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EFFECT OF EXTRACTION SOLVENT ON BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF *Cichorium intybus* L. GROWN IN KOSOVO

SUMMARY

The aim of this study was the investigation of the total phenolic content, flavonoid, and the assessment of the antioxidant potential of *Cichorium intybus* L. flowers grown in the Kosovo region. The flowers of *Cichorium intybus* L. were collected, dried, and extracted with solvents with different polarities (water, EtOH, MeOH, EtOAc, and acetone), using the extraction method. The total phenolic and flavonoid contents were analyzed by using Folin–Ciocalteu's and AlCl3 reagents, respectively. The antioxidant activity was assessed by DPPH in vitro assay methods. The obtained results revealed variation in the content of phenolic compounds (5.2 mg GAE/gDW in water extract, 6.8 mg GAE/gDW in ethanolic extract,72.1 0.3 mg GAE/gDW in methanolic extract, 45.3 0.1 mg GAE/g DW in ethyl acetate extract,and 31.3 0.1 mg GAE/gDW in acetone extract), flavonoid (2.9 mg CE/gDW in water extract, 19.0 mg CE/gDW in ethanolic extract,30.50 mg CE/gDW in methanolic extract, 27.1 mg CE/gDW in ethyl acetate extract, and 25.4 mg CE/gDW in acetone extract), and antioxidant activity (23.0 μMolTE/gDW in water extract, 124.7 μMolTE/gDW in ethanolic extract, 152.4 μMolTE/gDW in methanolic extract, 92.7 μMolTE/gDW in ethyl acetate extract,and 107.2 μMolTE/gDW in acetone extract). *Cichorium intybus* L. flowers from the Kosovo region are also a rich source of flavonoids. Further studies are recommended to quantify and isolate the pure phytoconstituents from *Cichorium intybus* L. flowers grown in the Kosovo region which might serve as natural antioxidants application in the food and drug industry.

Keywords: *Cichorium intybus* L., total phenolic content, flavonoid, antioxidant activity DPPH

INTRODUCTION

Medicinal plants have been the source of therapeutic agents since ancient times. These plants have played an important role in the discovery of medicines

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and still, most people in developing countries rely on herbal medicines for their primary health care (Kandil *et al*., 2019).

Cichorium intybus L., is a plant of the family *Asteraceae*, commonly known as *chicory*, with its natural distribution in many countries of Europe. The flower, roots, and leaves of *Cichorium intybus* L. have been used traditionally for human consumption. The gastronomic uses and medical properties of *Cichorium intybus* L. have been well known for a long time (Jancic *et al*., 2017).

Food vegetables are a significant part of the human diet worldwide since ancient times. In this context, the Mediterranean diet is recognized as a valuable source of health benefit constituents and represents one of the most peculiar examples of traditional cuisine with many dishes rich in healthy vegetables. The traditional recommendation for a healthy diet with salad vegetables including *Cichorium intybus* L. is relevant as a dietary source of natural antioxidants, which have been associated with a lower risk of cardiovascular diseases and cancer (Tardugno *et al*., 2018, Sinkovic *et al*., 2014).

Cichorium intybus L. has gained attention for its content of phytochemicals with nutraceutical potential. Phytochemicals, the plant-derived non-nutritive compounds, are one of the different types of dietary factors which play an important role in various functions of the human body (Lee *et al*., 2017). All parts of this plant possess great importance due to the presence of compounds with health benefits, such as phenolic acids, flavonoids, coumarin, cinnamic acid derivatives, and anthocyanins, alkaloids, inulin, sesquiterpene lactones, vitamins, chlorophyll pigments, unsaturated sterols, saponins, and tannins (Sahan *et al*., 2017, Abbas *et al*., 2015).

Chicory is widely used in herbal preparations which display a beneficial influence on bile excretion, diuretic action, gastric juice excretion, as well as stimulation of digestion and metabolism of food ingredients (Milala *et al*., 2009).In the last decades, an increasing interest has been observed in the health-promoting properties of particular food constituents, including dietary fiber-polyphenolic complex (Juskiewicz *et al*., 2011). Other health benefits of *Chicorim intybus* L. as anti-microbial, anti-inflammatory, anti-mutagenic, anticarcinogenic, anti-toxic, anti-hyperglycemic, anti-ulcerogenic activities, easing digestive problems and heartburn, reducing arthritis complaints and reducing the risk of liver, as well as supporting the immune system (Sahan *et al*., 2017).

