

Comparison of Commercial *Calendula officinalis* L. Samples with Pharmacopeial Drug: Antiradical Activities and Chemical Profiles

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ABSTRACT *Calendula officinalis* L. (Asteraceae) is traditionally used as a potent anti-inflammatory medicinal plant. The commercial petal samples of marigold are mostly used as herbal tea for its sedative and oral health effects due to its anti-viral and anti-bacterial properties. Its significant pharmacological activities are linked to the presence of distinct classes of compounds, particularly phenolic compounds, carotenoids, volatile oil, terpenoids and flavonoids. Taking into consideration that the amount and type of constituents could be variable in the commercial samples provided from Cyprus and Turkey compared to the standard due to various factors such as ecosystem and climate, time of harvesting, drying procedure and soil type, in this article the quality and safety of market samples was determined while ethanol and hexane extracts of standard and commercial *C. officinalis* samples were investigated for their anti-radical activity using ABTS and DPPH method. They both demonstrated dose-dependent manner anti-radical activity, further investigation by TPC (Total Phenolics Content) and HPLC provided evidence that both commercial and standard samples present similar fingerprint in terms of composition, and the total phenol equivalent to Gallic acid is not significantly different ($P < 0.05$); therefore, both samples can be considered to be good and safe antiradical candidates used alternatively with the pharmacopeial drug.

KEYWORDS: *Calendula officinalis* -1; phenolics -2; DPPH -3; ABTS -4; HPLC -5.

1. INTRODUCTION

Through centuries interesting therapeutic properties of plants have been found and transferred orally from generation to generation by tribesmen notably from Pacific countries, north to South America, and tropic region of Africa in which most of the valuable plant species are located.¹ Although in past the usage of herbal medication was limited to the rural population, nowadays they have gained more importance among people due to the fact that in contrast to chemical medications bearing countless adverse effects and side effects, natural products are considered as a safe treatment line.² In the pharmaceuticals modern age, again all the attention has been attracted to herbal drugs and natural products. In other words, plant-derived natural products along with their semi-synthetic and synthetic analogs play a key role in the new medications' discovery and development process used against various kinds of disease.³

Calendula officinalis L. (Asteraceae) is one of the therapeutic plants that has been used for topical treatments as a natural remedy for mild skin complications and wound healing. It is also available in modern dosage forms; ointment, lotion, and gargle in OTC shelves for patients to take as a hypoallergenic anti-inflammatory medication.^{4,5} *C. officinalis* also known as marigold is an annual flower, rarely found as a biennial, that appears in May to June and easily recognized by its single flower head of about 5-7 cm bright, yellowish-orange daisy-like flowers on the tip of each stem.⁶ *C. officinalis* and *C. arvensis* are the most medicinally important species of *Calendula* genus which proved to show anti-inflammatory and antipyretic activity.⁷ *Calendula* petals are composed of volatile and non-volatile contents. As non-volatiles content, it contains saponins (2-10%) classified in oleanolic acid such as calendasaponins. Moreover, it contains triterpenes (4.8%) that have a pentacyclic structure that may have one or two alcoholic groups like faradiol,

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arnidiol, Calenduladiol and triterpenes with three alcoholic groups like heliantriols and ursatriols. There are also flavonoids (0.3-0.8%) and other compounds like hyperosides, sesquiterpenes, and their glycosides and hydroxy coumarins, for example officinosides, loliolide and arvoside.^{5,8} Carotenoids, anthocyanins, flavonoids, and flavones are responsible for the yellow and orange color of these petals.⁶ Various extracts of *C. officinalis* have proven to exhibit anti-inflammatory, anti-tumor⁹, anti-bacterial¹⁰, anti-viral¹¹, immunostimulant¹², fungicidal¹³, anti-edematous¹⁴, anti-leishmanial¹⁵, anti-neoplastic¹⁶, spasmolytic¹⁷ and anti-fatigue activity¹⁸. Petal extracts are commonly used as the natural colorant and food flavoring agents.¹⁹ Moreover, extracts of calendula are widely used for the treatment of chronic gingivitis²⁰, seborrheic dermatitis²¹ and wound healing and skin injuries.²²

In this study, the quality of *Calendula* petal samples obtained from local markets, were examined and compared according to the Pharmacopeial drug. Antiradical activity and chemical composition analysis were used in this comparison. The aim of this study is to protect the public health by determining the quality of the samples found in the markets.

