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Effect of extraction solvent on bioactive compounds and antioxidant activity of *Achillea millefolium* L. grown in Kosovo region

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ABSTRACT

Achillea millefolium L. is a plant of the family *Asteraceae*, commonly known as yarrow, which grows and is being distributed in countries of Europe. *Achillea millefolium* L. has been traditionally used in medicine. The medical properties of *Achillea millefolium* L. have been known for a long time. This study aims at assessments of the total phenolic content, flavonoid content and antioxidant activity of *Achillea millefolium* L. flowers, grown in Kosovo region. Solvent extracts with different polarity (aqueous, ethanol-EtOH, methanol-MeOH, ethyl acetate-EtOAc, acetone) from the flowers of *Achillea millefolium* L. are analyzed. The total phenolic and flavonoid quantities were analyzed by using Folin–Ciocalteu's and $AlCl_3$ reagents, respectively. The antioxidant activity was assessed by DPPH in vitro assay methods. The extract obtained by methanol showed the highest total antioxidant activity and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity (51.9±0.3 µMol/g). The same extract also exhibited the highest phenolic content (52.6±0.3 mg GAE/g) and the highest flavonoid content (27.8±0.2 mg CE/g). Further studies need the assessment to quantify and isolate the phytochemicals from *Achillea millefolium* L. flowers, grown within the Kosovo region, which might serve as a cheap natural antioxidants in food and drug industry.

Introduction

Medical herbs are utilized in many domains in diverse areas that include medicine, food, pharmaceutical and cosmetics industries (Georgieva et al., 2015; Pacheco et al., 2019). WHO reported as approximately 80% of the world's population from developing countries use plant-derived medicines for their primary health care purposes (Haliloglu et al., 2017). The application of medicinal plants, among them plants from the Asteraceae family, within the food industry, cosmetology and pharmacology is being increasingly studied (Mekinić et al., 2014). The consumption of herbal medicines is widespread and is continuously increasing worldwide (Giorgi et al., 2009). With society being increasingly concerned with health and nutrition, medical plants emerge as an alternative to synthetic products, used not only in traditional medicine, but also a number of food and pharmaceutical products, thanks to their nutritional properties and bioactivity (Dias et al., 2013). *Achillea millefolium* L., commonly referred to as yarrow, belongs to *Asteraceae* family it's native in Europe, and also grows wild all around. *Achillea millefolium* L., recognized as a strong medicinal plant, has been used for centuries to treat several conditions, like gastrointestinal and hepatobiliary disorders, inflammation, and diabetes (Guz et al., 2019; Pereira et al., 2018). Ethanolic extract of *Achillea millefolium* L. has been widely utilized in Europe in the traditional medicine of several cultures thanks its to numerous pharmacological properties like treating digestive problems, diabetes, hepato-biliary diseases, and amenorrhea. *Achillea millefolium* L. also consumed for antitumor, antimicrobial, anti-inflammatory, antioxidant properties, and applied mainly in gastrointestinal disorders, as a bitter aromatic medicine during a temporary loss of appetite, to stimulate bile secretion,