An important class of natural bioactive compounds is represented by polyphenols which are considered secondary metabolites synthesized by plants, vegetables, and medical plants in their normal development and also as a response to stress factors (Cisneros-Zhevallos *et al*., 2020). As polyphenol compounds, flavonoids, and phenolic acids are natural antioxidants that have important roles in protecting biological systems against the harmful consequences of oxidative stress. A valuable source of polyphenolic compounds is the plant species from the Asteraceae family including *chicory* (Epure *et al*., 2021). They are characterized by aromatic rings and hydroxyl groups, and they can be divided

into several classes, such as phenolic acids, stilbenes, flavonoids, and lignans (Sinkovic *et al*., 2015). The great importance of phenolic compounds in the various physiological and morphological features, such as defense mechanisms, cell wall structure, interaction with phytohormones, proteins, and enzymes, with a high potential to neutralize free radicals by donating the electrons and signaling for gene expression (Petropoulos *et al*., 2017, Zhao *et al*., 2014).

Flavonoids, a subfamily of polyphenols compounds, exhibit several interesting biological, exert a wide range of biochemical and pharmacological effects that add to their well-known antioxidant capacity such: antibacterial, hepatoprotective, anti-inflammatory, anti-allergic, anti-cancer, and antiviral (Becker *et al*., 2016).Many studies revealed that antioxidant capacity is attributed to a combination of polyphenols and flavonoids (Balea *et al*., 2018), as was considered in our study because the total polyphenolic content and flavonoids content of the *Cichorium intybus* L. extracts was correlated with the antioxidant effects.

The chemical structure of the phenolic compounds and their concentration is related to the antioxidant capacity of the extracts. The mixture of phenolic compounds present in extracts can determine different supplementary effects such as synergistically, additively, or antagonistically actions, that influence the total capacity of the extract to neutralize free radicals. Both phenolic acids and flavonoids are known as antioxidant compounds, but their ability depends not only on the quantities but on the chemical structure also (Jacobo-Velázquez *et al*., 2009).

Antioxidant properties of *Cichorium intybus* L. have previously been reported in methanolic, water/acetone, and aqueous extracts, as also within the hydroalcoholic extract (Tusevski *et al*., 2013, Eray *et al*., 2020, Denev *et al*., 2014). Extraction of bioactive compounds from plant materials gains more and more interest within the food and drug industry. Extraction yield and antioxidant activity depend on the extraction method also the solvent used for the extraction process (Do *et al*., 2014). The presence of various antioxidant compounds with different chemical characteristics and polarities may or might not be soluble in a particular solvent. Polar solvents are frequently used for recovering polyphenols from plant matrices. The foremost suitable solvents are aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate (Sultana *et al*.,2009). Ethanol has been referred to as a suitable solvent for polyphenol extraction and is safe for human consumption. Methanol has been generally found to be more efficient in the extraction of lower relative molecular mass polyphenols, whereas aqueous acetone is suited for the extraction of high molecular mass flavanols (Do *et al*., 2014).

Ethnobotanical studies show that the medical plants still play a crucial role in the sphere of human health in Kosovo, especially in isolated rural areas (Mustafa and Hajdari, 2014).

Cichorium intybus L. preparation as infusion and decoction form, still consumed for the prevent hart disorders, atherosclerosis, bronchitis, urinary

system infections, anti-haemorrhoid, and hepatic disorders (Mustafa and Hajdari, 2014).

According an inventory of MAP (Medicinal and Aromatic Plants) and WB (Wild Berries) growing spontaneously in the territory of Kosovo,568.600 kg *Cichorium Intybus* L. grows wild in Kosovo (Millaku F., 2010).

Over the last few years, several studies have explain chemical composition and antioxidant activity of *Cichorium intybus* L. grown in Balkan Peninsula such: Denev *et al*., 2014; Dzharov *et al*., 2016, Bulgaria; Petropoulos *et al*., 2017, Greece; Epure *et al*., 2021, Romania; Jancic *et al*., 2017, Montenegro; Sinkovic *et al*., 2015, Slovenia; Tusevski *et al*.,2013, North Macedonia. According with our knowledge no any study in Kosovo about chemical composition and antioxidant activity of *Cichorium intybus* L.grown in Kosovo. Only previously studies are Faiku *et al*., 2016 about antibacterial activity of different solvent extract of *Cichorium intybus* L. grown in Kosovo.

