HPLC analysis and Antioxidant, Antibacterial and Cytotoxicity Activities of Various Solvent Extracts of *Erysimum kotschyanum* Gay. (Brassicaceae)

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(Received on 17th March 2017, accepted in revised form5th March 2018)

Summary: *Erysimum* is the second richest genus in Brassicaceae. *Erysimum* species have been used in some phytochemical and biological activities studies, as well as in the taxonomic, floristic and genetic works. In this study, some biological activities and phenolic contents of various solvents extracts (ethanol, acetone and distileted water) of *Erysimum kotschyanum* Gay. were evaluated..The antioxidant activity of the extracts was evaluated by β-carotene/linoleic acid, ferric reducing power assays (FRAP), radical scavenging assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS) and total phenolic and flavonoid contents in the extracts were determined. The brine shrimp lethality test was used to determine the cytotoxic activity of *E. kotschyanum* extracts and also, antibacterial potential of extracts obtained. Antioxidant activity was determined as β-carotene/linoleic acid (%80.47±1.83), DPPH (IC_{50}: 0.29±0.03) and ABTS (IC_{50}: 47.15±0.74), ferric reducing antioxidant power (FRAP) (IC_{50}: 18.79±0.15), total phenolic and total flavonoid contents was found as (4.883±0.47 mgGAE/g) and (93.322±1.57 mgQE/g), respectively ,and then 9 different phenolic compounds in ethanol extracts was carried out by HPLC. Also, ethanol extract's antimicrobial activity (17±1 zone diameter) and cytotoxic activity (Brine Shrimp LC_{50}: 315.48) of extracts was calculated. According the results, the extract of *E. kotschyanum* may be considered as a potential source of biological agents.

Keywords: *Erysimum kotschyanum* , antioxidant, antibacterial, HPLC.

Introduction

Turkey consists of European Turkey and Anatolia, i.e. that part of the country in Asia. The country is the meeting place of three phyto-geographical regions: Euro-Siberian, Mediterranean and Irano- Turanian. Their distinctive vegetation reflects differences in climate, geology, topography, soils and floristic diversity, including endemism [1]. The Turkish flora contains over 10,000 plant species and many of them are endemic [2]. Many wild edible plants are traditionally used in the human nutrition and consumed as vegetable in Turkey [3-5].

Brassicaceae are among the most important sources of food waste both due to the product size and to all pre-market machining processes required before sale. Brassica are of great interest for their nutraceutical properties as they are rich in antioxidants compounds including polyphenols, which are well regarded for their taste and particularly for their health-promoting effects, such as anticancer or antioxidant properties [6]. Therefore, the ingestion of health promoting compounds directly or by addition to pharmaceutical products (nutraceutical) or other foods (functional foods) after extraction from vegetables provides safe and effective protection against many common diseases [7-10]. *Erysimum* is the second richest genus in Brassicaceae according to the *Flora of Turkey*, in which this genus is the range of 290 and 350 species and they are mostly perennial and biennial plants, distributed throughout Europe, the Mediterranean area, the Near East, and East Asia as well as North and Central America [11-14].

Erysimum species have been used in some phytochemical and biological activities studies, as well as in the taxonomic, floristic and genetic works. *Erysimum cheiranthoides* L. is distributed over the northern world and is used as a folk medicine in China for treating cardiac diseases; weak cardio palmus, edema, and dyspepsia, etc. [15]. *Erysimum* species are reported to have many biological activities including antioxidant [16], antibacterial [17] and cytotoxic effects [18]. In addition some studies have identified effective compounds within *Erysimum* plants such as methylthioalkyl, methylsulfinylalkyl and methylsulfonylalkyl glucosinolates [19], sinigrin, progoitrin, glucoiberin, 3-(methylcarbonyl)propyl glucosinolate, glucocheirolin and glucoerysolin [20], 4-isothiocyanatobutanoic acid [21], cardiac glycoside [22].

This study aimed to evaluate the several biological activity tests, such as antioxidant (β-carotene/linoleic acid, ferric reducing power assays (FRAP), radical scavenging assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2'-azino-bis-3-ethyl

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benzthiazoline-6-sulphonic acid (ABTS)), total phenolic and flavonoid contents, antibacterial activity, cytotoxixity (Brine shrimp lethality test) and quantification phenolic contents of *E. kotschyanum* by HPLC method.