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externally in skin and mucous membranes inflammations, also as a wound healing remedy (Baczek et al., 2015; Hammad et al., 2014). The extraction of bioactive compounds from plant materials gains more and more interest within the industry. Recently, there has been much interest in potential health benefits of dietary plant polyphenols, especially as antioxidants. Flavonoids, a subfamily of polyphenols, exhibit several interesting biological activities that add to their well-known antioxidant capacity such as antibacterial, hepatoprotective, anti-inflammatory, anti-cancer, and antiviral (Becker et al., 2016). Flavonoids represent a large group of polyphenolic compounds. They exert a wide range of biochemical and pharmacological effects including antibacterial, antiviral, anti-inflammatory, antiallergic, and vasodilatory activities. These naturally occurring compounds widely spread in nature and they are consumed as a part of the human diet in significant amounts (Şabanoglu et al., 2017). In the past few years, interest in the antioxidant properties of plant-derived foods and medicinal plants has increased, since antioxidants contained in plants are involved in the preservation of human health. Moreover, plant extracts with antioxidant properties are becoming more and more attractive for the food industry, as they are considered to represent a ‘‘natural” alternative to synthetic antioxidants. Therefore, the assessment of antioxidant properties of traditional medicinal plants, which are widely used, is an important concern in the quest for new sources of natural antioxidants, and richness in bioactive compounds contributes to a wide range of medicinal properties (Giorgi et al., 2009). The global interest in food preservation has been recently greatly increased due to the high economic costs of deterioration and spoilage of food products through lipid oxidation as well as food pathogens. Currently, there is a growing interest in prolonging shelflife and the safety of food using natural antioxidant and antimicrobial compounds. The phenolic compounds like flavonoids and phenolic acids are considered together of the foremost important groups of pharmacologically active compounds present in *Achillea* species (Benetis et al., 2008). Antioxidant properties of *Achillea millefolium* L. have previously been reported in hydroalcoholic, methanolic, and aqueous extracts, as also within the essential oil (Bozin et al., 2008; Vitalini et al., 2011; Candan et al., 2010). Extraction yield and antioxidant activity not only depend on the extraction method, but also on the solvent used for extraction. 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. Ethanol has been referred to as a suited 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). *Achillea millefolium* L. is one of the richest sources of antioxidants. The aim of this study was the investigation of the effects of extraction solvent on major antioxidant phenolic compounds such as polyphenols, flavonoids, and antioxidant activity in *Achillea millefolium* L. grown within the Kosovo region.

Materials and methods

Reagent

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.

Extracts preparation

About 250 g of the *Achillea millefolium* L. flowers were collected from Kosovo region in July 2019. The flowers of *Achillea millefolium* L., after being washed under running water, were dried at the room temperature. The flowers of plant (1g) were ground and soaked in 30 mL boiling water and 30 mL organic solvents (EtOH, MeOH, EtOAc, Acetone) for 24 hours, allowed to filter. Then 20 mL water and 20 mL organic solvents were added sequentially a bottom flask and boiled on a water bath for 2 hours.

Determination of total phenolic content

Total phenolic content of the extract of flowers *Achillea millefolium* L. Was determined by Folin Ciocalteu Reagent using the method of Singleton et al. (1999), with minor modifications, with the help of Folin-Ciocalteu reagent. 100 μL of extract flowers of *Achillea millefolium* L. (10–100 mg L^{-1}) was added to 2 mL water. Afterwards, 200 μL Folin-Ciocalteu reagent was added. Then 500 μL 30% Na_2CO_3 was added and the mixture was made up to 5 mL with distilled water. The mixture was incubated for 2hours at room temperature and after that the absorbance was measured at 750 nm. Total phenolic content was expressed as gallic acid equivalent (μg GAE)/g.

Standard gallic acid (GA) in a concentration of 10-100 μg/mL was used to construct a calibration curve. Three determinations were made and at every determination 3 repeated readings. The results are the average of the 3

determinations. In the same conditions a calibration curve in gallic acid was built, using solutions in methanol with a concentration of 4.20 to 21.0 μg/mL. The results were calculated as the average values of gallic acid equivalent (GAE) with mean and standard deviation.

Determination of total flavonoid content

The total flavonoid content of the extracts was determined by colorimetric assay with the $AlCl_3$ according to reference by/of Miliauskas et al. (2004) with minor modifications. 1.0 mL extract of flowers *Achillea millefolium* L. was added to 1.0 mL of $AlCl_3$ (2%). After 1hour, the absorbance was measured at 420 nm. Catechin was used as a standard and therefore the flavonoid content was expressed as mg catechin equivalent g^{-1} dry weight of the material (mg CE/g). Three determinations were made and at every determination 3 repeated readings. The results are the average of the 3 determinations. In the same conditions a calibration curve with catechin was built, using solutions in methanol with a concentration of 4.08 to 20.4 μg/mL. The results were calculated as the average values of catechin (CE) with mean and standard deviation.

