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RESEARCH ARTICLE

Studies on the Conformity of *Hibiscus sabdariffa* L. Samples from Turkish Market to European Pharmacopeia

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Summary

Hibiscus genus which belongs to Malvaceae family, has more than 300 species in the world. Hibiscus sabdariffa L. is grown in the South and Southeast Asian countries. In-vivo and in-vitro studies have shown that it has many biological activities. The use of the red calyx of the plant as a colorant in alcoholic beverages has been approved by the U.S. Food and Drug Administration (FDA). Additionally, calyx of the plant is registered in the European Pharmacopoeia with the name "Roselle". Hibiscus sabdariffa is not a native plant in Turkey, but it is imported and sold for various purposes. The aim of this study is to compare the sample purchased from Egypt and the samples bought in the market and to determine if they conform to Hibiscus sabdariffa monograph in the European Pharmacopoeia. For this purpose, morphologic and microscopic analyses, TLC analyses, foreign matters, losses on drying, total ash quantities, colouring intensities, acid percentages in citric acid equivalent have been assigned on 8 samples brought in the market and the Egyptian sample, respectively. In addition, DPPH radical scavenging effects of the extracts and their total phenol contents were also investigated.

Key Words: *European Pharmacopeia,Hibiscus sabdariffa L., Malvaceae*

Türkiye Piyasasından Toplanan Hibiscus sabdariffa L. Örneklerinin Avrupa Farmakopesi'ne Uygunluğu Üzerine Çalışmalar

Özet

Dünyada 300'den fazla türü bulunan Hibiscus cinsi, Malvaceae familyasında yer almaktadır. In- vivo ve in-vitro çalışmalarda birçok biyolojik aktiviteye sahip olduğu bulunan Hibiscus sabdariffa L. Güney ve Güneydoğu Asya ülkelerinde yetiştirilmektedir. Bitkinin kırmızı renkli kaliksinin alkollü içkilerde renklendirici olarak kullanımı, Amerikan Gıda ve İlaç Dairesi (FDA) tarafından onaylanmıştır. Ayrıca bitkinin kaliksi Avrupa Farmakopesi'nde "Roselle" adı ile kayıtlıdır. Bitki, ülkemizde doğal olarak yetişmemekte fakat değişik kullanım amaçları için ithal edilerek satılmaktadır. Bu çalışmanın amacı, Mısır'dan getirilen ve piyasadan satın alınan H. sabdariffa örneklerini mukayeseli olarak incelemek ve örneklerin Avrupa Farmakopesi'nde yer alan Hibiscus sabdariffa monografına uygunluğunu tespit etmektir. Bu amaçla, piyasadan alınan 8 örnek ve Mısır'dan getirilen örnek üzerinde sırasıyla; morfolojik ve mikroskobik analiz, İTK analizi, yabancı madde, kurutmada kayıp, bütün kül, renk şiddeti, sitrik asit cinsinden % asit miktarı tayin edilmiştir. Ayrıca ekstrelerin total fenol miktarları ve DPPH radikal süpürücü etkileri de araştırılmıştır.

Anahtar Kelimeler: *Avrupa Farmakopesi,Hibiscus sabdariffa L., Malvaceae*

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INTRODUCTION

Hibiscus sabdariffa L. (Malvaceae) is an erect annual herb known as Roselle, rozelle, red sorrel, jelly okra, lemon bush in English speaking countries. It is called karkade, or carcadé in North Africa and Near East and these names are used in the pharmaceutical and food-flavouring trades in Europe. It has been cultivated worldwide throughout the tropics for its succulent, fleshy, edible calyx. The deep red tea from the thick, cup-shaped calyces is consumed as a cold beverage and as a hot drink (sour tea). On the other hand, calyces are commonly used to make jellies and jams. In Ayurvedic literature of India, different parts of the plant have been recommended for various ailments like hypertension, pyrexia and liver disorders. It is also used in folk remedy for treatment of gastric ulcers, hypercholesterolemia, kidney stones, and strangury, in Thailand. Additionally, in many countries, decoctions and infusions of calyces are used as antiseptic, aphrodisiac, astringent, cholagogue, demulcent, digestive, diuretic, emollient, purgative, refreshment, sedative, stomachic, and tonic, traditionally (1,2).