This is the first research of this type in Kosovo and it should give us a novel result of the *Cichorium intybus* L. as a richest and cheapest source of antioxidants.

The aim of this study was the investigation of the effect of extraction solvent on major antioxidant phenolic compounds such: phenolic compounds, flavonoids, and antioxidant activity by DPPH in the extract of the *Cichorium intybus* L. grown in the Kosovo region.

MATERIAL AND METHODS

Reagents

Gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin Ciocalteu, $AlCl_3$, $NaNO_2$, ethanol, methanol, ethyl acetate-EtOAc, acetone) and standard compounds, Gallic acid, Catechin, Trolox were purchased from Sigma-Aldrich Chemie GmbH, Germany. All reagents were of analytical grade.

Preparation of plant extracts

The *Cichorium intybus L*. flowers of about 250 g were collected from growing wild in Kosovo region within the month of July 2019. Locality was a location on central part of Kosovo (Lipjan 42° 31' 28.79" N, 21° 08' 11.40" E, elevation 559m). Voucher specimens (EE/2019/003) were deposited in the herbarium of the Laboratory of Botany in UBT-Higher Education Institution.

The flowers of *Cichorium intybus L*. after washing under running water were dried at room temperature. The flowers of the plant (1g) were ground and soaked in 30 mL of boiling water, and 30 mL organic solvents (EtOH, MeOH, EtOAc, Acetone). Then 20 mL water and 20 mL organic solvents were added sequentially during a bottom flask and soaked on an ultrasonic bath for 10 minutes.

Figure 1. *Cichorium intybus* L. growing wild in the Kosovo region

Table 1. Location information of the regions that samples were collected

No.	Locality	Latitude	Longitude	Elevation (m)	Voucher specimens no.
1	Lipjan	42° 31' 28.79" N	21° 08' 11.40" E	559 m	EE/2019/003

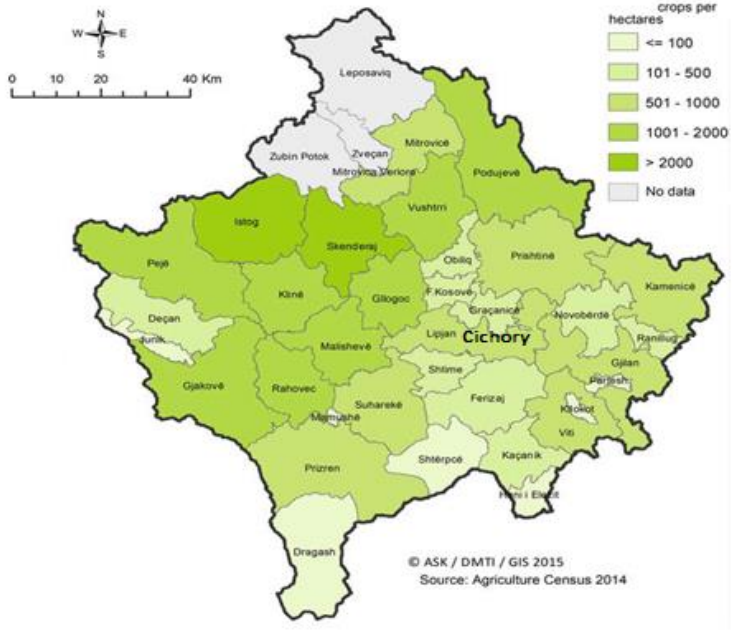

Figure 2. Map of the area where sample collection.

Determination of total phenolic content

The total phenolic content of the different extracts (water,EtOH, MeOH, EtOAc, Acetone) was estimated according to the previously reported method Singleton *et al*., (1999) and slight modifications, with the help of the Folin-Ciocalteu reagent. 200 µL of extract or standard gallic acid (5–100 mg L^{-1}) was added to 3 mL distilled water and then 250 µL Folin-Ciocalteu reagent/2 min was added. Then 750 µL sodium carbonate 20% was added and the mixture was made up to 5 mL with distilled water. The mixture was incubated for 2 h and the absorbance was measured at 765 nm in a GENESYS^{TM} 10S spectrophotometer. Total phenolic content was expressed as gallic equivalent (mg GAE/g DW). Standard Gallic acid (GA) in a concentration of 5-100 µg/mL was used to construct the external calibration curve.