2. Results and Discussion

2.1. Extraction yields and chemical compositions

Calendula officinalis samples and Pharmacopeial drug were extracted with *n*-hexane and ethanol and all extraction yields are given in the Table 1. According to Table 1, the ethanol extract's yields were found to be higher than *n*-hexane for both samples and standard. Pharmacopeial sample was found to contain more polar compounds than commercial samples, because its ethanol extract yield was higher than *n*-hexane extract. The total phenolic amounts of the extracts were measured spectrophotometrically, and then equivalently calculated according to gallic acid calibration curve. Total phenolics amounts were almost the same within the ethanol extracts of standard and sample plants ($P < 0.05$). However, this situation was slightly different in *n*-hexane extracts (Table 1).

Table 1. Extraction yields and total phenolics of *C. officinalis* extracts

Sample	Extraction yield [%]	Total phenolics [mg _{GAE} /g _{ext}]
S1E	18.226	12.830±1.525*
S2E	27.618	18.018±1.780
S3E	24.850	18.788±1.542
S1H	7.272	23.214±1.765
S2H	7.086	15.657±2.029
S3H	5.200	12.279±0.253

S1 and S2 are commercial samples, S3 is Pharmacopeial drug; E is ethanol extract and H is *n*-hexane extract; *mean ± SD (n=3)

The difference in antiradical activities presented by both plants depends on small differences in total phenolics amounts within the extracts. Previous studies concluded the amount of phenolics equivalent to gallic acid to be 1.34 mg GAE L⁻¹.²³ Moreover, in further investigation, rosmarinic acid was also identified to be present in the extracts.²³ HPLC studies on *C. officinalis* identified polyphenols present to be gallic acid, catechin, syringic acid, cinnamic acid, cholinergic acid, caffeic acid, ferulic acid, rutin and quercetin.²⁴

Reverse phase separation was used in the present evaluation and obtained HPLC chromatograms given in (Figure1). Each extract was investigated in different wavelengths to be identified, benzoic acids in 280 nm, hydroxycinnamic acids in 320 nm, and flavonoids in 360 nm. Although the HPLC fingerprints within the three wavelengths of the S1 and S2 extracts are very different from each other, the S1 extract was found to be quite similar to the standard (Figure 1). On the other hand, both extracts are similar to the pharmacopoeia sample in terms of flavonoid content. According to Figure 1, the majority of peaks in the S1 and S2 have the same retention time with slight variation in intensity and peak area than the standard plant. For the quantitative comparison of the S1 and S2 extracts with standard plant were injected in same concentration to the HPLC in the same conditions. The peak areas with same retention time in the extracts were calculated for quantification of the relative peak amount using Eq 1.

$$\text{Relative peak amount} = \text{sample peak area} / \text{standard peak area} \quad (\text{Eq 1})$$