According to Commission E Monographs, calyces of *Hibiscus sabdariffa* L. var. *sabdariffa* are useful to stimulate the appetite, for colds, for catarrhs of the upper respiratory tract, to dissolve phlegm, as a gentle laxative, diuretic, and for circulatory disorders (3). The phytopharmaceutical industry in Germany considers *H. sabdariffa* among the neutral products (4).

According to the literature survey, many alkaloids, amino acids, anthocyanins, flavonoids, lipids, naphthalenes, polysaccharides, quinones, sesquiterpenes, steroids, and terpenoids were identified from different parts of *Hibiscus* species (5). Many biological activity studies were conducted on *H. sabdariffa* and some of these compounds have been shown to have antibacterial (6), antifungal (6), antihypertensive (7), anti-inflammatory (8), antioxidant (9), antispasmodic (10), antitumor (11) and hypoglycaemic (12) activities.

The objectives of this research were to characterize the morphological, microscopic and chemical properties of dried calyces, to establish the chemical fingerprints by thin layer chromatography as well as to determine total phenol content and radical scavenging activities of the selected samples. Roselle monograph in the European Pharmacopoeia 7.0 (EP) was used to compare the results (13).

Table 1. Plant Material Data

Sample	Collection Date	Place
1	2008	Egypt
2	13.09.2010	Kızılay
3	08.09.2010	Söğütözü
4	14.09.2010	Tunalı Hilmi
5	13.09.2010	Kızılay
6	15.09.2010	Ulus
7	13.09.2010	Kızılay
8	15.09.2010	Ulus
9	09.08.2010	Söğütözü

MATERIAL AND METHODS

Plant Material

The calyx samples of *Hibiscus sabdariffa* L. were obtained from various herbalists in Ankara (n = 8) in 2010 and a reference sample were purchased from Egypt by the help of Prof. Dr. Ekrem Sezik. All samples were received as dried raw material (Table 1).

Morphological Analyses

The morphological properties of the inner and outer sides of the entire calyx samples and the reference sample were determined on a plotting paper according to the "Identification" part of EP 7.0 monograph and their photos were taken. General views of the samples and the colours, shapes, lengths of the calyces were determined.

Microscopic Analyses

In order to determine the anatomical characters of the samples, calyces were powdered and examined under a microscope, using chloral hydrate solution R. Characteristic elements were determined and their photographs were taken. A Motic BA200 model microscope was used in microscopic analyses (ocular:CPL W10X; objective: 4X, 10X and 40X).

Thin Layer Chromatography Analyses

1 g each of powdered calyx samples were extracted by 10 ml ethanol (60 % v/v), shaken for 15 minutes and

Table 2. Results of Pharmacopeia Analysis on Roselle Samples*

No.	Foreign Matter	Loss on Drying	Total Ash	Colouring Intensity	Acid Content
1	0.00	**11.15 ±0.47**	7.79 ±0.53	0.88 ±0.00	**8.60 ±0.04**
2	**3.94**	**11.52 ±0.30**	6.56 ±0.40	0.44 ±0.00	**8.38 ±0.13**
3	0.05	9.63 ±0.04	6.71 ±0.15	0.96 ±0.02	**6.45 ±0.02**
4	1.86	10.70 ±0.69	6.52 ±0.09	0.38 ±0.01	**7.66 ±0.30**
5	0.68	**11.16 ±0.08**	6.50 ±0.18	0.57 ±0.01	**6.69 ±0.06**
6	0.07	10.79 ±0.04	6.23 ±0.28	0.57 ±0.00	**8.70 ±0.06**
7	0.59	6.81 ±0.10	6.22 ±0.03	0.43 ±0.00	**7.15 ±0.21**
8	0.23	7.85 ±0.60	7.51 ±0.00	0.84 ±0.01	**6.60 ±0.04**
9	0.83	10.03 ±0.07	9.31 ±0.06	**0.28 ±0.00**	**8.56 ±0.08**
EP St.	**<2.0%**	**<11.0%**	**<10.0%**	**>0.35**	**>13.5%**