Determination of total flavonoid content

The total flavonoid content of the different extracts (water, EtOH, MeOH, EtOAc, Acetone) was estimated by colorimetric assay with the $AlCl_3$ method by Montefusco *et al*., (2015) with some modifications. On 1 mL aliquots of the samples added 2 mL distilled water and 0.3 mL of 2% $NaNO_2$. After 5min, 0.5 mL of 1% $AlCl_3$ was added, followed, after a further 6 min, by 2 mL of 1M NaOH and 2 mL of distilled water. The absorbance was read at 510 nm in a GENESYS^{TM} 10S spectrophotometer. Catechin was used as a standard and the flavonoid content was expressed as g catechin equivalent g^{-1} dry weight of plant material (mg CE/g DW).

Antioxidant activity

Antioxidant activity of the different extracts (water, EtOH, MeOH, EtOAc, Acetone) was evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazil radical) assay Brand-Williams *et al.*, (1995) and Petropoulos *et al*., (2017) with some modifications. A brief description of 1,1-diphenyl-2-picrylhydrazyl (DPPH) was dissolved in methanol to prepare a 100 µM working standard solution. To 1 mL of various concentrations of the different extract (25, 50, 100, and 200 µg/mL) 2 mL of DPPH solution was added and the resulting mixture was mixed well. After incubation in dark at room temperature for 45 min, the absorbance of these solutions was measured at 515nm in a GENESYS^{TM} 10S spectrophotometer. $A_{control}$ was prepared in a similar manner by replacing the amount of sample with methanol.

% Inhibition of DPPH radical was calculated using the following formula:

$$\% \text{ inhibition of DPPH} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

Where; $A_{control}$ and A_{sample} are absorbance of control and sample, respectively. All determinations were performed in triplicates.

Statistical Analysis

For all the analyses, three samples were analyzed for each treatment and all of the assays were carried out in triplicate. The results were expressed as mean values and standard deviations (Mean ± SD). Statistical analysis of data was

applied using SPSS v. 22.0 program through a one-way analysis of variance (ANOVA) while, for means where a statistical difference was detected, means comparisons were carried out using Tukey's test ($P < 0.05$).

RESULTS AND DISCUSSION

Total phenolic content

The total phenolic content of both extract-water and organics extract (EtOH, MeOH, EtOAc, Acetone) from flower of the *Cichorium intybus* L. was analyzed using the Folin–Ciocalteu method are shown in table 2,and figure 3a and 4 which ranged from 5.2± 0.2 mg GAE/g in water extract, 6.8± 0.2 mg GAE/g in ethanolic extract, 72.1± 0.3 mg GAE/g in methanolic extract, 45.1± 0.3 mg GAE/g in ethyl acetate extract, and 31.3± 0.2 mg GAE/g in acetone extract. From our results, we showed the methanolic extract gave higher total phenolic content compared to those water, and other organics solvent extract ($p > 0.05$). The lowest amount of the total phenolic contents was determined in the water extracts (5.2± 0.2 mg GAE/g). The total phenolic content of the water extract is significantly less than that of other organics solvents ($p<0.05$). The total phenolic contents in water and organic solvents are ranged: Water<EtOH<Acetone<EtOAc<MeOH. Our results correspond with the results of the work Morales *et al*.,(2014), which reported that the total phenolic content of flower extract of *Cichorium intybus L*. in methanolic extract in the range from 51.1± 0.8 mg GAE/g. Tusevski *et al*.,(2013) reported that the total phenolic content of *Cichorium intybus L*. in methanolic extract range from 33.36± 0.14 mg GAE/g. Our results were the higher of these studies. Piluzza *et al*.,(2014) reported a higher amount of total phenolic compounds in the acetone/water (7:3) extract of *Cichorium intybus* L. range about 33.76 to 43.89 g GAE/Kg.Our results were the lowest of these studies. Eray *et al*.,(2020) reported total phenolic content in herbal parts of *Cichorium intybus* L.water extract range about 138.8±0.87 µg GAE/mg,and methanolic extract range about 186.3±3.281 µg GAE/mg. Also, our results were the lowest of these studies.The other hand Denev *et al*.,(2014) reported that the total phenolic content of *Cichorium intybus* L. in water extract range from about 3.7± 0.4 mg GAE/g to 6.7± 0.9 mg GAE/g, and in ethanolic extract range about 4.3± 0.5 mg GAE/g. Our results were the higher of these studies. In EtOAc extract no have any previously publication results (EtOAc=No).

