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Research Article

Antioxidant and Anticancer Activities of *Ocimum basilicum* L. *cv. Dark Opal* (Lamiaceae)

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ABSTRACT: Background: Plants used in folk and traditional medicines have been accepted as therapeutic drug development in modern medicine. Although many studies have been focused on Lamiaceae family, few studies on medical effects of purple basil have been performed until now. **Objective:** *Ocimum basilicum cv. dark opal* was chosen for this study as it has been used in Persian traditional medicine and many Iranian dishes. It was considered important to determine the cytotoxicity effect and the reductive capacity of the purple basil oils and extracts, as this may indicate their potential as antioxidants. **Materials and Methods:** The reducing power activity of both essential oils of the leaves (before flowering) and the seeds and also methanolic extracts of roots and aerial parts (stem-leaf) (collected prior flowering), and flowers were determined by utilizing of FRAP. Also the MTT assay has been used in order to consider *in vitro* cytotoxicity of essential oils and extracts on cancerous cell line (MCF-$_7$). Moreover, the extracts were analyzed by HPLC todetermine the levels of some phenolic compounds. **Results:** The purple basil extracts have more powerful antioxidant activity than the essential oils. The MCF-$_7$ cell proliferation was significantly inhibited compared with the controls, and the oils were found to be more effective than the extracts. The phytochemical analysis of the extracts has led to the identification of 3 phenolic. **Conclusion:** Our study, partially validates the traditional use of this medicinal herb as complementary and alternative medicine.

KEYWORDS: purple basil, antioxidant, MCF-$_7$, HPLC.

INTRODUCTION

The *Ocimum* genus belongs to the Lamiaceae family[1] that includes approximately 150 species.[2] The species have variation in phenotype, oil content, composition, and possibly bioactivity.[3] Although the taxonomy of basil is complicated by the existence of numerous botanical varieties within the species that may not differ significantly in morphology,[4] a system of standardized descriptors, which include volatile oil, has more recently been proposed by Paton and Putievsky.[5] This permits easy identification of the different forms of *O. basilicum*.Dark Opal is one of the basil cultivars that is a rich source of anthocyanin. Purple basils are highly marketable herbs, not only for culinary purposes but also for their ornamental value. Interspecific hybridization and polyploidy are common within *Ocimum* genus,[6] and purple types such as 'Dark Opal', is a possible hybrid between *O. basilicum* and *O. forskolei*, which has lobed-leaves, with a sweet basil plus clove-like aroma.[7] The anthocyanins present in purple basils have been analyzed using high performance liquid chromatography, spectral data, and plasma-desorption mass spectrometry. Fourteen different anthocyanins have been identified by these analysis.[8] Apart from role of anthocyanins as pigments there are many functions performed by these flavonoid compounds in these plants (e.g. UV protection, defense against pathogens and pests, protecting DNA) and numerous scientific studies have shown that these active compounds act also as antioxidant.[9]

Recently scientists have carried out extensive research into production of herbal drugs using bioactive compounds from herbs. The application of essential oils and

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extracts of plants in the anticancer therapy may appear unconventional; however, their easy availability, pleasant aroma of the oils and low or insignificant toxicity make them more attractive candidates for the long term treatment of various chronic ailments.[10] Therefore, there has been growing interest in study of antioxidant and anticancer properties of plants. Lamiaceae family (rosemary, sage, oregano, marjoram, basil, thyme, and mints) is well known for antioxidant activity and present antioxidants such as phenolic compounds.[11] Various species of *Ocimum* have acquired special attention due to their medicinal properties and different parts of the plant (root, stem, flower, leaves) are used in the treatment of a wide range of disorders from centuries.[12]

Ocimum basilicum L. is considered to be promising essential oil crops and there is a great variation of essential oil composition (and aroma) among basil cultivars currently on the international market. The basil essential oil contains pleasant aroma and is known to possess antimicrobial, antioxidant[13,14] and insecticidal[15] activities and hasbeen used traditionally as a medicinal herb.[3] Iranian basils are also used to treat fevers, throat congestions, and stomachache.[16]

Basil is also used for pharmaceutical and cosmetic preparations due to the high content of phenolic compounds that are well-known phytochemical molecules found in all plants and act as antioxidants to prevent heart disease,[17] reduce inflammation,[18,19] lower the incidence of cancers[20,21] and diabetes,[22,23] as well as reduce rates of mutagenesis in human cells.[24,25] The antioxidant activity of phenolic compounds is mainly caused by their redox properties, which permit them to act as reducing agents, hydrogen donors and singlet oxygen quenchers.[26] Rosmarinic acid (RA) is one of the most abundant caffeic acid esters present in *Ocimum* spp. and have several important biological properties, including antioxidant, antibacterial, antiviral and anti-inflammatory activities.[27,28] *P*-Coumaric acid (CA) is also reported as an abundant plant phenolic acid and has dietary chemo protectant and antioxidant activity.[29] Moreover, it has been shown that ferulic acid (FA) may have potential as a preventative agent against colon tumor development.[30] Therefore it is important to examine the phenolic acid (PhA) constituents in purple basil extracts along with the assessment of anticancer and antioxidant properties.