Experimental

Materials

Plant material and Preparation of Plant Exracts

E.kotschyanum Gay.(Brassicaceae) species were collected from Honaz Mountain in Denizli in May 2015. The species identified by the Laboratory of Botanic of Pamukkale University. The aerial parts were dried in shade at room temperature, powdered with a blender and prepared for the experiment.

10 g of air-dried parts of *E. kotschyanum* were extracted with 100 mL of three different solvents (ethanol, acetone and distilted water) in a shaker water bath at 55°C for 6 h. In this process, three different erlenmeyers (total 30 g dry plants and 300 mL solvent) were used for each solvent in order to obtain sufficient extracts. The extraction was repeated twice at the same condition. These extracts were filtered and the solvents were removed in vacuum by a rotary evaporator at 42-49°C. The water in each extract was frozen in freeze-drying machine and then drawn out. Anhydrous extracts were stored at -20°C until analysis [23].

β –Carotene/Linoleic Acid Assay

The antioxidant activity of the crude extracts was evaluated using the β-carotene-linoleic acid test system with slight modifications [24, 25, 26]. β-Carotene (0.2 mg) in 1 mL of chloroform was added to 20 µL of linoleic acid and 200 mg of Tween-20 emulsifier mixture. The mixture was then evaporated at 40°C for 10 min by means of a rotary evaporator to remove chloroform. After evaporation of chloroform, 100 mL of distilled water saturated with oxygen, 4.8 mL of this emulsion was placed into test tubes which had 0.2 mg of the sample and 0.2 of the extract in them. For control, 0.2 mL of solvent (ethanol, acetone and water) was placed in test tubes instead of the extract. As soon as the emulsion was added into the test tubes, initial absorbance was measured with a spectrophotometer (Shimadzu UV- 1601, Japanese) at 470 nm. The measurement was carried out at 30 min intervals for 2 h. All samples were assayed in triplicate. BHT was used as standard. The antioxidant activity was measured in terms of successful bleaching of β-carotene by using the following equation. The measurements were made using the equation below:

$$[\ 1- (A_0-A_t\ /\ A_0^o\ -\ A_t^o)]\ x\ 100$$

where A_0 and A_0^o are the absorbance values measured at the initial incubation time for samples and control, respectively, While A_t and A_t^o are the absorbance values measured in the samples or standards and control at t½ 2 h.

Scavenging Activity on DPPH (2,2-diphenyl-1-picrylhydrazyl) Free Radical

Free radical scavenging activity of the extracts was determined using method of Wu et. al [27]. In this method, 0.1 mM solution of DPPH in methanol was prepared and 4 mL of this solution was added to 1 mL (0.2 - 1.0 mg) of extracts in, ethanol, acetone and dH_2O at different concentrations. 30 minutes later, the absorbance was measured at 517 nm. Synthetic antioxidant BHT (butylated hydroxytoluene) was used as a positive control. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. Free radical scavenging activity was measured using the equation below:

$$[A_{blank} - A_{sample}/\ A_{blank}]\ x\ 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound.

Reducing Power

The reducing power of the extracts was determined according to the method of Oyaizu [28]. Extracts (0.10 mg) in phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) were added to potassium ferricyanide (2.5 mL, 10 g/L) and the mixture was incubated at 50°C for 20 min. Trichloroacetic acid (2.5 mL, 100.0 g/L) was added to the mixture, which was then centrifuged at 650 *x g* for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL, 1.0 g/L), and then the absorbance was read spectrophotometrically at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power.

ABTS radical cation decolorization assay

The determination of ABTS radical scavenging was carried out as described by Re et. al [29]. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was

produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the study of phenolic compounds and food extracts, the $ABTS^{\bullet+}$ solution was diluted with ethanol, to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30°C. Stock solutions of phenolics in ethanol, carotenoids in dichloromethane and plasma antioxidants in water were diluted such that, after introduction of a 10 µl aliquot of each dilution into the assay, they produced between 20%–80% inhibition of the blank absorbance. After addition of 1.0 ml of diluted $ABTS^{\bullet+}$ solution (A_{734nm} = 0.700 ± 0.020) to 10 µl of antioxidant compounds or Trolox standards (final concentration 0–15 µM) in ethanol the absorbance reading was taken at 30°C exactly 1 min after initial mixing and up to 6 min. Appropriate solvent blanks were run in each assay. All determinations were carried out at least three times, and in triplicate, on each occasion and at each separate concentration of the standard and samples. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of concentration of antioxidants.