Total Flavonoids content

The total flavonoids content of both extract-water and organics extract (EtOH, MeOH, EtOAc, Acetone) from flower of the *Cichorium intybus* L. analyzed using the $AlCl_3$ method by spectrophotometry are shown in table 2,and figure 3b and 4 which ranged from 2.9± 0.2 mg CE/g in water extract, 19.0± 0.3 mg CE/g in ethanolic extract, 30.5± 0.2 mg CE/g in methanolic extract, 27.1± 0.3 mg CE/g in ethyl acetate extract, and 25.4± 0.2 mg CE/g in acetone extract. From our results, we showed the methanolic extract gave higher total flavonoid content compared to those water, and other organics solvent

extracts (p >0.05). The lowest amount of the total flavonoid contents was determined in the water extracts (2.9± 0.2 mg CE/g). The total flavonoid content of the water extract is significantly less than that of other organics solvents (p<0.05). The total flavonoid contents in water and organic solvents are ranged: Water<EtOH<Acetone<EtOAc<MeOH. Our results correspond with the results of the work of Denev *et al.*,(2014), which reported that the total flavonoid content of flower extract of *Cichorium intybus* L. in water extract in the range from 2.7± 0.2 mg CE/g to 2.8± 0.2 mg CE/g. Tusevski *et al.*,(2013) reported that the total flavonoid content of *Cichorium intybus* L. in methanolic extract range from 8.06± 0.24 mg CE/g. Our results were the higher of these studies.The lowest values of total flavonoids content were obtained by Malik *et al.*,(2017) on methanolic extract (13.5± 0.70 mg RE/g) and ethanolic extracts (8.49± 0.08 mg RE/g DW.) from chicory leaves. Piluzza *et al.*,(2014) reported a higher amount of total flavonoid compounds in the acetone/water (7:3) extract of *Cichorium intybus* L. range about 27.13 to 36.94 g CE/Kg.Our results were the lowest of these studies. Eray *et al.*,(2020) reported total flavonoid content in herbal parts of *Cichorium intybus* L.water extract range about 388.3±5.77 µg CE/mg, and methanolic extract range about 550±14.53 µg CE/mg. Also, our results were the lowest of these studies.On the other hand, Kandil *et al.*,(2019) reported that the total flavonoid content of *Cichorium intybus L.* in methanolic extract range about 167.47 ± 5.83 µg QE/g,whiles in ethanolic extract range about 36.56 ± 5.95 µg QE/g.Our results were the higher of these studies.Also,the assessment flavonoid in EtOAc extracts does not have any previously publication results (EtOAc=No).

Antioxidant activity by DPPH

DPPH free radical scavenging method is a widely used and reliable method to evaluate the *in vitro* antioxidant activity of natural products and plant extracts. The natural or synthetic antioxidants such as ascorbic acid, tocopherol, cysteine, glutathione, gallic acid, etc., have the ability to reduce the DPPH radial (purple color) to a yellow-colored compound. The extent of color change depends on the hydrogen donating ability of the antioxidants.

Therefore, in the current study, we have used the DPPH method to evaluate the antioxidant activity of the *Cichorium intybus* L. flowers extracts. All extracts of the *Cichorium intybus* L. flowers exhibited higher antioxidant activity. In this study, the extracts of the undertaken of the *Cichorium intybus L.* flowers extract were assessed for antioxidant potential by utilizing the above principle of the DPPH radical scavenging method. Table 2 and figure 3c and 4 represents the DPPH radical scavenging abilities of the *Cichorium intybus L.* flowers extract used in this study. The antioxidant activity of the extract (water and organics solvent extract) from the flower of the *Cichorium intybus L.* was analyzed using the DPPH method, and are shown in table 1, and figure 3 which ranged from 23.0± 0.4 µMolTE/g in water extract, 124.7± 0.2 µMolTE/g in ethanolic extract, 152.4± 0.3 µMolTE/g in methanolic extract, 92.7± 0.3 µMolTE/g in ethyl acetate extract, and 107.2± 0.4 µMol TE/g in acetone extract. From our results, we