However, few studies on antioxidant and anticancer activities of purple basil essential oils and extracts have been performed until now. The objective of the current study was to evaluate the anticancer activity of essential oils (leaves and the seeds) and methanolic extract of roots, aerial parts (stem-leaf) and flowers of *Ocimum basilicum L. cv. Dark Opal* against human breast cancer cell lines (MCF-$_7$), in addition to antioxidant activity using reducing power assay. In this work, high performance liquid chromatography (HPLC) was utilized to identify and quantify RA, CA and FA in the purple basil methanolic extracts.

MATERIALS AND METHODS

Plant material

The fresh roots, stems, leaves and flowers and the seeds of *O. basilicumcv. Dark Opal* were collected from a farm at Shahr e Ray city nearby Tehran, prior to flowering and flower development stages, in May 2013. After collection, the herb materials were washed with the fresh water and drained under shadow at 18–20°C for 15–25 days and then refrigerated at 4°C.

Extraction

500 mg of dried leaf, stem, and root (prior flowering) and flower materials were pulverized separately in a mortar and pestle, suspended in 5 ml of absolute methanol, and left overnight at 4°C under dark conditions. All supernatants were decanted and filteredusing a Whatman syringe filter with a 0.45 μm pore size. Samples were rotary-evaporated (45°C, 10) to near-dryness and then were stored at -20°C until used.[2]

Essential oils isolation

Essential oils were extracted from Purple basil dried leaf (prior to flowering) and seed powder samples. 100 g of each dried sample were hydro-distilled in Clevenger-type apparatus (Council of Europe, 1997). Essential oils were distilled for at least 2 h and after collection stored in the dark at -20°C until antioxidant and anticancer assessments.

Phytochemical studies

Rosmarinic acid and other phenolic acids including p-coumaric acid and ferulic acid used as standards were purchased from Sigma-Aldrich Chemicals Co., USA. The HPLC system consisted of a P580 pump (Dionex Co., Sunnyvale, CA), connected to an ASI-100 automated sample injector. A reverse phase C18 column (5 μm particle size, 25 cm × 4.6 mm) was used. The absorbance at 280 nm was measured by a PDA-100 Photodiode array variable UV/vis detector (Dionex Co.).

For HPLC analysis of phenolic compounds, mobile phase solution A consisted of 0.1% TFA (trifluoroacetic acid) in water, and absolute acetonitrile was used as solution B (Fisher Co., USA). A multistep gradient was used for all

separations with an initial injection volume of 10 µL and a flow rate of 1 mL min^{-1}. The multistep gradient was as follows: 10% B, 0–5 min; 35% B, 5–20 min; 70% B, 20–40 min; 90% B, 40–41 min; 50% B, 41–42 min; 25% B, 42–43 min; 5% B, 43–44 min; 100% A, 45 min. Phenolic acids in each sample were identified by comparing retention times to those of authentic standards and were further quantified by comparison of peak area of the standard runs.[2]

Antioxidant activity

The reducing power of the oils and extractions was measured according to the method used by Oyaizu with some modification.[31] This method is based on the abilities of a sample to reduce ferricyanide to ferrocyanide and produce a Prussian blue-colored complex (Fe^{3+}) 4[Fe^{2+} (CN-) 6]3, an intense bluish green color, that is detectable spectroscopically.[32,33]

A volume of 0.5 ml of the extracts and essential oils with different concentrations (1–5 mg/ml) was mixed with 1.25 ml of 0.2 M phosphate buffer (pH 7) and 1.25 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min followed by the addition of 1.25 ml 10% TCA and then centrifuged for 10 min at 1800 g. Then after, 0.5 ml of the supernatant was mixed with as equal volume of distilled water, followed by adding 0.1 ml of 0.1% ferric chloride ($FeCl_3$). After 10 min the absorbance of the resulting solution was measured at 700 nm. All experiments were performed in triplicate.

MTT assay

Cell culture of MCF-$_7$ cells

The MCF-$_7$ cells (IUMS, Cellular and Molecular Research Center, Tehran) were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml of penicillin, and 100 mg/ml of streptomycin. The cell line was maintained at 37°C in an incubator with an atmosphere of 5% CO_2. After counting, almost 5000 cells were transferred to flasks. In all any cases, 4 repeats have been conducted for each test.[34]

MTT staining

The cells were incubated at 37°C for 18 hours in 5% *carbon dioxide* in order to full adherence of cells to the plate. Different concentrations of essential oils and extracts (0 as control) were subsequently added to cells and plates were incubated for 72 hours at 37°C and 5% CO_2.

MTT solution (0.5 mg/ml) in PBS (0.15 M) was prepared and then autoclaved. After 72 hours incubation of cancerous cells with different concentrations of the essential oils and methanolic extracts, the plates incubated at 37°C with 5% CO_2 then stained with MTT 0.5 mg/ml and after 2–4 hours incubation at 37°C, the supernatant liquid was removed and replaced by 100 µl DMSO (dimethyl sulfoxide) (Merck, Germany). The absorbance of the plates was subsequently recorded using a micro titer plate reader (ELISA-reader, Organon- Teknika, Netherland) at 570 nm. Cytotoxicity level was calculated by the following formula:

$$\text{Cytotoxicity \%} = 1 - \frac{\text{mean absorbance of toxicant} \times 100}{\text{Mean absorbance of negative control}}$$

$$\text{Viability \%} = 100 - \text{Cytotoxicity \%}$$

To diminish the level of test error, MTT strain was added to some wells without cells and the absorbance level was read and ultimately subtracted from the test absorbance.[34]

Statistical analysis

All data were expressed as means ± SE for three experiments. The difference between the means ± SE of the antioxidant and anti-proliferative activity between the control (without plant extract) and experimental group were assessed using the one way ANOVA and Tukey Post Hoc test. P values < 0.05 were considered statistically significant. Statistical analyses were performed using Excel software and SPSS version 18.0 for Windows 2007.