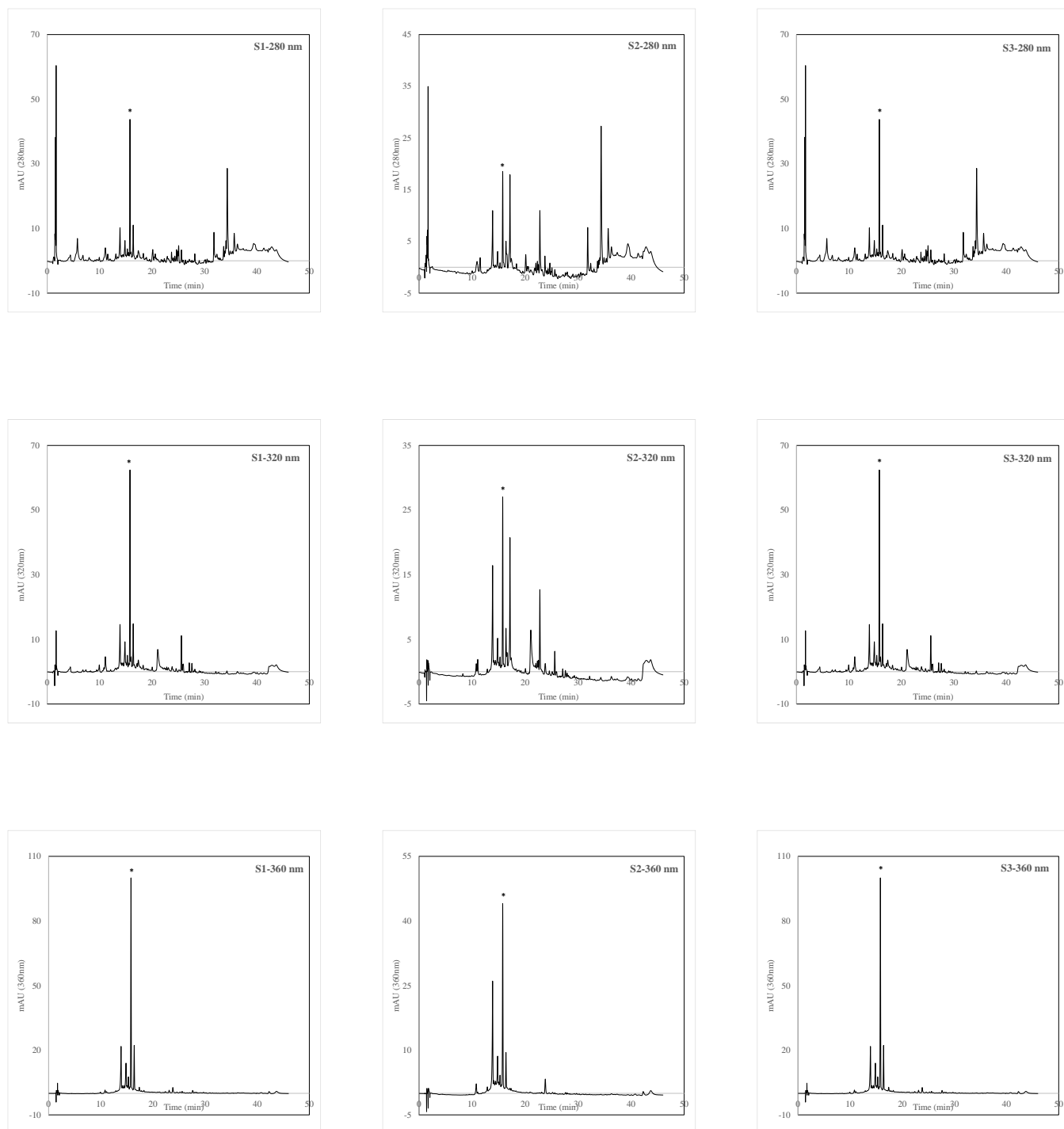


Figure 1. HPLC Chromatograms of *C. officinalis* samples and Pharmacopoeial plant (*: main peak, 17.151 min)

Three wavelengths were used in the calculations for benzoic acids, hydroxycinnamic acids and flavonoids at 280, 320, and 360 nm respectively. These phenolic derivatives were classified according to their

UV spectra collected by HPLC-PDA system. The calculated relative peak amounts are given in Table 2. According to the peak areas within the Table 2, there was no distinctive difference was observed for the major constituents of commercial sample provided from Turkey (S1) when compared to standard. In both extracts, there is a noticeable main peak at 17.151 min in 320 nm wavelength.

Table 2. Relative peak amounts of S1E and S2E extracts of *C. officinalis*

Time (min)	S1E	S2E	calculation wavelength (nm)
5.77	0.1	0.0	280 nm
11.099	0.5	0.5	320 nm
11.1	0.5	0.6	280 nm
11.555	0.7	1.4	280 nm
13.887	0.5	1.2	360 nm
14.836	0.4	0.5	360 nm
15.291	0.3	0.5	360 nm
15.793	0.4	0.4	360 nm
16.408	0.4	0.4	360 nm
17.151	4.7	11.4	320 nm
17.385	0.0	0.8	280 nm
18.346	1.1	0.5	280 nm
20.136	0.7	0.8	280 nm
20.453	1.1	1.1	280 nm
20.614	0.6	0.7	280 nm
21.089	0.8	1.0	320 nm
23.821	1.5	1.4	360 nm
24.349	0.0	0.6	280 nm
24.681	0.6	0.6	280 nm
25.076	0.3	0.4	280 nm
25.602	0.4	0.5	320 nm
25.608	0.3	0.3	280 nm
27.105	0.8	0.4	320 nm
27.601	0.4	0.4	320 nm
31.811	1.3	1.1	280 nm
32.372	2.1	1.0	280 nm
33.664	0.6	0.4	280 nm
33.904	0.8	0.9	280 nm
34.101	0.6	0.3	280 nm
34.346	1.1	1.0	280 nm
34.889	1.7	1.1	280 nm
35.676	1.3	1.0	280 nm
36.289	1.1	1.0	280 nm
39.403	0.0	1.0	280 nm