* All experiments were done in triplicate and results were expresses as mean ±S.E.M.

filtered. Quinaldine red and sulfan blue dissolved in methanol were mixed and used as references. Merck Silica gel TLC plates (Aluminium Sheets, 20x20 cm 60 F_{254}) were used. A mixture of anhydrous formic acid, water, butanol (10:12:40 v/v/v) was used as the mobile phase.

Foreign Matters

100 g each of whole dried calyx samples were spread in thin layers on a white paper. Foreign matters were detected by inspecting with naked eye, separated and weighed. Then the percentages of foreign matters were calculated (Table 2).

Losses on Drying

Powdered Roselle were accurately weighed (1.000 g) in glass weighing bottles previously dried in an oven to a constant mass. The percentages of weight losses were calculated after drying powdered drugs in an oven at 105°C for 2 h.

Total Ash

Powdered Roselle were weighed (1.000 g) in silica crucibles previously ignited by a furnace to a constant mass. Samples were incinerated at 600°C for 3 h and weighed after they were allowed to cool in desiccators.

Colouring Intensities

Samples were coarsely powdered and 10 g of each sample was reduced to a powder. To 1.000 g of the powdered Roselle, 25 ml boiling water were added in flasks. Flasks were heated for 15 min on a water bath, shaken frequently. Hot extracts were filtered into 50 ml graduated flasks. First flasks were washed with hot water for 3 times (5 ml each) and these parts were added to filtrates. After cooling, extracts were diluted to 50 ml with water and the absorbance values were measured at 520 nm using water as the compensation liquid.

Acid Percentages

Carbon dioxide free water (100 ml) were added to powdered Roselle (1.000 g) and shaken for 15 min. They were then filtered and 100 ml of carbon dioxide free water were added to 50 ml of these filtrates. They were titrated with 0.1 M sodium hydroxide to pH 7.0. The end-points were determined potentiometrically and the acid percentages were calculated in citric acid equivalent (1 ml of 0.1 M sodium hydroxide is equivalent to 6.4 mg of citric acid.)

Analyses on Roselle Extracts

Preparation of the Extracts

Powdered calyx samples were extracted with 50 ml hot water, shaken for 3 h. Filtered extracts were dried in a freeze-dryer. Yield percentages of the extracts were calculated and are given in Table 3.

Total Phenol Contents

Total phenol content of the Roselle extracts were determined, using Folin-Ciocalteu's method,

Table 3. Total Phenol Contents and Radical Scavenging Activities of Roselle Extracts

No.	Yield %	Total Phenol Content ±SD	DPPH Radical Scavenging Activity (Inhibition % ±SD)		
			0.5 mg/ml	1 mg/ml	2 mg/ml
1	39.9	37.2 ±2.0	69.3 ±0.6	55.8 ±2.7	21.2 ±3.2
2	41.1	16.4 ±3.1	60.9 ±0.8	71.9 ±0.3	64.6 ±1.1
3	39.7	49.1 ±2.7	58.8 ±2.1	27.0 ±1.2	44.6 ±9.8
4	45.9	22.4 ±0.7	68.4 ±1.4	64.2 ±0.5	47.4 ±2.3
5	38.9	29.5 ±0.7	67.5 ±0.9	54.1 ±2.4	15.1 ±2.4
6	37.7	24.0 ±0.7	60.4 ±1.4	66.3 ±0.7	50.6 ±0.6
7	43.1	27.8 ±1.5	65.5 ±1.8	59.4 ±1.6	42.0 ±2.9
8	36.4	27.6 ±2.1	65.5 ±0.9	47.1 ±2.4	6.5 ±1.8
9	42.0	18.0 ±0.4	65.5 ±0.9	66.3 ±0.7	57.1 ±0.6
BHT			**0.05 mg/ml**	**0.1 mg/ml**	**0.2 mg/ml**
			61.8 ±2.7	72.5 ±1.3	76.1 ±0.3