Determination of Total Flavonoid Content

Total flavonoid content was determined as described by Arvouet Grand *et al.* with some modifications. 1 milliliters of 2% aluminum chloride ($AlCl_3$) in methanol were mixed with the same volume of each sample solution of plant extract (1mg mL^{-1}) [30]. Absorption readings at 415 nm were taken after 10 min against a blank solution. The mean of three readings was used and expressed as mg quercetine equivalent (QE) per gram of extract. All experiments were done in triplicate and the results are reported as mean ± SD.

Determination of Total Phenolic Content

The concentrations of total phenolic content in the crude extracts were expressed as gallic acid equivalents (mg/gGAE extract) were determined with Folin-Ciocalteu reagent (FCR) according to the method of Slinkard and Singleton [31]. Briefly, 1 ml of the solution extracts (1 mg) was added to 46 ml of distilled water and 1 ml of FCR and was mixed thoroughly. After 3 min, the mixture was added to 3 ml of sodium carbonate (2%) and shaken intermittently for 2 h. The absorbance was measured at 760 nm with spectrophotometer (Shimadzu UV-1601, Japanese). The total phenolic contents were calculated using standard gallic acid graph. The results were expressed as gallic acid equivalent in milligram per gram dry weight.

Determination of Antibacterial Activity

The extracts or fractions were weighed and dissolved in phosphate buffer saline (PBS) (pH=7.0–7.2) and DMSO (%0.1) at a 1:1 ratio to prepare extract solution of 5000 μg/ml.

Each microorganism was suspended in sterile saline and diluted at 10^6 colony forming unit (cfu) per ml. They were 'flood-inoculated' onto the surface of Columbia blood agar plates (Unipath, UK) which were then dried. Six millimeter diameter wells were cut from the agar using a sterile cork-borer, and 0.1 ml of the plant extract solutions were delivered into the wells. After incubation at 37°C overnight, plates were examined for any zones of growth inhibition.Ampicilin (10 μg/ml) and penicillin (10 μg/ml) served as control antibiotics. *Pseudomonas aeruginosa* ATTC 27853, *Escherichia coli* ATCC 25922 and *Staphylacoccus aureus* subsp. *aureus* Rosenbach ATCC 12598 were used as standard [32].

Determination of Cytotoxic Activity

Brine shrimp lethality test (BSLT) was applied to analyze the possible cytotoxic activity of the extracts. The test was performed as described by Krishnaraju et al. [33]. *A. salina* eggs (Sera Artemia Mix, 18g, USA) were incubated in 500 mL of seawater under artificial light at 28ºC, pH 7-8. After incubation for 24h, nauplii were collected with a pasteur pipette and kept for an additional 24h under the same conditions to reach the metanauplii (mature larvae) stage. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5mL of brine solution. In each experiment, 0.5mL of the plant extract was added to 4.5mL of brine solution and maintained at room temperature for 24h under the light and then dead nauplii were counted. Experiments were conducted along with control and four different concentrations (100, 250, 500 and 1000µg/mL) of the extract in a set of three tubes ±data was performed by StatPlus Pro Analysis Program (version 5.9.8) to determine the LC_{50} and LC_{90}.

Quantification of the Phenolic Compounds with HPLC

Phenolic compounds were analyzed by high performance liquid chromatography (HPLC) according to the method of Caponio *et al.* with some modification [34]. Detection and quantification were performed with a diode array detector (SPD-M20A), a LC-20AT pump, a CTO-10ASVp column heater,

SIL-20ACHT auto sampler, SCL-10Avp system controller and DGU-14A degasser. Separations were carried out at 30°C. The eluates were identified at 280 nm. The mobile phases were A: 3.0% formic acid in distilled water and B: methanol. The elution gradient applied at a flow rate of 1 ml min^{-1} was: 95% A/5% B for 5 min, 80%A/20%B in 15 min, 60%A/40%B in 10 min, 50%A/50%B, in 10 min, 40%A/60%B in 10 min, 30%A/70%B 10 min, 100%B in 10 min until the end of the run. Methanol was used to dissolve samples, and then 20 µL of this solution was injected into the column. Gallic acid, 3,4- hydroxybenzoic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, cinnamic acid (Sigma-Aldrich Co., USA) were used as standards. The differentiation and quantitative analysis were made by comparing the standards. The quantity of each phenolic compound was expressed as µg per gram of the extract.

