

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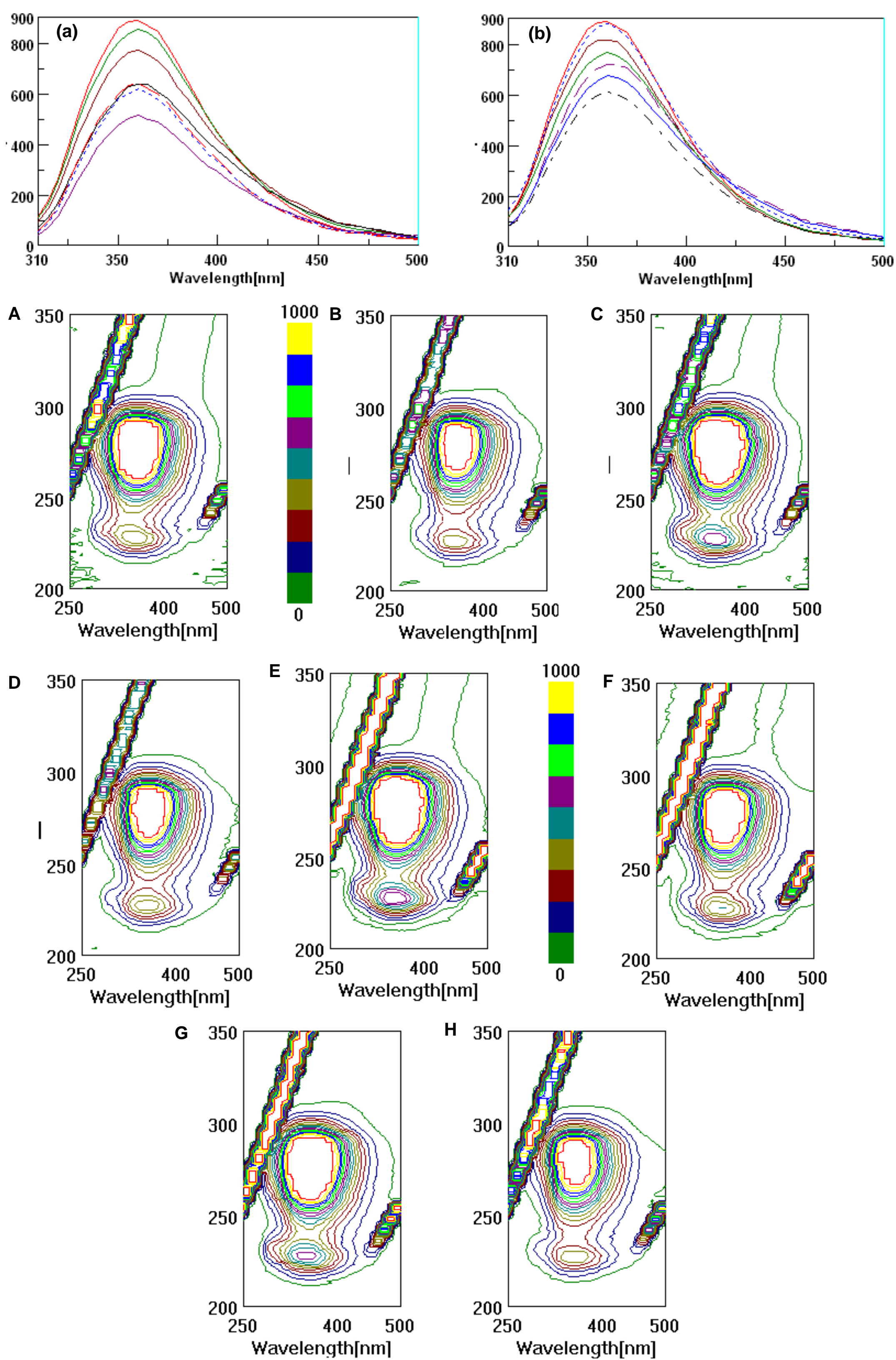