RESULTS

Phytochemical Analysis

According to the phytochemical analysis results, rosmarinic acid was the major component identified in purple basil extracts in comparison with two other compounds. *P*-coumaric acid and ferulic acid were also identified. These two phenolic acids levels were higher in the flower extract, although they were still only present in low quantities compared to rosmarinic acid level (Table 1) (Figures 1 A-D).

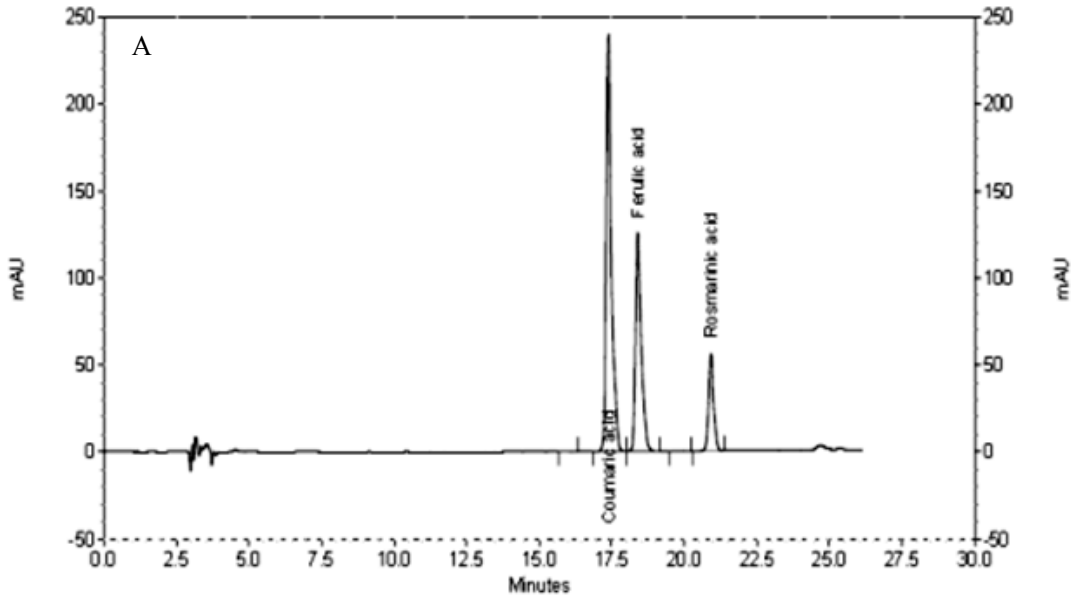
Antioxidant activity

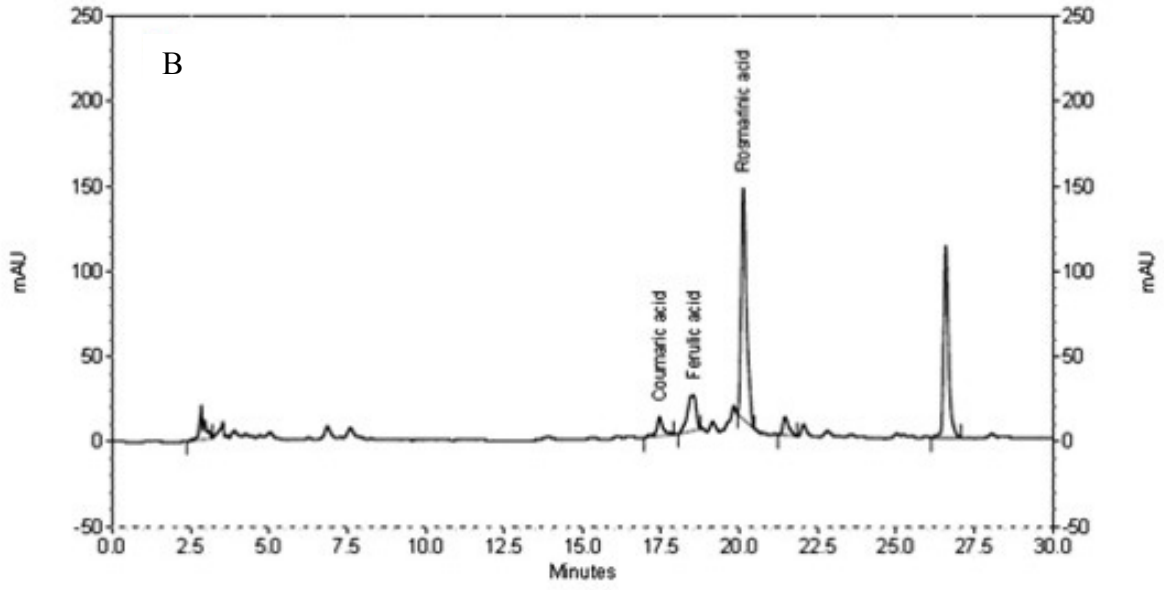
The presence of reductant (antioxidants) in the tested samples would result in the reduction of Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). The ferrous ion can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm.[35] As can be seen from Figure 2, although all the three extracts and two oils showed some degree of reduced Fe^{3+} to Fe^{2+}, the extractswere the more effective at reducing the iron (III), with an absorbance reading in range of 0.273 ± 0.008 to 1.344 ± 0.055 rather than the oils. The activity for the oils was in range of 0.05 ± 0.010 to 0.410 ± 0.010 (P <