Results and Discussion

In this research, there are some methods for determining the biological activity (antioxidant, antibacterial, cytotoxic activity) and phenolic compounds of *E. kotschyanum.* Antioxidant properties of ethanol, acetone and water exracts of *E. kotschyanum* were determined by four methods, namely, β –Carotene/Linoleic Acid Assay, DPPH, Reducing power, ABTS (Table-1). Also, total phenolic and total flavonoid contents were identified as gallic acid and quercetin equivalents (Table-2). The content of common phenolic compounds of *E. kotschyanum* were quantified with nine standards by HPLC analysis (Table-4).

According to antioxidant capacity tests, while water extract had more active than acetone and ethanol exract in β –Carotene/Linoleic Acid Assay, FRAP value of ethanol extract more higher than other. In DPPH assay, ethanol extracts was exhibited the highest activity. On the other hand, acetone extract had more ABTS radical scavenging activity than ethanol and water extracts.

The total phenolic concentrations of extracts of *E. kotschyanum* are as shown in (Table-2). Water extracts had the highest amount of total phenolic compounds while ethanol extracts have the lowest amount. Total flavanoid content of exracts of *E.kotschyanum* were determineted by quercetine equivelant and results are given Table-2. According to this data, the highest flavanoid amount was determined respectively, in ethanol, acetone and water exracts.

The phenolic compound were separately by comparing the retention time of their peaks with HPLC method. The results were showed in Fig. 2, Fig. 3 and Table-4. According this data, vanilic acid is the common in ethanol extract of *E. kotschyanum* while 3-4hidroxybenzoic acid is the minor compound.

Results of antibacterial activity of *E.kotschyanum*'s extracts that prepared in ethanol, acetone, water against *P. aeruginosa,E. coli and S. aureus* was reported in Table-3. Results showed that higher activity was observed in ethanol exract against *P. aeruginosa.*

Cytotoxic activity of extracts of *E. kotschyanum* was determined by Brine shrimp lethality test. LC_{50} and LC_{90} values of the brine shrimp obtained for ethanol and water extracts are given in Fig. 1. Water extract of *E. kotschyanum* showed most prominent activity with LC_{50} 315.48 µg/mL. Maximum mortalities took place at a concentration of 1000 µg/mL whereas least mortalities were at 100 µg/mL concentration. LC_{50} values of ethanol and water extracts were determined by a plot of percentage of the shrimp nauplii killed against the concentrations of extracts.

Table-1: Antioxidant properties (mean ± SD) of *E. kotschaynum* extract.

	***E. kotschyanum* exracts**			**BHT standard**		
Antioxidant Methods	**EKE**	**EKA**	**EKW**	**BHTE**	**BHTA**	**BHTW**
β –Carotene/Linoleic Acid Assay (% Inhibition)	**50.86±0.58**	**70.33±0.61**	**80.47±1.83**	**96.76±0.010**	**95.62±0.01**	**97.05±0.02**
DPPH (IC_{50})	**0.29 ±0.03**	**0.43 ±0.03**	**0.98 ±0.13**	**0.18±0.003**	**0.40±0.002**	**0.44±0.005**
FRAP (IC_{50})	**18.79±0.15**	**22.37±0.41**	**21.78±0.25**	**18.43±0.13**	**22.24±0.45**	**21.69±0.25**
ABTS (IC_{50})	**47.77 ±0.14**	**47.15 ±0.74**	**47.83 ±0.01**	**45.83±0.05**	**46.76±0.32**	**48.47±0.27**

*EKE: *E. kotschyanum* ethanol; EKA: *E. kotschyanum* acetone; EKW: *E. kotschyanum* water; BHTE: BHT ethanol; BHTA: BHT acetone; BHTW: BHT water.

Table-2: Total phenolic and flavanoid contents (mean ± SD) of *E.kotschyanum* exracts in different solvents.