* All experiments were done in triplicate and results were expresses as mean ±S.D.

adapted to 96 well-plates by Zongo et al. (2010) (14). 20 µl each of Roselle extracts were mixed with 100 µl Folin Ciocalteu's reagent in 96 well-plates. After incubation for 5 min at room temperature, 80 µl of sodium carbonate solution each were added to the wells. The plates were shaken and incubated in the darkness, at room temperature for 30 min. The absorbance values were measured at 735 nm, utilizing an ELISA microplate reader (VersaMax, Molecular Devices, USA). The measurements and calculations were evaluated using Softmax PRO 4.3.2.LS software. Gallic acid was used as standard and the equation was $y = 5.306x+0.0587$ ($r^2 = 0.9986$), where y is the absorbance value read at 735 nm, and x is the concentration of the gallic acid (µl/ml). Total phenol contents of the extracts were expressed as gallic acid equivalent per gram of Roselle extract.

Radical Scavenging Activities

The DPPH radical scavenging activities of the extracts were determined in 96 well-plates, according to the method reported by Jung et al (2011) (15). 160 µl of Roselle extracts (in 2, 1, 0.5 mg/ml concentrations) were mixed with 40 µl of DPPH solution and were incubated in darkness for 30 min. Then the absorbance values were measured at 520 nm, utilizing an ELISA microplate reader. BHT was used as a positive control at 0.05, 0.1 and 0.2 mg/ml concentrations.

Statistical Analyses

All experiments were done in triplicate. The mean of three results were calculated, standard deviation and standard error of the mean were also given in tables.

RESULTS AND DISCUSSIONS

Hibiscus sabdariffa is a native of Old World Tropics, probably a native of West Africa or the East Indies. Although does not grow in Turkey, it is well known and widely consumed as a colorant in herbal tea preparations. It is very popular and cultivated in some parts of North Africa, especially in Egypt and Sudan. Hence, identified plant material purchased from Egypt is used as a reference and conformities of both the reference (Egyptian sample) and the samples (bought in Turkish market) to European Pharmacopeia were evaluated.

The pictures of calyx samples are given below (Figure 1). They were fleshy, dry, easily fragmented and bright red or purple, somewhat lighter at the base of the inner part. The calyces were joined in the lower half to form an urceolate structure. The upper half divided to form 5 long acuminate recurved tips. The epicalyx consisted of 8-12 small leaflets adnate to the base of the calyx. Lengths of all the calyx samples were between 5-8 cm and their widths ranged from 3 to 4 cm. According to morphological analyses, all