Table 1: "Comparison of Phenolic compound levels in purple basil extracts, by HPLC method"

Samples	CA (µg/ml)	FA (µg/ml)	RA (µg/ml)
Flower extract	1.86 ± .0152	7.54 ± .0173	93.52 ± .0070
Root extract	<1	<1	97.59 ± .0141
Leaf-stem extract	<1	<1	53.35 ± .0100

CA: *P*-Coumaric Acid, FA: Ferulic Acid, RA: Rosmarinic Acid. Values are presented as mean ± SE (n =3) (P<0.05).





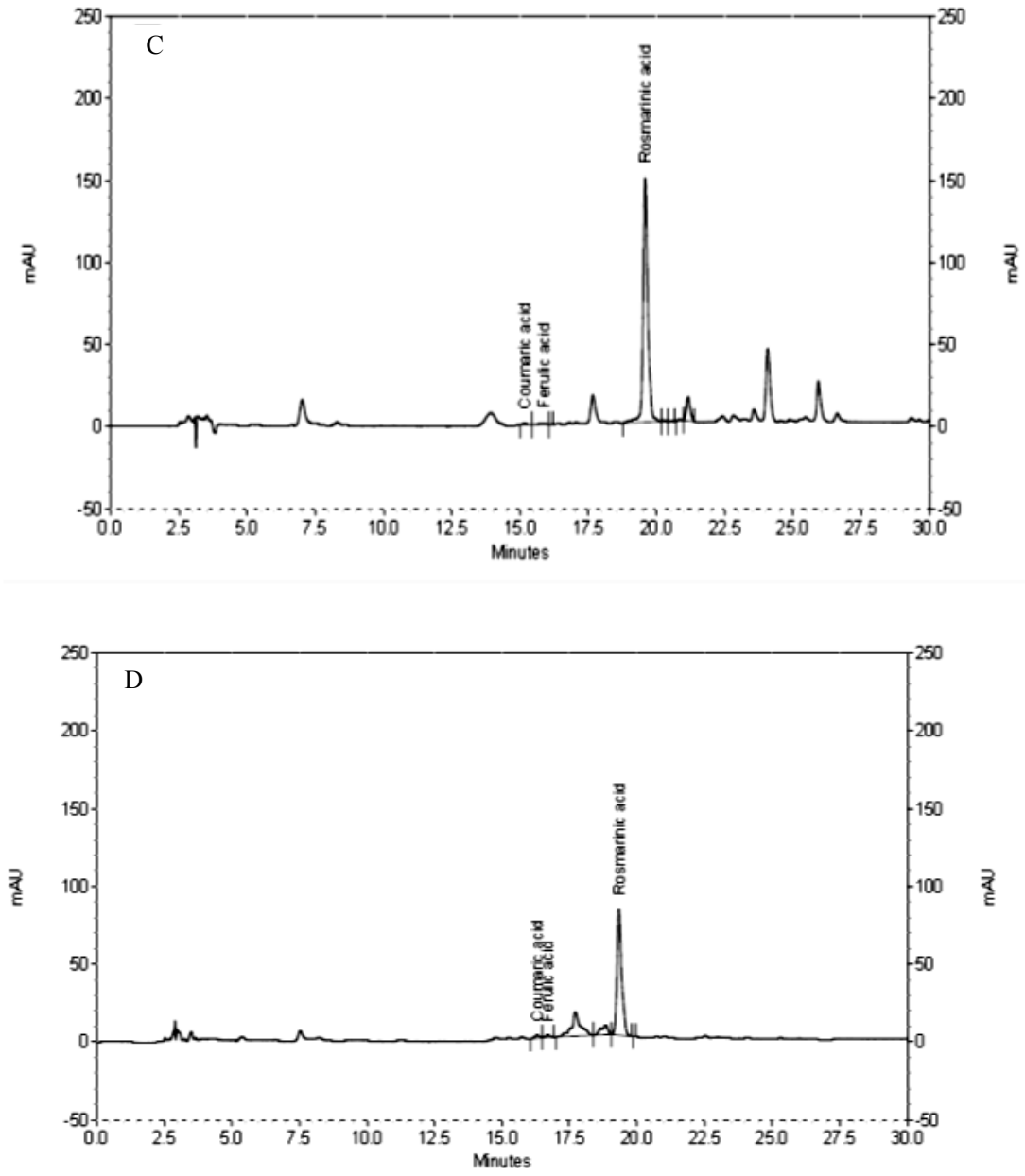


Figure 1. HPLC chromatograms of rosmarinic acid, ferulic acid and p-coumaric acid pure standards (A) and of purple basil flower (B), root (C) and leaf-stem (D) extracts.

0.05). It seems the methanolic extracts have a considerable ability to react with free radicals to alter them into more stable non-reactive species and to terminate radical chain reaction.