Determination of antioxidant activity by DPPH radical scavenging assay

Antioxidant activity was analyzed using the DPPH (1,1-diphenyl-2-picrylhydrazil radical) assay according to Georgieva et al. (2015) with some modifications. 1,1-diphenyl-2-picrylhydrazyl (DPPH) was dissolved in methanol to prepare a 200 μM working standard solution. To 2 mL of varied concentrations of ethanolic extract (25, 50, 75,100 and 200 μg/mL) 4 mL of DPPH solution was added and after that, the resulting mixture was mixed well. After incubation in dark at room temperature for 45 min, the absorbance of those solutions was measured at 515nm. Analyses were performed in triplicate. The DPPH radical scavenging activity was presented as a function of the concentration of Trolox and expressed as the μM Trolox per g (μMol/g).
A control was prepared a similar manner by replacing the quantity of sample with methanol. The % inhibition of DPPH radical was calculated using the subsequent formula:

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

where; an impact and a sample are absorbance of control and sample.

Statistical analysis

For all the analyses, three samples were analyzed for every treatment and each of the assays was performed in triplicate. The results were expressed as mean values and standard deviations (Mean ± SD). Statistical analysis of experimental data is performed using OriginLab program through a one-way analysis of variance (ANOVA), while, for those means where a statistical difference was detected, means comparisons were administered using Tukey's test ($P<0.05$).

Results

Different *Achillea* species had been reported to contain large amounts of polyphenolic compounds, especially flavonoids, phenolic acids and tannins, which can effectively exert antioxidant and radical scavenging activities (Hammad et al., 2014). Phenolic compounds are liable for the antioxidant activity of the material. Therefore, within the present study, total phenolic compounds, total flavonoid content and antioxidant activity of Achillea millefolium L. aqueous, alcoholic, EtOAc, and acetone extract were determined.

Total phenolic content

The total phenolic content in the extracts of *Achillea millefolium* L. was determined spectrophotometrically by using Folin Ciocalteu reagent. Phenolic compounds react with Folin Ciocalteu reagent under basic conditions (pH = 10). In this condition, the phenolic proton dissociates to a phenolate anion, which may reduce Folin Ciocalteu Reagent (Haliloglu et al., 2017). The results can give recommendations on the potential of *Achillea millefolium* L. as a possible source of antioxidants. The entire phenolic contents were presented as a function of the concentration of gallic acid and expressed as the mg of GAE/g. The experimental data of the total phenolic contents in *Achillea millefolium* L. extracts are presented in Table 1 and Figure 1. Analyses were performed in triplicate.

Total flavonoid content

Total flavonoids in *Achillea millefolium* L. extracts were determined using the spectrophotometric method with $AlCl_3$ and the obtained results varied from 5.2±0.2 to 27.8±0.2 mg of CE/g extract. The total flavonoid contents were presented as a function of the concentration of catechin and expressed as 174 mg of CE/g. The experimental data of the total flavonoid contents in *Achillea millefolium* L. extracts are presented in Table 1 and Figure 2. Analyses were performed in triplicate.

Table 1. The total phenolic contents, total flavonoid content, and Antioxidant activity DPPH of *Achillea millefolium* L. extracts

Extracts	Total phenolic content (mg of GAE/g)	Total flavonoids content (mg of CE/g)	Antioxidant activity DPPH (µMol/g)
Aqueous	18.2±0.2	5.2±0.2	24.3±0.4
EtOH	27.2±0.2	11.0±0.3	30.0±0.2
MeOH	52.6±0.3	27.8±0.2	51.9±0.3
EtOAc	30.1±0.3	4.9±0.3	38.5±0.3
Acetone	41.3±0.2	17.8±0.2	43.7±0.4