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Figure 1 Change in the relative fluorescence intensity (RFI) in two-dimensional fluorescence (2D FL) spectra as a result of binding affinity of HSA, quercetin (Q, 0.79 9 10^{-6} M), water and methanol extracts of berries: (a) fluorescence spectra of HSA (2.0 9 10^{-6} M), HSA and water extracts (WE) of blueberries (BLUEB), WE of cranberries (CRAN), WE of gooseberries (GOOSEB), HSA and WE BLUEB and quercetin (Q), HSA and WE CRAN and Q; HSA and WEGOOSEB and Q (lines from the top to the bottom with RFI of 890.21, 640.45, 774.65, 855.14, 518.74, 623.66 and 642.97). (b) fluorescence spectra of HSA (2.0 9 10^{-6} M), HSA and methanol extracts (MeOHE) of BLUEB; MeOHE of CRAN; MeOHE of GOOSEB; HSA and MeOHE BLUEB and Q, HSA and MeOHE CRAN and Q; HSA and MeOHE GOOSEB and Q (lines from the top to the bottom with RFI of 890.21, 767.52, 878.87, 818.87, 611.73, 676.80 and 722.15). A, B, C, D, E, F, G, H, three dimensional fluorescence spectra of WECRAN and HSA, WECRAN and HSA and Q; WEGOOSEB and HSA; WEGOOSEB and HSA and Q; MeOHECRAN and HSA, MeOHECRAN and HSA and Q; MeOHEGOOSEB and HSA; MeOHEGOOSEB and HSA and Q.

Table 3 Matching of the peaks (%) in the FTIR spectrum of extracted polyphenols in water from gooseberries (GB, *Physalis peruviana*), cran- berries (CB, *Vaccinium macrocarpon*) and blueberries (BB, *Vaccinium corymbosum*) and standards

Range of bands	3200–3000 cm^{-1}			2500–2000 cm^{-1}			1800–1500 cm^{-1}		
Matching of standards/samples (%)									
Standards	GB	BB	CB	GB	BB	CB	GB	BB	CB
Gallic acid	64	64	64	37	33	36	0	1	0
Ferulic acid	23	23	23	26	19	25	2	3	3
Fisetin	20	19	16	35	34	35	3	2	3
Hesperidin	78	77	78	5	5	5	28	28	28
Tannic acid	87	87	87	41	40	40	6	6	6
Caffeic acid	52	48	53	40	35	40	26	26	26
Quercetin	23	22	22	26	26	26	1	1	1

Table 4 Matching of the peaks (%) in the FTIR spectrum of extracted polyphenols in methanol from gooseberries (GB, *Physalis peruviana*), cranberries (CB, *Vaccinium macrocarpon*) and blueberries (BB, *Vaccinium corymbosum*) and standards

Range of bands	3500–3100 cm^{-1}			3000–2800 cm^{-1}			1800–1600 cm^{-1}			1500–700 cm^{-1}		
Matching of standards/samples (%)												
Standards	GB	BB	CB	GB	BB	CB	GB	BB	CB	GB	BB	CB
Quercetin	70	71	70	1	5	6	1	3	3	5	8	5
Ferulic acid	17	17	18	14	12	11	18	8	18	6	5	5.5
Fisetin	10	17	17	10	15	12	3	22	3	7	7.5	7.5
Hesperidin	69	69	70	34	35	40	20	26	9	6	4	3.5
Tannic acid	84	84	84	14	16	14	9	35	2	35	35	37
Caffeic acid	34	38	35	27	30	24	16	17	3	11	10	16
Gallic acid	59	57	57	11	13	11	19	28	16	27	18	26

similar to the results obtained in this research. Total phenolic content of four berry fruits (strawberry, saskatoon berry, raspberry and wild blueberry), chokecherry and seabuckthorn ranged from 22.83 to 131.88 g kg^{-1}, which corresponds with our results as well. Conclusions made in the report of Elberry *et al.* (2010) are in line with our results about the high antioxidant activity of berries. Our results are in accordance with You *et al.* (2011), where four rabbiteye blueberry cultivars (Powderblue, Climax, Tifblue and Woodward) grown organically and conventionally were compared regarding their chemical profiles and

antioxidant capacity in terms of total phenolic content

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and antioxidant values determined by ABTS, DPPH, FRAP and CUPRAC assays. The comparison of the results of different solvents in dabai fruit parts (metha- nol, ethanol, ethyl acetate, acetone and water) and total phenolics, total flavonoids and antioxidant capac- ity ($ABTS^+$ and FRAP assays) were in accordance with our data (Khoo *et al.*, 2012). The acetone extract had maximum phenol and flavonoid content and showed the best DPPH free radical scavenging activity and reducing capacity assessment. Ethyl acetate extract showed best superoxide radical scavenging activity, while aqueous extract showed best hydroxyl radical scavenging activity (Chanda & Kaneria, 2012). Rop