Similar studies on *C. officinallis* found in the literature and different variations were recorded on the chemical composition of *C. officinalis* due to collection area, season and plant parts used.^{25,26} Quantitative differences within the constituents were significant between different extracts because of different solubility

of the compounds.²⁵ Methanol extracts of *C. officinalis* collected in Hungary were investigated by HPLC. The results represent that flavoxanthine is the most abundant constituent, its presence is measured up to 21.09%.²⁷ 19 carotenoids were identified in *C. officinalis* collected from National Institute of Floricultural Science (Tsukuba, Japan) and extracted by acetone. The most abundant carotenoids were flavoxanthin, luteoxanthin and lycopene present in respective amount of 28.5%, 11.0% and 8.7%.²⁸

2.2. ABTS^{•+} assay

ABTS^{•+} assay is a simple method to investigate the antioxidant activity of extracts based on the decolorization of ABTS^{•+} solution and its absorbance inhibition at 734 nm. Therefore, the lower absorbance indicates better antiradical effectivity. Different concentrations of *C. officinalis* extracted with hexane showed almost no antiradical activity against ABTS^{•+} and their associated graph remained constant over a 30-minute time interval. On the other hand, ethanol extract showed a higher decrease in the concentration of ABTS^{•+} free radical and consequently higher antiradical activity over a specific time in comparison with the *n*-hexane plant extracts.

According to Figure 2 and 3, the linear correlation was found between concentration of *C. officinalis* extracted by ethanol and the inhibition percentage of ABTS^{•+}. The IC₅₀ value for ethanol extract of the commercial samples were 15.9 µg/ml and 17.94 µg/ml which were close to standard (16.07 µg/ml). This means that the ABTS^{•+} scavenging activity of the commercial samples have been almost the same as the standard. Similar study in the literature on ethanol extract of *C. officinalis* resulted in IC₅₀ 6.5 µg/ml, however, in this study the plant was collected from the Botanical Garden in Ooty, Nilgiris.²⁹ Further studies on *C. officinalis*, reported IC₅₀ value to be 154.77 mg/ml³⁰ in ethanol extract while supercritical fluid extract recorded highest IC₅₀ value by ABTS^{•+} assay at 442.40 mg/ml.³⁰