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

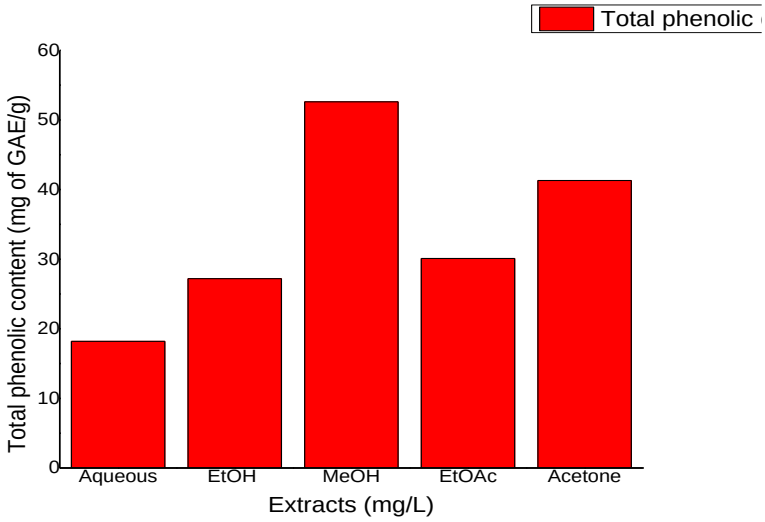

Fig. 1. The total phenolic contents of *Achillea millefolium* L. extracts

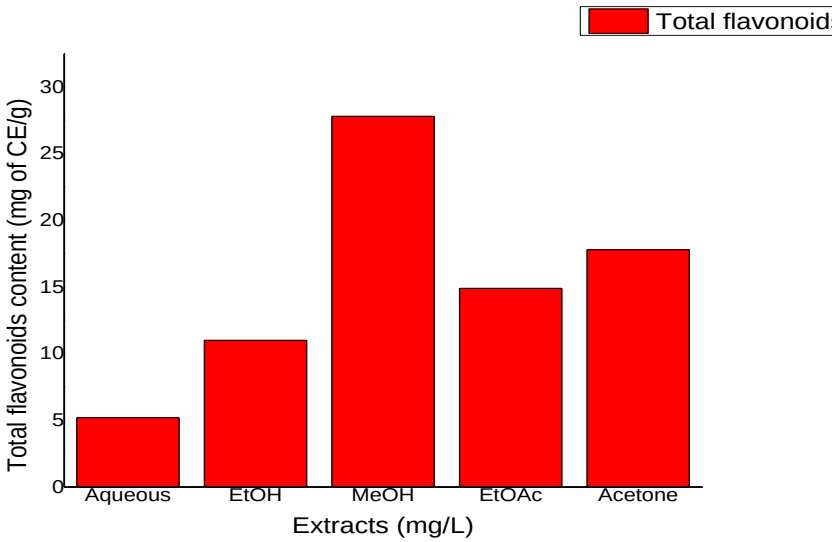

Fig. 2. The total flavonoids contents of *Achillea millefolium* L. extracts

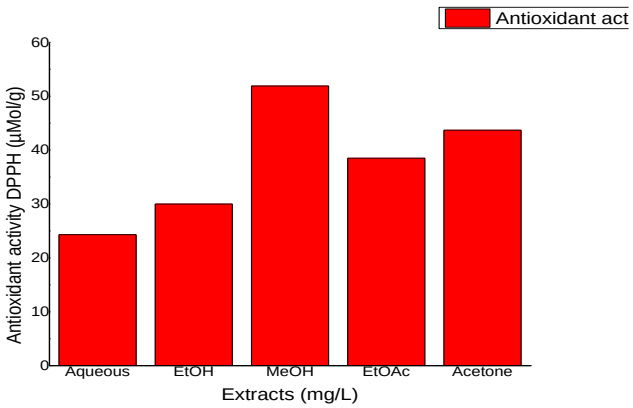

Fig. 3. Antioxidant activity by DPPH method of *Achillea millefolium* L. extracts

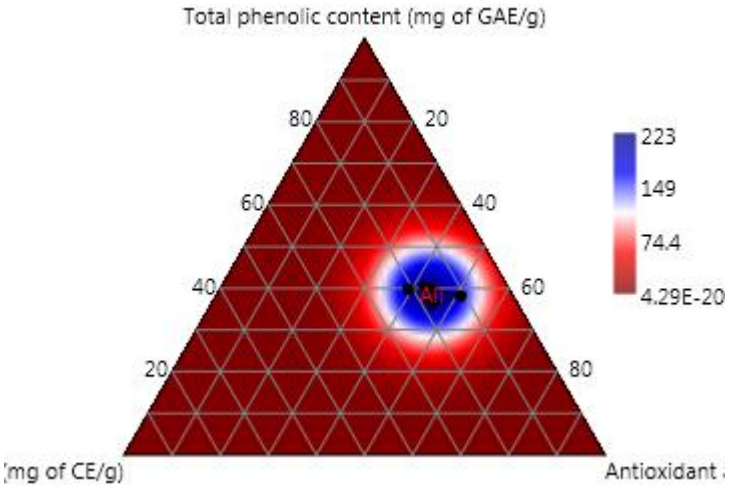

Fig.4. Total phenolic contents, total flavonoids contents and Antioxidant activity of *Achillea millefolium* L. extracts on different solvents