Anticancer activity

The MTT assay, which measures cell viability, was performed with different concentrations of both oils and methanolic extracts of purple basil for screening of toxic (or anti-proliferative) activity against MCF-$_7$ cell line at the 72 h time point. Figures 3 and 4 present the plots of toxicity/anti-proliferation (%) of MCF-$_7$ cell line versus concentrations of extract and oil samples respectively. Table 2 presented the IC_{50} values determined from the graphs of the extracts and oils on MCF-$_7$ cell line.

Flower and leaf-stem extracts gave the IC_{50} values of 232.19 ± 1.16 and 242.28 ± 8.17 (µg/ml) (Table 2), and the total mean toxification (or anti-proliferation) were 47.76 ± 4.87 and 48.66 ± 5.22, respectively. The root extract showed no apparent toxification activity, thus indicating that it is nontoxic (or very low toxicity) (Figure 3). Seed

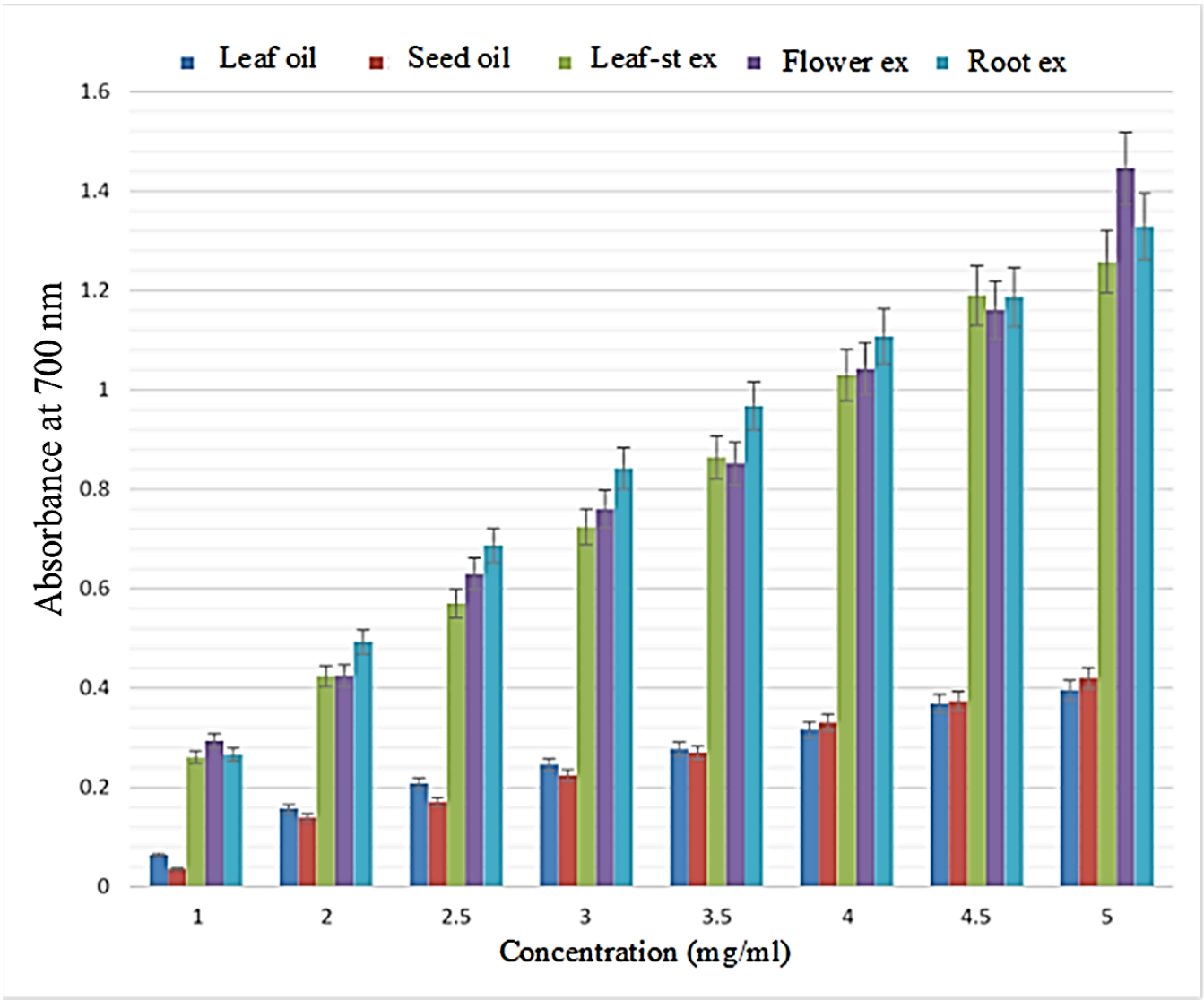


Figure 2. Comparison of purple basil oils and extracts effect on iron (III) reduction; ex: extract, st: stem. Values are presented as mean ± SE (n = 3) ($P<0.05$)

Table 2: The IC50 values (µg/ml) determined from the plot of percent toxicity on MCF-$_7$ cell line versus the concentrations of extract and essential oil

Samples	IC_{50} (µg/ml)
seed essential oil	52.45 ± 2.46
leaf essential oil	98.51 ± 6.49
flower extract	232.19 ± 1.16
leaf-stem extract	242.28 ± 8.17
root extract	NA

NA, the IC_{50} value was not founded even the highest extract concentration was used