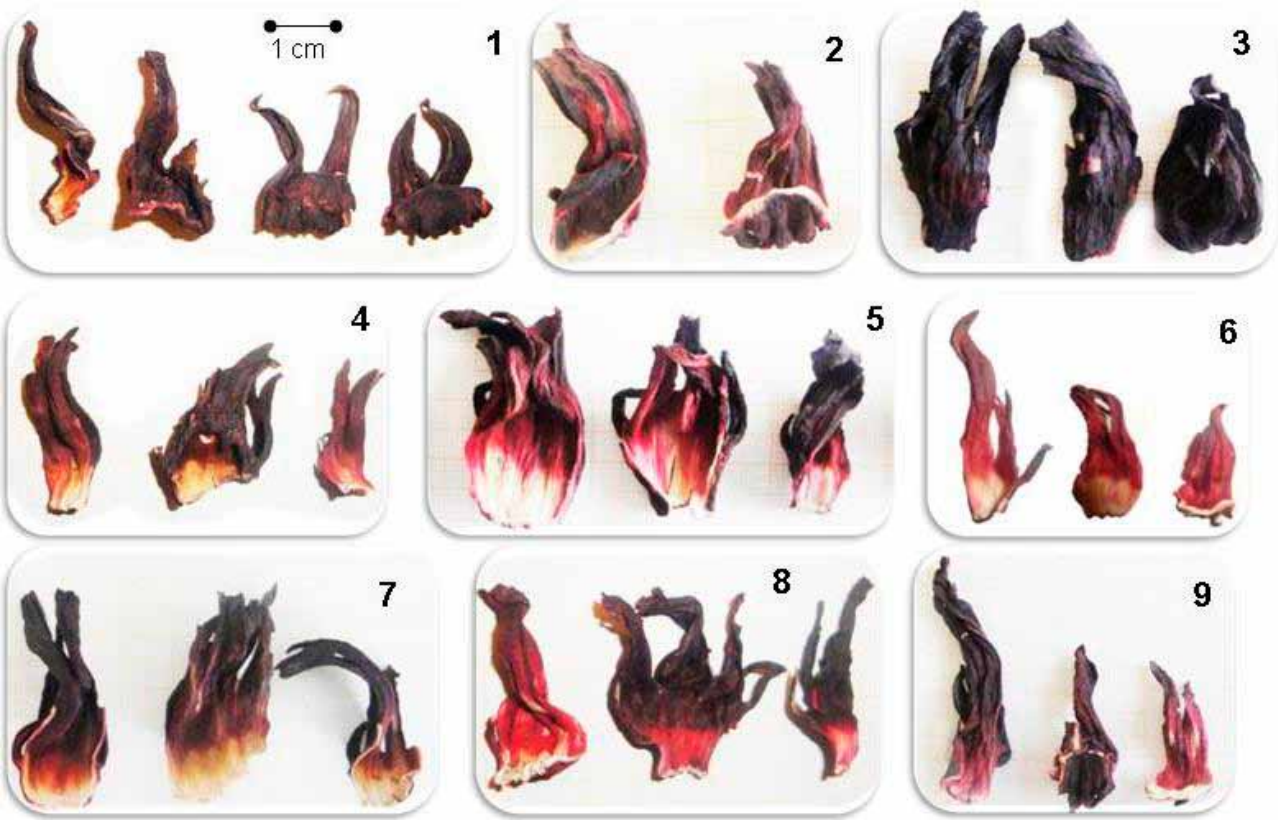


Figure 1. General views of the collected Roselle samples

the samples and the Egyptian sample were found to conform to EP monograph.

All the examined powdered samples were homogeneous, red or purplish-red coloured, with strong acidic taste, and had characteristic, aromatic odour. Powdered samples were examined by a microscope and the properties of their elements were described. Photos of the diagnostic characters were taken and given below in Figure 2. In the examinations, mainly red fragments of parenchyma cells [1] containing calcium oxalate crystals [11] and with mucilage-filled cavities [6], epiderma cells of the calyx [2], polygonal epiderma cells [3] with anisocytic stoma [4], sclerenchymatous fibres, rarely pitted vessels [7], fragments of unicellular smooth bent and stellate covering trichomes [5, 8, 9], glandular trichomes [12], and numerous fragments of vascular bundles with spiral and scalariform vessels [10,13] were seen in all samples. In EP monograph, it is mentioned that rounded pollen grains with spiny exine could be found in Roselle powder, but no pollen was observed in our examinations. All specific tissues and elements of the Roselle except pollen grains were determined in all samples. Additionally, there was no plant tissue belonging to a foreign plant.

After thin layer chromatography analyses, plates were examined immediately in daylight. In accordance with the EP monograph, zones of quinaldine red were found higher than the intense violet zones of roselle antocyanins, and zones of sulfan blue were found lower than these antocyanin zones. Second antocyanin zones (violet-blue) were found under the sulfan blue zones. Intense violet (first) antocyanin zones were seen in every sample but violet-blue (second) antocyanin zones were dark in samples 1, 3 and 8, and weak in sample 5, but they were not below the sulfan blue zone, as mentioned in EP monograph. This second antocyanin zones were not seen in other samples (Figure 3). According to the literature, another plate was developed in ethyl acetate-glacial acetic acid-formic acid-water (100:11:11:26) mobile phase. Two major bands were examined