showed the methanolic extract (152.4±0.3μMolTE/g) gave higher antioxidant activity content compared to other extracts. A good correlation was observed between total phenolic content and antioxidant activity. It is observed from the results of this study that the highest phenolic and flavonoid content of chicory extracts exhibited high antioxidant activities (Kandil *et al*.,2019). The higher DPPH antioxidant activity in methanolic extract of *Cichorium intybus* L. is significantly higher than that of other solvents ($p > 0.05$). The DPPH antioxidant activity of the water extract is significantly less than that of other solvents ($p < 0.05$).

Previous publications found that the methanolic extract of *Cichorium intybus L*. flowers possessed the highest content of phytochemicals and antioxidant activity. Antioxidant activity obtained by organic solvents are ranged:water<EtOAc<Acetone<EtOH<MeOH. Our results of the antioxidant activity content as compared to those reported by Tusevski *et al*., (2013), reported that the total antioxidant activity of *Cichorium intybus* L.flower in methanolic extract range about 154.40± 0.2 μM TE/g. Similar results also obtained Epure *et al*.,(2021), they reported the DPPH radical scavenging activity of the methanolic extracts 164.98± 5.93 μg/mL and 336.35± 11.77 μg/mL for the ethanolic extract.Denev *et al*.,(2014), reported that the DPPH antioxidant activity of *Cichorium intybus* L. flower in water extracts range about 16.1± 2.8 mM TE and 29.1± 0.8 mM TE. The DPPH antioxidant activity of *Cichorium intybus* L. flower in ethanolic extracts range about 29.2± 0 mM TE and 31.3± 0.1 mM TE. Our results were the lowest of these studies. On the other hand, Piluzza *et al*.,(2014) reported the total antioxidant activity by DPPH in the acetone/water extract of *Cichorium intybus* L. flower range about 15.63 to 23.86 mMol/g.Our results also were the lowest of these studies.The results of the study of the total antioxidant activity by DPPH in the water extract,and methanolic extract of *Cichorium intybus* L. flower presented Eray *et al*.,(2020),they reported the total antioxidant activity by DPPH calculation as IC_{50} in the water extract range about 7.5± 0.1, whiles in the methanolic extract range about 3.59± 0.18. Our results were the highest of these studies.Not found any previous publication about total antioxidant activity by DPPH in EtOAc.

Until now, no any detailed information was available for antioxidant activity of *Cichorium intybus* L. flowers grown on the Kosovo region. This is the first research about total phenolic content, total flavonoid content, and antioxidant activity of *Cichorium intybus* L. flowers grown on the Kosovo region. In other view point the best solvent for extraction biological active compounds such: phenolic content, flavonoid content, and antioxidant activity is methanol. From results obtained in this study, different extracts of *Cichorium intybus* L. flowers can be considered a potential source of antioxidants for food and drug industry application.