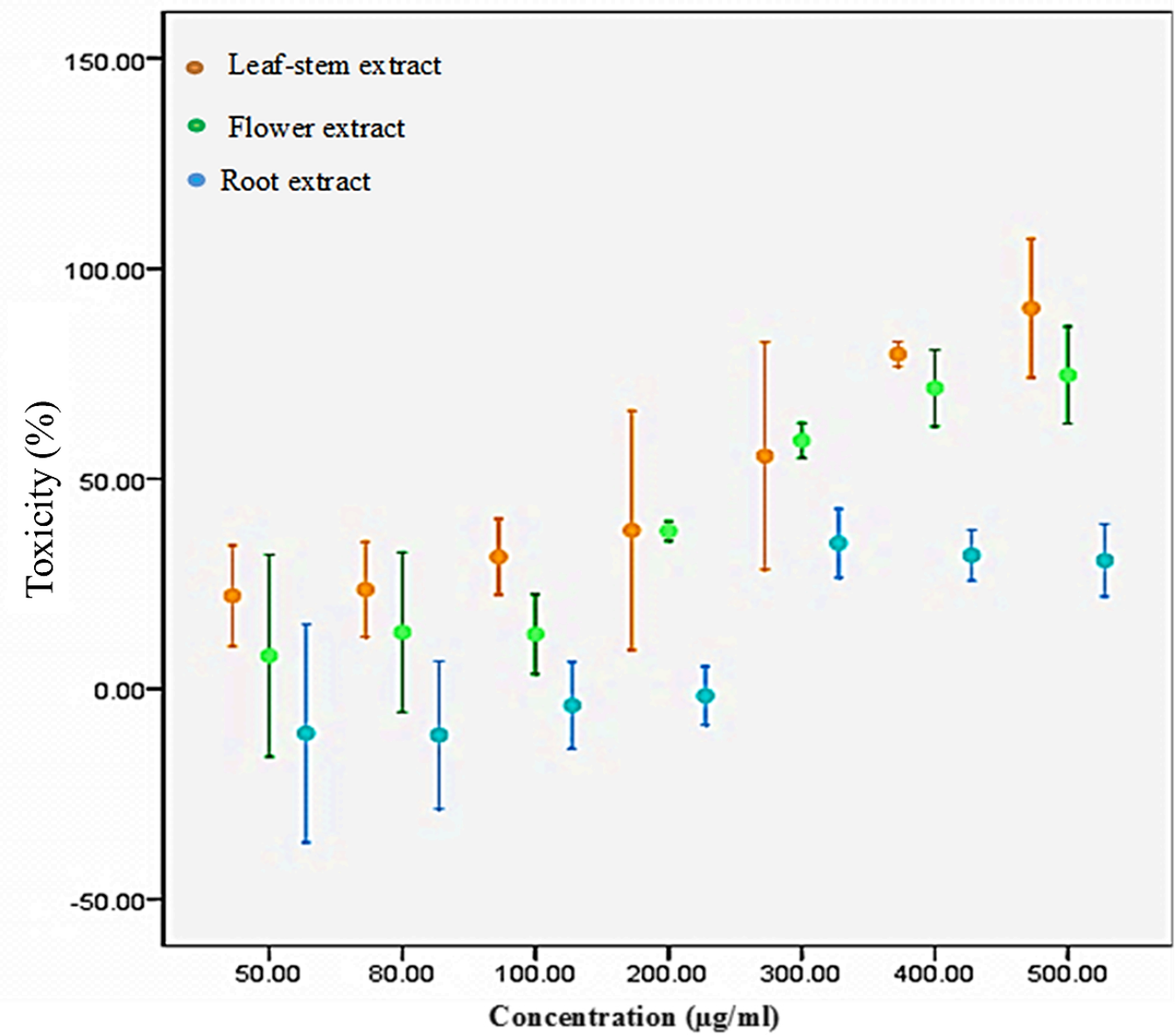


Figure 3. Comparition of the methanolic extracts toxic (anti-proliferative) effect on MCF-7 cell line after 72 hours of incubation ($P < 0.05$)

and leaf essential oils showed potent toxic effects with the IC_{50} values of 52.45 ± 2.46 and 98.51 ± 6.49 (µg/ml) in MCF-$_7$ cell line (Table 2), and total mean toxification of 48.38 ± 5.04 and 43 ± 4.48, respectively (Figure 4).

In addition to the significant anti-proliferative activity, as mentioned above, the methanolic extracts of leaf-stem and flower of purple basil presented a high antioxidant activity. The events of oxidative cell damage are often correlated with the oxidative stress, so that the presence of both properties in these extracts could be beneficial for preventive or therapeutic purposes.

DISCUSION

The contribution of new products from potential bioactive plants or their extracts and oils for disease treatment and prevention is one of the most important approaches in new drug development. Plants contain an almost unlimited capacity to generate compounds that fascinates researchers in the quest for new chemotherapeutics.[36] In recent years, there has been more emphasis on complementary and alternative [CAM] forms of medicine for the treatment of various cancers, among which herbal medicine is now being explored for cancer therapy.[37]