***E. kotschyanum* exracts**	**Quantitation of Secondary Metabolites**	
	Total Phenolic Content (mgGAE/g)	**Total Flavanoid Content(mgQE/g)**
Acetone	**4.069±0.28**	**79.37±0.23**
Ethanol	**3.798±0.29**	**93.322±1.57**
Water	**4.883±0.47**	**31.73±0.90**

Table-3: The dilitution values (mean ± SD) of standards and *E. kotschyanum* extracts (zone size, mm).

	Standard		Inhibition zone of exracts of *E. kotschyanum*		
Bacterial strain	**Amphicilin**	**Penicillin**	**Water**	**Ethanol**	**Acetone**
E. coli	**24±**	**ND**	**13±2**	**10±2**	**15±1**
S.aureus	**ND**	**ND**	**11±2**	**16±0**	**12±0**
P. auruginosa	**ND**	**ND**	**12±2**	**17±1**	**9±1**

ND = Not detected, All results are mean values of 3 replicates ± SD

Table-4: Phenolic components in the ethanolic extracts of *E. kotschyanum.*

Phenolic Components	*E. kotschyanum* (µg/g)	Retention Time (min)
Gallic acid	52,97	7.8
3,4-dihydroxybenzoic acid	18,05	12.2
4-hydroxybenzoic acid	113,67	18.1
Chlorogenic acid	233,29	19.9
Vanillic acid	753,21	22.1
Caffeic acid	91,83	23
p-Coumaric acid	47,2	30.3
Ferulic acid	26,71	35.7
Cinnamic acid	23,87	71.1