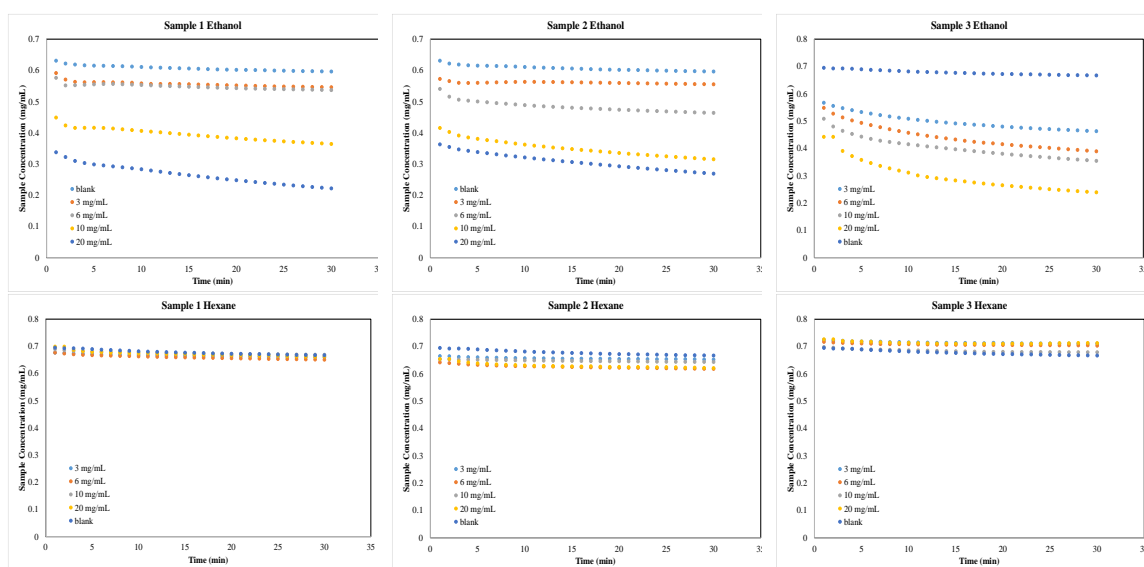


Figure 2. Concentration depended kinetic curves of *C. officinalis* extracts for 30 min

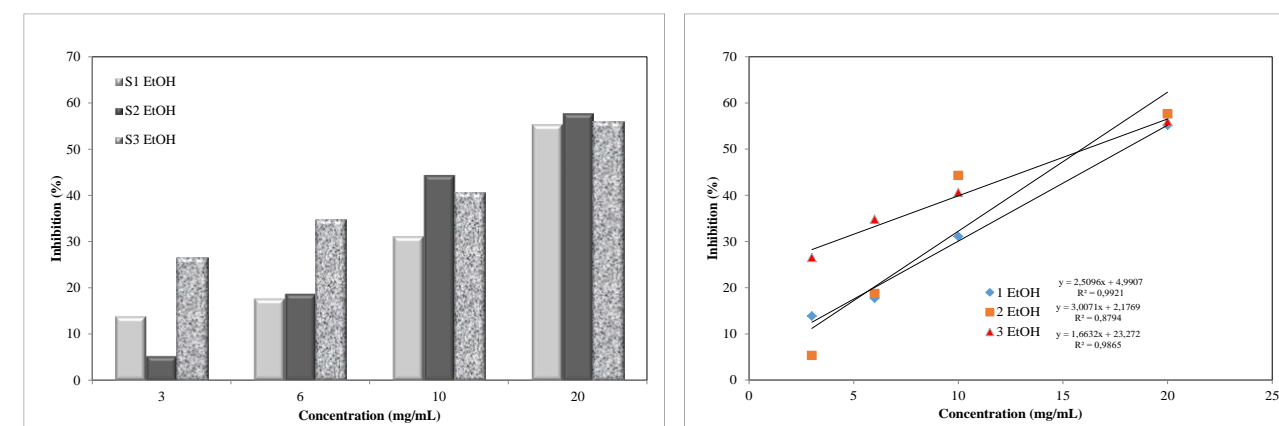


Figure 3. ABTS⁺ radical inhibition (%) values of *C. officinalis* extracts (left graph: bar graph for inhibition values of the extracts; right graph: linear regressions for concentration depended inhibitions of the extracts)

2.3. DPPH• assay

The purple color of DPPH• within the methanol solution turns colorless in an antioxidant dose-dependent manner. IC₅₀ values of the sample and standard extracts were calculated according to regression equations given in (Figure 4). The regression coefficient of ethanol extracts was almost one and the linearity of the ethanol extracts was found to be higher than hexane extracts of both standard and sample plants.

The IC₅₀ values for the ethanol extract of commercial samples were calculated to be 11.81 µg/ml, 13.13 µg/ml, which is slightly lower than the standard (23.19 µg/ml) because of their differences in phenolic configurations. It is worth mentioning that 50% ethanol extract showed IC₅₀ values at 97.1±2.1 µg/ml in the other independent research on *C. officinalis* collected from India.²⁹ Similar studies on *C. officinalis* and *C. arvensis* concluded that leaf extracts confirmed good antiradical activity on DPPH• assay. For instance, methanol extract of *C. arvensis* exerted antioxidant activity at 500 µl/ml with IC₅₀ value measured to be 19.48±82 while similar extract from flower petals exerted antioxidant activity of 27.83±1.75. Interestingly *C. arvensis* demonstrated far better antioxidant activity when compared with *C. officinalis*.³¹ Harvesting time and climate conditions effect the composition of plants prominently. Thereby wild *C. officinalis* collected in Serbia exert different level of activities, yet the level of activity shows similar behavior. Antiradical activity increased in a dose-dependent manner from 15.63% to 95.34% when the concentration increases from 0.1 to 0.9 mg/ml.³²