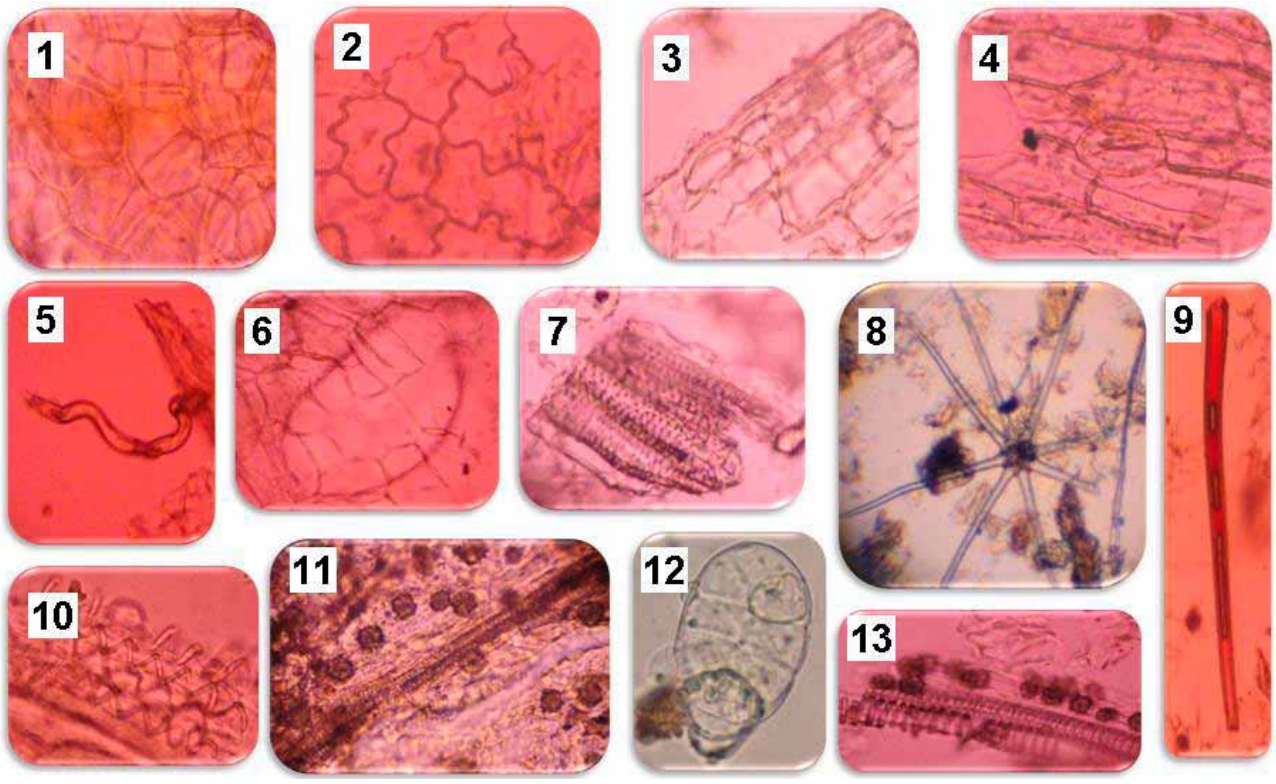
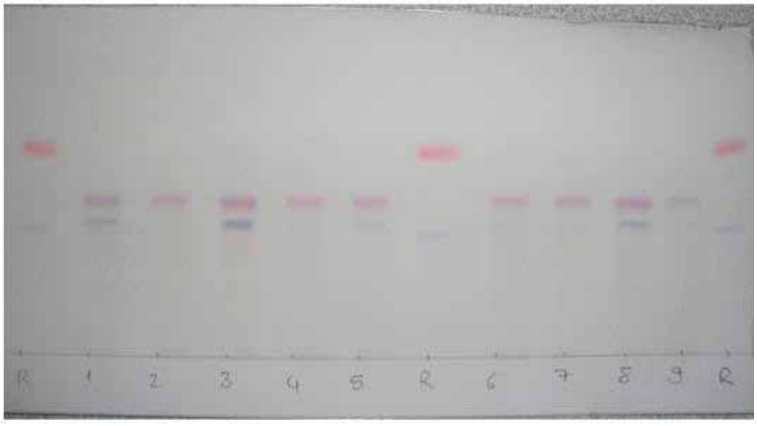