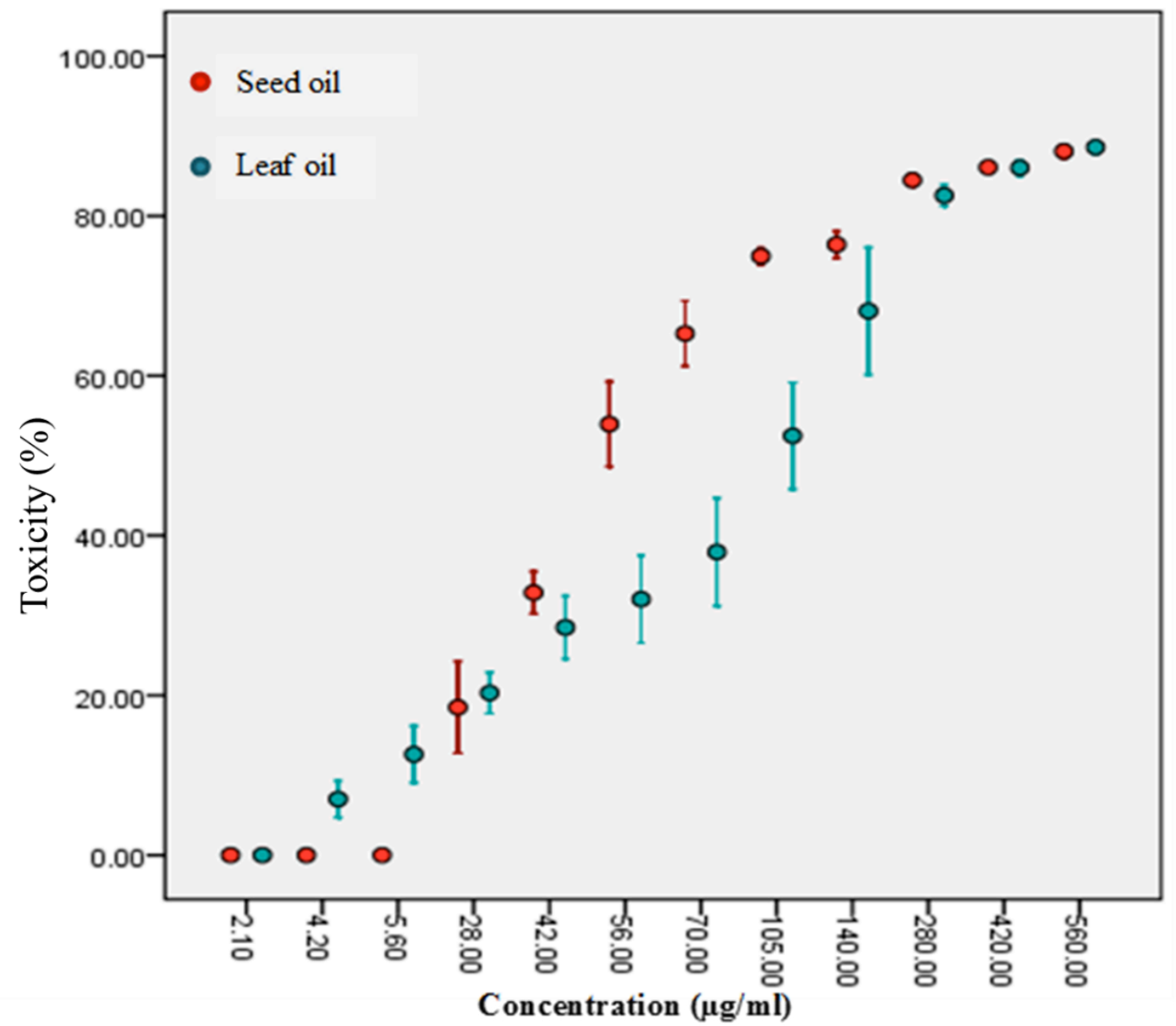


Figure 4. Comparison of the oils toxic (anti-proliferative) effect on MCF-7 cell line after 72 hours of incubation ($p < 0.05$)

Studies have observed the presence of a large number of bioactive compounds in the extracts of *Ocimum* plants including flavonoids and phenolic compounds. Similar compounds have been shown to prevent chemical-induced skin, liver, oral, and lung cancers and to mediate these effects by increasing the antioxidant activity, altering the gene expressions, inducing apoptosis, and inhibiting angiogenesis.[38] The expression of antioxidant activity is thought to be concomitant with the development of reductions, as these species are known to be free radical chain terminators.[39] Moreover, phenolic compounds and flavonoids such as ferulic acid, *p*- coumaric acid, and rosmarinic acid are potent antioxidants, free radical scavengers and metal chelators and have previously been reported in *O. basilicum*.[2, 40, 41]

In this study we identified and quantified rosmarinic acid in all 3 the extracts of purple basil. According our results, rosmarinic acid was the major component identified in purple basil extracts in comparison with other compounds. This is in general agreement with previous studies which reported that rosmarinic acid is the most abundant component in *O. basilicum*.[2] *P*-coumaric acid and ferulic acid were also identified, although they were found in very low quantities. These two phenolic acids levels were higher in the flower extract although they

were in low quantities compared to rosmarinic acid level (Figure 1).

We detected significant levels of rosmarinic acid (RA) in methanolic extract of roots (collected prior to flowering) (97.59 ± .0141 μg/ml), which was the highest concentration of RA observed of the parts of purple basil tested. This may explain the high antioxidant activity of the root extracts (Table 1). This result is similar with Kiferle study[42] in which the level of RA in root extracts was higher than the leaves extracts in *Ocimum basilicum,* and also is similar with Jayasinghe[43] study in which the major antioxidant compound was confirmed as RA. However, the flower extract was also rich in RA and the level was close to that of root extract which explains its powerful antioxidant effect (Figures 1B and 2).