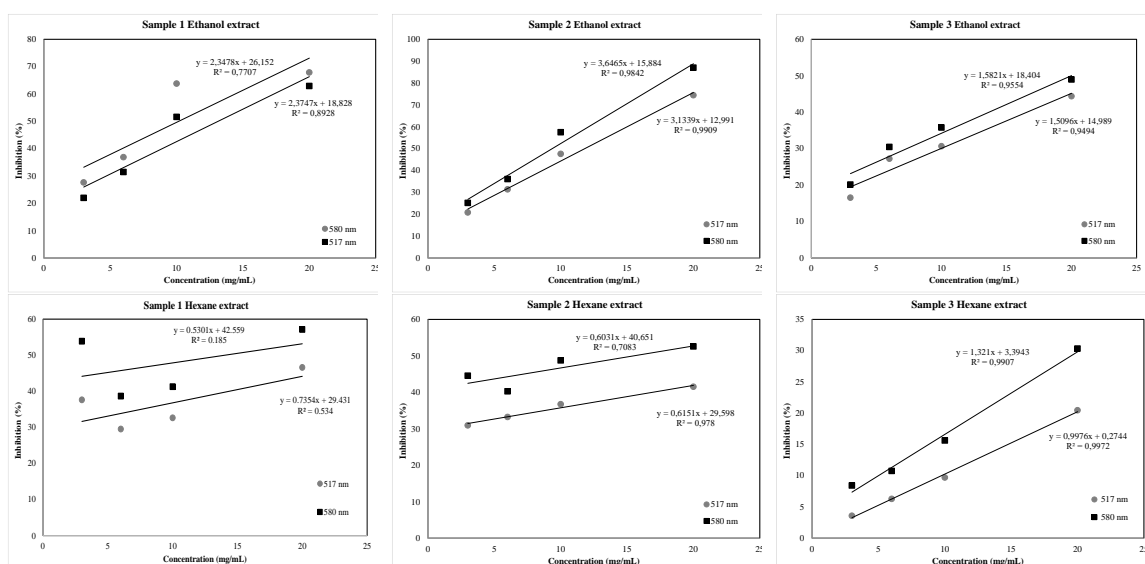


Figure 4. Concentration depended IC₅₀ values of *C. officinalis* extracts in DPPH• assay

3. CONCLUSION

C. officinalis is a self-seeding plant found in a warm and humid climate; hence it is abundantly seen in Middle Eastern countries especially in Cyprus, Turkey, and Iran. Commercially provided petals are used in folk medicine for their wound healing and anti-inflammatory activities. Petals are rich in phenolics thereby they reflect decent antioxidant activity. When the commercial samples, purchased from different countries, were compared with standard pharmacopeial plant, they demonstrated close results in antiradical activities towards ABTS^{•+} and DPPH[•]. Furthermore, almost similar fingerprints in terms of composition were obtained when investigated by reverse phase HPLC and TPC (Total Phenolics Content) method. Therefore, commercial petal samples provided from Turkey and Cyprus are considered to be safe candidates to be used alternatively instead of pharmacopeial drug. This is the first study for comparison with pharmacopeial drug. Although, further investigation might be required.

4. MATERIALS AND METHODS

4.1. Materials

Two different *Calendula officinalis* L. commercial samples were purchased from local markets in Turkey (sample 1) and Cyprus (sample 2) and were used further in assays after separation of their petals. Pharmacopeial Standard *C. officinalis* drug (sample 3) was provided from European Pharmacopoeia by Prof. Dr. Fatih Demirci (Anadolu University, Faculty of Pharmacy, Eskisehir, Turkey). All chemicals used in all experiments were analytical grade.