DPPH radical scavenging activity

DPPH free radical scavenging method is a widely used and a reliable method to evaluate in vitro antioxidant activity of natural products and plant extracts. The DPPH radical is a stable, organic, free radical with an absorption maximum band around 515 to 528 nm. The natural or synthetic antioxidants such as ascorbic acid, tocopherol, cysteine, glutathione, gallic acid, etc., have the ability to reduce the DPPH radical (purple color) to a yellow-colored compound. The extent of color change depends on the hydrogen donating ability of the antioxidants (Singleton et al., 1999). Therefore, in the current study, we have used the DPPH method to evaluate the antioxidant activity of the *Achillea millefolium* L. flower extracts. Both extract (aqueous and organic solvents) of the *Achillea millefolium* L. flowers exhibited high antioxidant activity. In this study, the extracts undertaken of the *Achillea millefolium* L. flower extracts were assessed for antioxidant potential by utilizing the above principle of the DPPH radical scavenging method. Antioxidant activity of *Achillea millefolium* L. extracts was determined by the DPPH method using spectrophotometric and the obtained results varied from 24.3± 0.4 to 51.9± 0.3 µMol/g extracts. The experimental data of the antioxidant activity of *Achillea millefolium* L. extracts are presented in Table 1. and Figure 3. Analyses were performed in triplicate. The DPPH radical scavenging activity was presented as a function of the concentration of Trolox and expressed as the µM Trolox per g (µMol/g).

Discussion

Total phenolic content

As shown in Table 1 and Figure 1, the flower extracts of *Achillea millefolium* L. prepared with methanol had high phenolic contents (52.6±0.3 mg GAE/g), significantly higher than that of other solvents ($p>0.05$). The lowest amount of the total phenolic contents was determined in the aqueous extracts (18.2±0.2 mg GAE/g). The total phenolic content of the water extract is significantly less than that of other solvents ($p<0.05$). The total phenolic contents in organic solvents are ranged: aqueous<EtOH<EtOAc<Acetone<MeOH. Our results correspond with the results of the work of Haliloglu et al. (2017). They reported that the total phenolic content of flower extract of *Achillea millefolium* L. in different solvents ranges from 51.1±0.8 mg GAE/g in the methanolic extract, 28.5±1.0 mg GAE/g in EtOAc extract, and 17.9±0.2 mg GAE/g in the aqueous extract. Dias et al.(2013) reported a higher amount of total phenolic compounds in the methanoli extract of wild *Achillea millefolium* L.,range about 128.36± 0.0 mg GAE/g. While the methanolic extract of the commercial sample of *Achillea millefolium* L. about 95.02 ± 1.0 mg GAE / g. Our results were the lowest of these studies. Georgieva et al. (2015) reported that the total phenolic content of *Achillea millefolium* L. in water extract ranged from 2.77±0.03 mg GAE/g to 7.92±0.09 mg GAE/g. Our results were similar of these studies. Wojdylo et al. (2007) reported total polyphenolic content in herbal parts of *Achillea millefolium* L. water extract ranged about 9.55±0.11 mg GAE/100g. Also, our results were higher of these studies.

Total flavonoid content

As shown in Table 1 and Figure 2, the methanolic extract flowers of *Achillea millefolium* L. (27.8±0.2 mg of CE/g) possessed the highest content of flavonoid compared to aqueous and ethanolic extracts (5.2±0.2 mg of CE/g and 11.0±0.3, respectively). Higher total flavonoid content (27.8±0.2 mg of CE/g) in flower methanolic extracts of Achillea millefolium L. significantly higher than that of other solvents ($p>0.05$. The lowest amount of the total flavonoid contents was determined in the aqueous extracts (5.2±0.2 mg CE/g). The total flavonoid content of the water extract is significantly lower than that of other solvents ($p<0.05$). The total flavonoid contents in organic solvents are ranged: Aqueous<EtOH<EtOAc<Acetone<MeOH. The effect of extraction solvents on total flavonoid content is similar to that on total phenolic content of *Achillea millefolium* L.