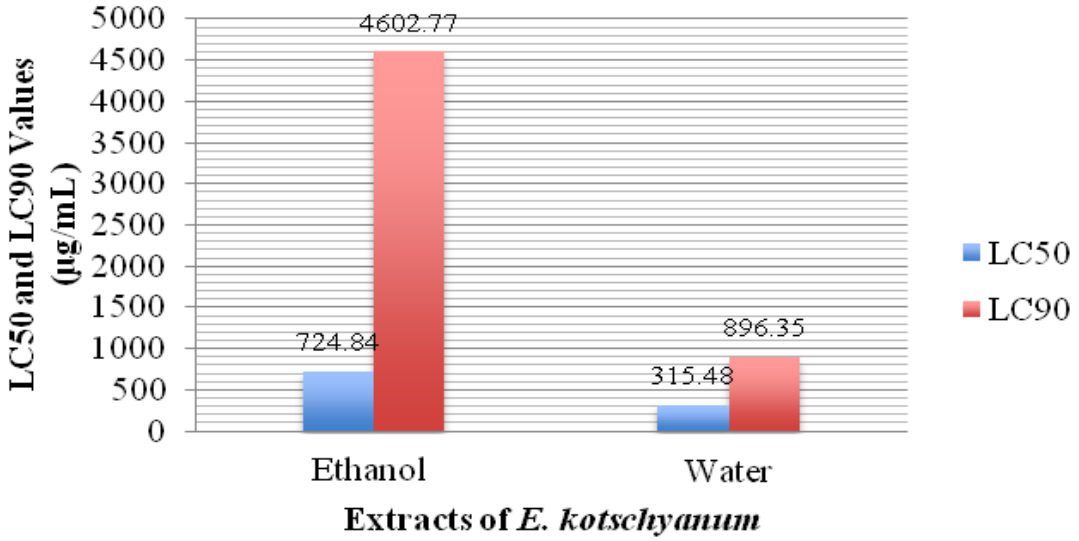

Fig. 1: Brine shrimp lethality data of extracts of *E. Kotschyanum.*

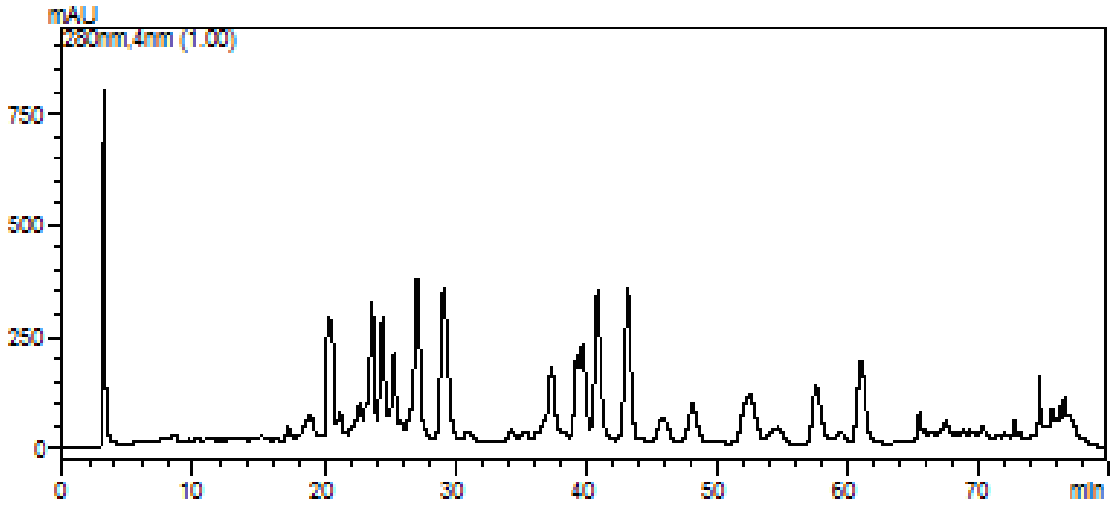

Fig. 2: HPLC Chromatogram of *E. kotschyanum.*

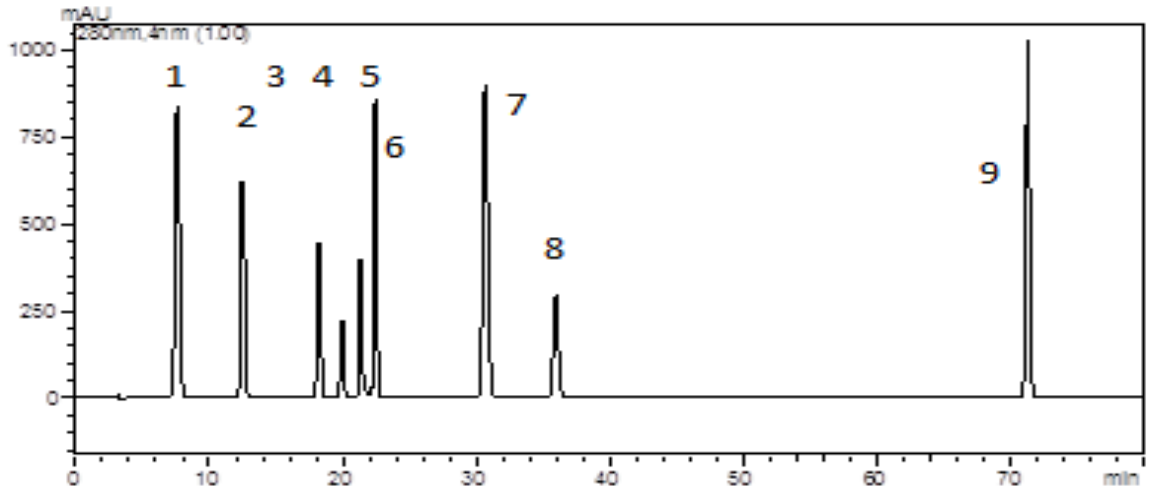

Fig. 3: HPLC Chromatogram of the standards ([1]gallic acid; [2]3-4 hydroxybenzoic acid; [3]4-hydroxybenzoic acid; [4]chlorogenic acid; [5]vanillic acid; [6]caffeic acid; [7]p-coumaric acid; [8]ferulic acid; [9]cinnamic acid)

In this study, some biological activity and phytocemical compounds obtained from exracts of *E. kotschyanum* collected from in Turkey. Several *in vitro* methods have been developed to measure antioxidant capacities of food, beverages and biological samples. Single assay will not accurately reflect all antioxidants [35]. Antioxidant capacity of *E.kotschyanum* are determined with four spectrophotometric methods: β –Carotene/Linoleic Acid Assay, DPPH, ABTS, FRAP (Table-1). The highest antioxidant activity that were used with β-carotene assay were determined in water extract (80.47±1.83 %) and the lowest antioxidant activity was found in ethanol extract (50.86±0.58 %). Ozay *et al.* [36] reported that total antioxidant activity of *Alyssum simplex* that belong to the same family with *Erysimum* were found % 83.58 ± 0.07 in methanolic extract with same method. Reducing power of *E. kotschyanum* was determined by FRAP assay and results show that ethanol exract had 18.79±0.15 IC_{50} value. In the same research of Ozay *et al.* showed that *Alyssum foliosum var. megalocarpum* had.0.149 ±0.005 EC_{50} value for ferric reducing power activity in methanol extract.