4.2. Preparation of Extracts

5 g of petals of each commercial *Calendula* sample and 2 g of standard *Calendula* petals were extracted using Soxhlet apparatus with *n*-hexane and same procedure was followed with absolute ethanol until the solution turned colorless. After extraction, the solvents were evaporated by rotary evaporator and kept in -20°C until experiments.

4.3. Total phenolics content

Standard gallic acid was diluted in 25, 50, 100, 150, 200, 250, 500, 750 and 1000 µg/ml concentrations and each concentration (20 µL) were added to 6 vessels in three repetitions. After that, 100 µL Folin Ciocalteau solution and 80 µL sodium bicarbonate solution was added. Blanks were prepared by adding methanol instead of gallic acid. The well plate was kept in dark for 30 min and then analyzed by (Varioskan®) UV-Vis multi-read photometer device at 760 nm. The calibration curve of gallic acid was drawn using absorbance against each concentration and correlation coefficient was calculated accordingly.

Calendula extracts were diluted in 3 and 6 mg/ml concentration using methanol. 50 µL of each concentration of extracts were mixed with 3 ml of distilled deionized water and 250 µL Folin Ciocalteau solution in 3 repetitions. After 1 min, 750 µL 20% sodium bicarbonate solution and 950 µL of distilled water were added and kept in a dark for 2 hours. Absorbance was measured in 96 well plates by UV-Vis multi-read photometer (Varioskan®) at 760 nm.

Results were evaluated with One way ANOVA test (Tukey's with 95% coefficient).

4.4. High Performance Liquid Chromatography (HPLC)

The chemical compositions of extracts were analyzed using HPLC (Agilent® 1200 infinity) with diode array detector and the compounds were eluted with formic acid/water solvents on C-18 column (15 mm×4 mm, 5µm, Agilent® 1200) using gradient elution. Gradient program of mobile phase A (2.5 % formic acid in water) and B (acetonitrile) was; 0-10 min 98% A, 10-20 min 82%A, 20-30 min 60%A, 30-35 min 30%A, 35-40 min 20%A, 40-41min 20%A, 41-46 min 98%A.

Phenolic compositions within the extracts were evaluated using different wavelengths according to the phenolic classes such as benzoic acids at 280 nm, hydroxycinnamic acids at 320 nm and flavonoids at 360 nm.

4.5. Antiradical Activities

4.5.1. ABTS^{•+} assay

ABTS⁺ solution was prepared by dissolving 36 mg of ABTS⁺ ((2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) and 6.6 mg of potassium persulfate to 10 ml water and the solution was kept in dark and under room temperature for 16 hours. The absorbance of ABTS⁺ was controlled under UV-Visible spectrometer at 734 nm before experiment and the absorbance were adjusted to 0.7-0.8 by adding water.

Extract solutions were prepared in 3,6,10 and 20 mg/ml concentrations in methanol from commercial samples and standard drug. Each sample was then transferred to 96 well plates and 145 µL ABTS⁺ were added. Blanks were prepared by adding methanol instead of sample to the same plate and gallic acid was used as standard. Samples and blanks were prepared as three repetitions. The absorbances were measured over 30 minutes (1 min intervals) at 734 nm and radical scavenging activities were calculated.

4.5.2. DPPH[•] assay

Tris buffer (7.4 PH) was prepared by adding 1.513 g Tris (Sigma Trisma®) and dissolved in 200 ml of deionized distilled water. pH was adjusted by adding 1% HCl to 7.4. 2,2-Diphenyl-1-picryl-hydrazyl-hydrate (DPPH[•]) solution was prepared by adding 9.86 mg DPPH[•] crystal powder to 250 ml of methanol and gradually stirring in the dark until dissolving. This solution was kept in dark and room temperature.

Extract solutions were prepared in 3,6,10 and 20 mg/ml concentrations in methanol from commercial samples and standard drug. Each sample was placed in 5 consecutive vessels in 50µL, and 450 µL Tris was added to all. Blanks were prepared by adding methanol instead of sample to the same plate and gallic acid was used as standard. After adding 1ml DPPH[•] solution, the well plate was kept in the dark for 30 minutes and then absorbance was measured by UV-Visible multiple read spectrometer (Varioskan®) at 517 (absorbance of DPPH[•] solution) and 580 (absorbance of carotenoids for correction) nm respectively.

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