In our study, *p*-coumaric acid and ferulic acid were quantified in the flower extracts, which is similar with Javanmardi[2] study where these phenolic compounds levels were higher in flower extracts than that of leaves samples. This result is also similar with Benedec[44] in which *p*-coumaric acid and ferulic acid, were found in small quantities in leaf and stem samples of *Ocimum basilicum,* which explains the antioxidant ability of flower extract. Although the antioxidant effect of flower extract was close to that of root sample, the antioxidant activity of flower sample was increased, by using higher concentration (5 mg/ml) of the extract. It seems that the presence of more quantity of CA and FA in flower sample must be the reason (Figures 1B and 2).

Leaf extract of purple basil showed a lower amount of RA whilst also having a powerful antioxidant activity. This is in general agreement with Dorman[45] who explained *Ocimum basilicum* L. leaf extracts have capable antioxidant effect.

Our results revealed that methanolic extracts of purple basil have antioxidant activity which is concentration-dependent, and that phenolic compound are responsible for this effect. This is similar with previous studies, where different species of Ocimum have been investigated.[26, 46]

Purple basil essential oils at various concentrations were determined. As can be seen in Figure 2, both two purple basil oils possess considerable reducing power and through the increasing of the concentrations, absorbance level was growing up. This result is similar with Trevisan et al study.[47] Statistical analysis indicated that the antioxidant activity of the leaf (prior flowering) and seed oils were similar. However the antioxidant activity of extracts with the mean absorbance level of 0.733 ± 0.081 was 3.14 fold more than that of oils (Figure 2). Juliani and Simon[48] evaluated the antioxidant activity of different basil essential oils and in all basils the essential oil contribution to the total antioxidant activity was low. In conclusion, all extracts and oils were found to exhibit a good anti-oxidant activity in the selected *in vitro* antioxidant assay. Overall, the oils tend to possess lesser activity in comparison to the extracts. However essential oils and their main components possess a wide spectrum of biological activity[49] and the main advantage of essential oils is that they can be used in any foods as their maximum effect is attained with the minimum change in the properties of the food.[50] In agreement with Hakkim et al,[46] although further research would be required, potential activity of all these samples in the tested antioxidant assays is a promising factor for their application as an effective preservative for the food and cosmetic industries.

In order to understand the characteristic of the toxic effect of *Ocimum basilicum cv. dark opal* on cancerous cells, the MTT assay was performed with different concentrations of both oils and methanolic extracts for screening of anti-proliferative activity against MCF-$_7$ cell line. Almost all of the samples reduced tumor cell proliferation, and the inhibition in cell growth was dose dependent (Figures 3 and 4). The investigation provides evidence for anti-proliferative effect towards MCF-$_7$ cells which may be due to existing phytochemicals in the *Ocimum basilicum cv. dark opal* extract. This result is similar with Chang et al[51] study where have been shown a phenolic compound like caffeic acid of *Ocimum gratissimum* could suppress the proliferation of HeLa cancerous cells line. Moreover, our results are similar with previous studies where extract of *Ocimum gratissimum* and *Ocimum sanctum* leaves inhibited tumor cells proliferation.[52–54]

Seed and leaf essential oils showed potent anti-proliferative effects (Figure 4 and Table 2). This result is similar to Kathirvel et al.[55] study, in which examined the *in vitro* anticancer activity of the leaves essential oil from *Ocimum basilicum* against the human cervical cancer cell line (HeLa), human laryngeal epithelial carcinoma cell line (HEp-2) and NIH 3T3 mouse embryonic fibroblasts. Manosroi has mentioned that the difference in sensitivity of cancer cell lines to substances containing in Sweet Basil oil must be the reason, since sweet basil (*Ocimum basilicum* L.) leaf oil gave the highest anti-proliferative activity with the IC_{50} value of 36.2 μg/ml in P388 cell line and 303.3 μg/ml in KB cell line.[56] Essential oils rich in monoterpenes are natural antioxidants[57] with anti-proliferative active against certain cancers.[58] Indeed, a number of dietary monoter-

penes have antitumoral activity that can prevent the formation or progress of cancer and cause tumor regression.

Based on the IC_{50} of essential oils and extracts through the MTT assay, they can be divided into two groups: (1) essential oil samples that had IC_{50} values of less than 100 µg/ml as possible candidates for further development to cancer therapeutic agent; (2) extract samples that had the IC_{50} values more than 100 µg/ml as moderate possibility to be developed to cancer therapeutic agent (Table 2).

CONCLUSION

The events of oxidative cell damage are often correlated with the oxidative stress, so that the presence of both properties in these extracts could be beneficial for preventive or therapeutic purposes. Since the preliminary results reported here are very promising, it is very important to explore the isolation of pure compounds responsible for these activities. Furthermore, basil essential oils are possible sources of antioxidant and anticancer compounds. These observations prompt the necessity for further studies, focusing on the isolation and structural elucidation of their antioxidant and anticancer compounds, since they have potential use as therapeutic agents in managing diseases associated with free radicals.[59]

Although these results clearly refer to the possibility of using purple basil or its essential oils as antioxidant and anticancer agents, studies need to be carried out on human patients. These results can be used as basis for development of a disease-oriented drug-discovery. The screening of those oil and extracts in other cell lines and study on chemical constituents of purple basil oil must be under investigation.

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