Figure 2. Microscopic analyses of Roselle samples with chloral hydrate solution.

in all samples at Rf 0.15-0.2 that are probably due to delphinidin-3-glucosyl-xyloside (hibiscin) and delphinide-3-glucoside-xyloside reported as major pigments (16).

In the EP monograph, it is said that foreign matter in the Roselle sample has to be maximum 2% of the fragments of fruits (red funicles and parts of the 5-carvened capsule with yellowish-grey pericarp; flattened and reniform seeds with a dotted surface). Loss on drying level has to be 11.0% maximum and total ash should not more than 10.0%. Roselle is used as colorant in beverages widely so colouring intensity is an important parameter for the quality. In colouring intensity experiment, the absorbance has to be 0.350 minimum for the whole drug and not less than 0.250 for the cut drug. Acid content of Roselle has to be 13.5 % minimum expressed as



Top of the plate	
Quanildine red: an orange red zone Sulfan blue: a blue zone	An intense violet zone An intense violet-blue zone
Reference solution	**Test solution**

Figure 3. Thin layer chromatogram of the samples and the TLC standard in EP monograph

citric acid for dried drug. The results with respect to foreign matters, losses on drying, total amounts of ash, colouring intensities and acid contents are given in Table 2.

Roselle is rich in phenolic acids, anthocyans (cyanidin, delphinidine and their glucosides) and flavonoids (gossypetine, hibiscetine, sabdaretine) (2,) so that total phenol content of Roselle extracts were determined. Additionally antioxidant effects of phenolic compounds are well known. Therefore, antioxidant potentials of the Roselle extracts were compared by DPPH radical scavenging activity results. Butylated hydroxy toluen (BHT) was used as positive control. Total phenol contents (mg gallic acid/1g extract) and DPPH scavenging activity results are given in Table 3.

CONCLUSION

Many studies were conducted on nutritional and therapeutic properties of Roselle, as well as the natural antioxidant compounds isolated from its calyces, especially anthocyanins and protocatechuic acid, this plant may be a source of therapeutically useful products for the treatment of various chronic and degenerative diseases such as diabetes, hypertension, hypercholesterolemia as well as certain types of cancers (17).

In this study 8 roselle samples purchased from different herbalists of Ankara were examined according to EP standards. Before the experiments, labels of the products were carefully examined. Turkish and Latin names of the plant were wrong in the labels of some samples. Roselle is called "Afrika bamyası" in Turkish, but four of the Roselle samples used in this study were named as "Nar çiçeği" and "Bamya çiçeği" incorrectly. Also Latin names were mentioned as "*Hibiscus esculentus*" in three labels. On the contrary, according to results of the morphological, microscopic, chemical characters of the samples purchased from the herbalists, that all the samples have been proven to be *Hibiscus sabdariffa*.

However, it has been seen that the morphological and microscopic characters of the samples were all suitable to the officinal drug standards in European Pharmacopoeia, while the acid contents of all samples were lower than the standards. Total ash levels of all samples matched the EP standards. Loss on drying value of one sample and foreign matter value of another sample were higher than the expected.

Additionally, total phenol contents and radical scavenging activities of Roselle extracts were determined. Total phenol contents of the extracts were found to be between 16.4-49.1 mg gallic acid/g extract. Inhibitory effects of the extracts in DPPH radical scavenging assay, were not found to be dose dependent. Also there was no correlation between the phenol content and radical scavenging activities of the extracts.

According to results of all experiments, the samples were not found to be suitable to the officinal drug standards in European Pharmacopoeia.

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