Our results were in concordance to those found by Haliloglu et al. (2017), who reported that the total flavonoids content of *Achillea millefolium* L. flower extract in different solvents ranged from 27.1± 2.0 mg CE/g in the methanolic extract, 14.5± 4.3 mg CE/g in EtOAc extract, and 3.7± 0.2 mg CE/g in the water extract. Guz et al. (2019) reported that the total flavonoids content of *Achillea millefolium* L. flower in water extract ranged from 18.90 mg CE/g to 23.10 mg CE/g, in ethanolic extract from 45.20 mg CE/g to 49.00 mg CE/g, and hexane/acetone extract from 32.40 mg CE/g to 35.20 mg CE/g. Our results were the lowest of these studies. Dias et al. (2013) reported that the total flavonoid content of *Achillea millefolium* L. flower in the methanolic extract of wild *Achillea millefolium* L. ranged about 24.56±0.36 mg CE/g and in water extract about 22.96±0.10 mg CE/g.Then the commercial sample methanolic extract of *Achillea millefolium* L. ranged about 28.63±1.01 mg CE/g and in water extract 33.78±1.98 mg CE/g. Our results were higher of these studies in the methanolic extract, whereas our results were the lowest in water extract of these studies.

DPPH radical scavenging activity

As shown in Table 1 and Figure 3, the methanolic extracts flower of *Achillea millefolium* L. (51.9±0.3 μMol/g extract) possessed the highest antioxidant activity compared to aqueous and ethanolic extracts (24.3±0.4 μMol/g and 30.0±0.2 μMol/g respectively). The aqueous extracts displayed relatively lower antioxidant activity in comparison to methanol extracts. Higher DPPH antioxidant activity in methanolic extract of Achillea millefolium L. 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 ($p<0.05$). Evaluation of the antioxidant potential of the tested Achillea millefolium L. extracts should be performed by taking into consideration the total phenol and flavonoid contents. As can be seen from Table 1, the highest content of phenolic compounds was measured in methanolic extract (52.6±0.3 mg of GAE/g). The highest flavonoid content was detected in the methanolic extract (27.8±0.2). It is known that the antioxidant potential of natural products is related to phenolic compound content. Antioxidant activity obtained by organic solvents are ranged: aqueous<EtOH<EtOAc<Acetone<MeOH. Our results revealed higher antioxidant activity in both extract (aqueous and organic solvents) contents as compared to those reported by Georgieva et al. (2015), who

reported that the total antioxidant activity of Achillea millefolium L.flower in water extract ranged about 24.15±0.15 μM TE/g. Results of DPPH antioxidant activity reported by Guz et al. (2019) showed that the DPPH antioxidant activity of Achillea millefolium L. flower in water extracts was about 71.22% to 76.60% inhibition, 79.90% to 87.48% inhibition in the ethanolic extract, and 18.11% to 22.53% inhibition in hexane/acetone extract. These results were higher of these studies. On the other hand, Haliloglu et al. (2017) reported that the total antioxidant activity by DPPH in the water extract of Achillea millefolium L. flower ranged about 4.88±3.13 IC50, mg/mL, in methanolic extract 0.38±0.055 IC50, mg/mL and in EtOAc had no results (EtOAc=No). Our results were the highest of these studies. The antioxidant activity was observed to increase with increasing the concentration and the highest activity was observed at 200 μg/mL. A good correlation was observed between total phenolic content and antioxidant activity. Until now, no detailed information was available for the antioxidant activity of Achillea millefolium L. flowers grown in the Kosovo region. This is the first research of this type in Kosovo and it should give us a novel result of the Achillea millefolium L.as the cheapest source of antioxidants.

Conclusion

This study is currently the first comprehensive report that presented detailed information for phenolic content, flavonoid content and antioxidant activity of *Achillea millefolium* L. flower extracts grown in the Kosovo region that has been examined. The result showed that *Achillea millefolium* L. flower extracts had a higher content of total phenolic contents and higher content of total flavonoids, also antioxidant capacity and 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 *Achillea millefolium* L. flower extracts and a direct relationship between antioxidant capacities and total flavonoid content in the *Achillea millefolium* L. flower extracts. Further studies are recommended to quantify and isolate the pure phytochemicals from *Achillea millefolium* L. flower extracts which might serve as the cheapest source of natural antioxidants in food and drug industry.

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