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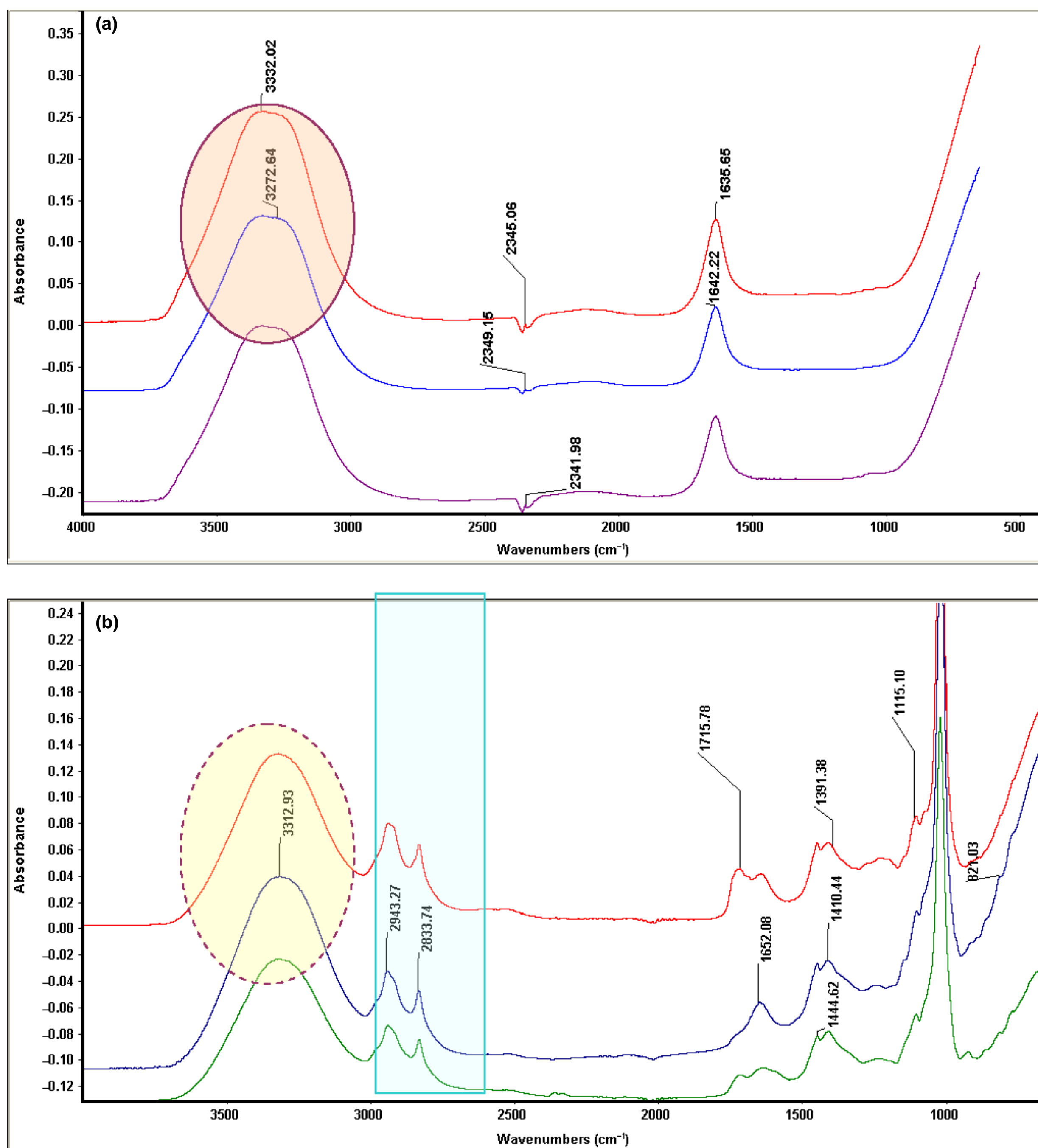