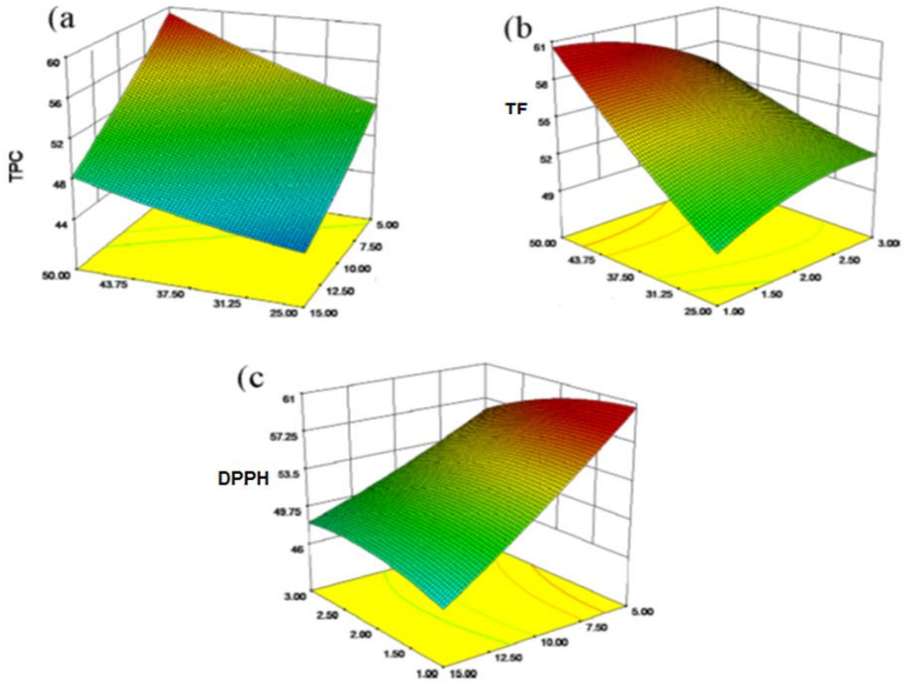

Figure 3. 3D visualization of the a) total phenolic contents b) total flavonoids contents and c) Antioxidant activity by DPPH method of *Cichorium intybus* L. extracts on different solvents

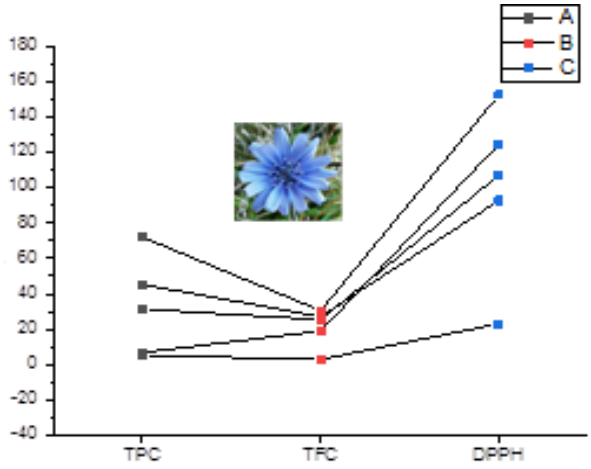

Figure 4. Correlation between total phenolic contents, total flavonoids contents, and Antioxidant Activity of *Cichorium intybus* L. extract on different solvents

Table 2. The total phenolic contents, total flavonoid content, and Antioxidant activity DPPH of *Cichorium intybus* L. extracts

Extracts	Total phenolic content (mg of GAE/gDW)	Total flavonoids content (mg of CE/gDW)	Antioxidant activity DPPH (µMolTE/gDW)
Water	5.2±0.2	2.9±0.2	23.0±0.4
EtOH	6.8±0.2	19.0±0.3	124.7±0.2
MeOH	72.1±0.3	30.5±0.2	152.4±0.3
EtOAc	45.1±0.3	27.1±0.3	92.7±0.3
Acetone	31.3±0.2	25.4±0.2	107.2±0.4

[1] Data are presented as average value ± standard deviation of three replicates

CONCLUSIONS

This study is currently the first comprehensive report that presented detailed information for phenolic content, flavonoids content, and antioxidant activity of *Cichorium intybus* L. flowers extract grown in the Kosovo region that has been examined. Very limited information is available on compositional and health-enhancing properties of *Cichorium intybus* L. grown in the Kosovo region. The result showed that *Cichorium intybus* L. flowers extract had a higher content of total phenolic contents and higher content of total flavonoids, also antioxidant activity. The highest total phenolic and flavonoid contents were determined in the flower methanolic extract. The results of our study indicate the presence of major classes of phytochemicals in *Cichorium intybus* L. flowers extract and a direct relationship between antioxidant capacities and total flavonoid content in *Cichorium intybus* L. flowers extract. Further studies are recommended to quantify and isolate the pure phytoconstituents from *Cichorium intybus* L. flowers extract which might serve as the cheapest source of natural antioxidants application in the food and drug industry. In conclusion, chicory, due to its phenolic and antioxidant contents as well as the bio accessibility of these compounds, provides important health benefits for consumers, while remaining an inexpensive vegetable. However, before this product is incorporated into a dietary supplement or as a natural food antioxidant, it is important to further study toxicity and in vivo activity.

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