Duran *et al.* [16] were found %70.074 DPPH scavenging activity in methanol extact of *E. kotschyanum*. In this work, ethanol extract of *E. kotschyanum* had higher scavenging activity (%89.39±0.61 and IC_{50}: 0.2967±0.03) with DPPH assay. In another free radical scavenging assay, we were determined acetone extract of *E. kotschyanum* had (%96.30±0.12 and IC_{50}: 47.15±0.74) the most ABTS scavenging activity. Boudouda *et al.* [37] determined the highest ABTS scavenging activity (%90.34±0.15) of *Biscutella raphanifolia* (Brassicaceae) in butanolic extract. This two species that are been in same family, belong to different genus, so their scavenging activity can be different.

In addition to the total polyphenolic-content determined by Folin–Ciocalteu method. Duran et al. [16] were determined 0.767 mg GAE/ml in 90 % methanol + 9 % water + 1 % acetic acid mix of *Erysimum kotschyana.* Our results showed that the highest phenolic content amount was respectively obtained in acetone (4. 883±0.47 mgGAE/g).Our results were served higher phenolic content, this difference may be result from the habitat, edaphic and climatic factor and used solvent variety. Also, ethanol exract of *E.kotschyanum* give that high total flavonoid content as 93.322±1.57 mgQE/g. another study was served that water extracts of *E. aurantiacum* had about 50 µg/ml by Lee *et al.* [17].

Lee *et al.* [17] determined the effect of characteristic antimicrobial activity on water extracts of *E. aurantiacum*. Their study showed that *E. aurantiacum* had over 30% antimicrobial activity on the bacteria causing a respiration disease. In our study, ethanol extract had most effective antibacterial activity (17±1 mm) of *E. kotshyanum* against for *P. aeruginosa* (Table-3).

The results of cytotoxic activity of *E. kotschyanum* exracts by brine shrimp lethality test are present in Fig. 1. Significant cytotoxicity was obtained from ethanol exract of *E. kotschyanum* with LC50 value of 724.84 µg/ml. In other study, Ozay *et al.* [36], LC50 value of *Alyssum simplex* (Brassicaceae) was determined as 29.22 µg/ml. This results are shown that *E. kotschyanum* exracts had remarkable cytotoxic activity in this method.

Erysimum species was used in some phtytochemical studies and was indicated that their involves several chemical compounds. For example, Fresh leaves, roots and ripe seeds of *Erysimum corinthium* Boiss. (Brassicaceae) were investigated six glucosinolates were identified for the first time in this plant; namely, sinigrin, progoitrin, glucoiberin, 3-(methylcarbonyl)propyl glucosinolate, glucocheirolin and glucoerysolin by Al-Gendy *et al.* [18]. Ateya *et al.* [38] indicated that pyrogallol is the major compound for ethyl acetate fraction while epicatechein is the major one for total extract(1147.09 ppm). Our research showed that 9 phenolic compound was determined with standards by HPLC analysis. Results was indicated that the highest phenolic compound amount is vanilic acid (753.21 μg/g), the lowest phenolic compound amount is 3,4-hydroxybenzoic acid (18.05 μg/g) (Fig. 2).

Conclusions

In conclusion, such detailed studies on biological activity and chemical composition of *E. kotschyanum* have been performed with several spectroscopic methods. Thus, their chemical composition was determined indicating significant amounts of phenolic compounds. In terms of the biological activity assays, antioxidant, antibacterial, and cytotoxic activity and phenolic contents of the extracts were obtained. For antioxidant activities, β – Carotene/Linoleic Acid Assay, FRAP, ABTS and DPPH were used. Total phenolic and flavonoids contents were determined. Antibacterial activity was evaluated against *P. aeruginosa*, *E. coli* and *S. aureus* subsp. *aureus*. For determination of cytotoxixity activity was used Brine Shrimp Lethality Test. Also, phenolic components of *E. kotschyanum* were revealed by HPLC. Our all results show that *E. kotschyanum* may be used as potential antioxidant source for pharmaceutical studies.

Acknowledgements

Financial support from Scientific Research Projects Coordinatorship of Pamukkale University, Turkey is gratefully acknowledged.

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