Figure 2 FTIR spectra of: (a) water extracts of gooseberries, blueberries and cranberries; (b) methanol extracts of gooseberries, blueberries and cranberries from the bottom to the top. The elliptical symbols showed the similar range of the spectra in two extracts.

et al. (2012) showed that gooseberry (*Physalis peruvi- ana*) fruit is one of the less used raw materials of plant origin, which can be used for human nutrition and can be promoted as a food additive in fresh and processed food, as an extract from fresh or frozen fruits. The

methanolic extracts of three cultivars expressed high antioxidant activity and correlated with the amount of polyphenols. We have investigated the binding proper- ties of quercetin in aqueous and methanol media, using UV/vis and fluorometry, which is one of the

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major phenolic compounds found in berries. Our results were in accordance with Guo *et al.* (2007), who demonstrated that quercetin and other phenolic compounds can effectively modulate iron biochemistry under physiologically relevant conditions, providing insight into the mechanism of action of bioactive phenolics (Guo *et al.*, 2007). Our results are in agreement with Xiao *et al.* (2011a) as well that dietary flavonoids are important polyphenols in berries as they are of great interest for their bioactivities, which are related to the antioxidative property. The binding affinities with HSA were strongly influenced by the structural differences of dietary polyphenols from berries. The HSA–polyphenol interaction weakened with the free radical scavenging potential of polyphenols. The structural difference of flavonoids strongly affects the binding process with plasma proteins. Flavonoids played as a hydrogen bond acceptor when bound to HSA (Xiao *et al.*, 2011a,b). The relatively high binding properties of gooseberries are important from the point of view of their incorporation in food products as an important ingredient. Our *in vitro* fluorometry studies are in agreement with others, who investigated the properties of berries *in vivo*. So, the drinking of cranberry juice for 4 months affected antioxidant capacity and lipid profile in orchidectomised rats. Orchidectomy depressed plasma antioxidant capacity of plasma and increased triglyceride and cholesterol values of liver and plasma (Deyhim *et al.*, 2007). Rats fed with goldenberry (*Physalis peruviana*) juice showed lower levels of total cholesterol, total triacylglycerol and total low-density lipoprotein cholesterol, as well as higher levels of high-density lipoprotein cholesterol in comparison with animals fed with HCD and cholesterol-free diet (Ramadan, 2012). It is possible to supplement food products with the extracts of the studied berries, as it was shown in the study by Lastawska (2010). The selected products were in the form of hard gelatin capsules. They contained the extracts from chokeberry, cranberry and blueberry. All studied preparations showed antioxidant properties and may provide substantial antioxidant protection. The *in vitro* antioxidant capacity varied considerably and was associated with the content of polyphenols in the capsule. The studied gooseberry can be used as dry or fresh material or as water extracts. The most important aspect is the prevention of antioxidant properties during the food processing. Our present results are in correspondence with the previous results, where only aqueous extracts were used, with other kinds of berries. In our previous report, it was shown that aqueous extracts of investigated berries were subjected to different times of thermal processing. Only thermal treatment of studied berries influences their quality: berries after 10 and 20 min of thermal processing preserved their bioactivity (Arancibia-Avila *et al.*, 2012). This is in accordance with Reyes *et al.* (2011), who showed that ascorbic acid content was decreased in freeze-dried blueberries compared with fresh fruit, while polyphenols were decreased in atmospheric freeze-drying unlike in vacuum freeze-drying, where this nutritional property was increased. The results show promising perspectives for the exploitation of berry species with considerable levels of nutrients and antioxidant capacity in foods. Our data add valuable information to current knowledge of the nutritional properties of berries, such as the considerable antioxidant and binding capacities that were found. In conclusion, the bioactivity of gooseberries is lower and comparable with blueberries and cranberries. Gooseberries are a promising exotic fruit that could be made into many novel dishes. 3D fluorescence spectroscopy and FTIR spectroscopy were used as additional tools for the characterisation of the polyphenol extracts in different berry cultivars. The analytical methods used in this study can be applied for any food analysis.

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