DETERMINATION OF THE CYTOTOXIC EFFECT ON HUMAN COLON CANCER AND PHENOLIC SUBSTANCE CONTENT OF THE ENDEMIC SPECIES *SIDERITIS OZTURKII* AYTAÇ & AKSOY

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(Received 28th Feb 2019; accepted 3rd May 2019)

Abstract. *Sideritis* L. species that we based on in this study are annual or perennial flowering plants which are widely used as herbal medicine, and also called mountain tea or shepherd’s tea. They are common in the Mediterranean Region, the Balkans, Iberian Peninsula, Greece, Central Asia and Anatolia. They are xerophytic shrub or herbs between 8 and 90 cm. They usually grow on arid cliffs, calcareous slopes, high-altitude mountainous regions (650-1400 m). Full bloom usually occurs in July. It is commonly used in Albania, Greece, Kosova, Bulgaria, Macedonia, Asia, Central Europe and Anatolia as herbal tea and for therapeutic purposes. It is usually used to aid digestion, strengthen the immune system, suppress common cold, allergy, sinusitis and influenza and as therapeutic for pain and mild anxiety. The methanol extracts obtained from the flower and leaf parts of the species *Sideritis ozturkii* Aytaç & Aksoy, which is endemic in Turkey, were applied at different concentrations and for different times on DLD-1 human colorectal cancer cells cultured in RPMI-1640 medium. The cytotoxicity measurements were made through the use of MTT and the cytotoxicity indices were calculated using an Elisa Reader. As a result of the study, it was determined that the flower and leaf extracts obtained from the species *Sideritis ozturkii* exhibit cytotoxic activity on DLD-1 cells in a dose- and time-dependent manner. It is believed that the extracts obtained from the species *Sideritis ozturkii* could be beneficial as a natural anti-proliferative agent.

Keywords: *Sideritis*, cytotoxicity, cancer, phenolic, DLD-1, MTT

Introduction

Cancer is characterized by abnormal growth of cells that have the potential to spread to other parts of the body, and the body’s normal control mechanism is a disease that develops when it stops working and causes death. Today more than 100 types of cancer affect people. Cancer is a global health problem; it is known that 9% of deaths in developed countries and 21% of deaths in developing countries are caused by cancer. The World Health Organization estimates that in the year 2050, about 27 million new cases of cancer will occur annually and 17.5 million people will die from these cases (Hussain et al., 2016).

The word cancer means the growth of malignant tumors resulting from the irregular division and proliferation of the cells in an organ or tissue. In the general sense, the cancer refers to a group of more than 100 diseases caused by the uncontrolled proliferation of the cells in various regions of our body. Although there are a great variety of cancer types, all begin with an uncontrolled proliferation of the abnormal cells. These may cause serious disorders, even death, in case left untreated (Horner et al., 2009).

Colon cancer is the third most common type of cancer in the world, and colorectal cancer is the second most common cause of death. Although colon cancer is a disease that can be seen at any age, its incidence is generally higher in people aged 40 years and older. Among all cancers, the incidence is in the third place in females and the second in males (Giardiello, 2014). According to the Ministry of Health data, it is among the five most
common types of cancer in Turkey. Globally, more than a million people per year are caught in colorectal cancer. In 1990, 490,000 people per year of death of the colon cancer, which led to the death of 715,000 people annually was announced to cause death. Colorectal cancer is the second largest cause of male and female deaths in the United States. In 2011, there were 141,210 cases per year in the United States and 41,000 cases in the United Kingdom. Environmental factors are the major factors in the incidence of colon cancer in the community. Especially in developed countries, unhealthy eating habits of people with unbalanced life and high fat increase the incidence of colon cancer. In addition, the genetic predispositions and characteristics of people as well as environmental factors increase the incidence of colon cancer.

Increase in the incidence of cancer cases, chemotherapy and radiotherapy used in the treatment of many cancer patients to damage the normal cells and reducing the percentage of success of these treatments in targeted cancer cells has encouraged scientists to develop new anticancer drugs from plants (Shah et al., 2013).

It is seen that in many archaeological findings, people have been using plants for treatment and nutrition purposes for thousands of years (Koçyiğit, 2005). The belief in the power of plants to treat diseases is almost as old as the history of humanity. Throughout the history of humanity, plants with many different forms have been used in the treatment of diseases. There is much historical evidence that plants are used for treatment in all continents of the world. The first written information about the ethnobotanical field, which refers to the use of plants for medical purposes, dates back to 3000 BC, namely the periods of Egypt, Hittite, Greek and Roman periods (Dağcı et al., 2002).

At the end of the 5th century BC, the book De Mataria Medica rod by Dioscorodidas in the 1st century AD is shown as the basis of modern pharmacology. Plants still present in these works and new species of these plants continue to be used in traditional treatment (Cowan, 1999).

In recent years, especially after the 1980s, the chemical industry began to take the place of herbal medicines as a result of the rapid progress of the synthetic (chemical) drugs. However, because of the occurrence of many side effects of these synthetic drugs and the high prices of many of them, people have turned to prefer treatment with herbal remedies (Yücel, 2010).

As in all countries of the world, plants are seen important from a medical point of centuries people in Turkey, tea, ointments, spices and so on. used in the treatment of diseases in the shapes. Nowadays, interest in natural herbal medicines has increased again because microorganisms have more resistance to synthetic drugs (Nakipoğlu and Otan, 1992).

The genus Sideritis L. constituting the basis for our present study are the annual or perennial flowering plants, which are also called the mountain tea or the shepherd’s tea and are commonly used as herbal medicine (Barber et al., 2015). They are abundant in Mediterranean region, the Balkans, the Iberian Peninsula, Greece, Central Asia and Anatolia. These are xerophilous shrubs or herbs with a height in the range of 8-90 cm. They usually grow on the arid rocks, on calcareous slopes and in mountainous regions with high altitude (650-1400 m). The inflorescence is generally verticillate. The blooming is usually observed in July (Aytaç and Aksoy, 2000). It is commonly used as herbal tea for therapeutic purposes in Albania, Greece, Kosovo, Bulgaria, Macedonia, Asia, Central Europe and Anatolia. It is generally used to aid digestion, strengthen the immune system, suppress common cold, allergies, sinusitis and flu and treat pain and mild anxiety (Todorova and Trendafilova, 2014).
Studies have been made regarding the plant’s antimicrobial, antibacterial, anti-inflammatory, antioxidant and cytotoxic effects on the diseases. Diterpenes and flavonoids were identified as the predominant active ingredients as the result of the extraction process (Petreska et al., 2011).

The cytotoxicity is the quality of being toxic to cells (Tokur and Aksoy, 2017). Cytotoxicity determines the effect of the active ingredients, particularly flavonoids, glucosides, alkaloids, terpenoids, which are obtained as a result of the extraction of the medicinal plants, on the cancer cells (Kuntz et al., 1999). The most important stage in the research related to the anticancer drugs is the determination of the in-vitro cytotoxicity. The cultures of the human or animal cancer cells are performed in-vitro in the culture plates where the reproducing cells are grown. Then, the effective dose is determined as a result of a study that is based on the steps of collecting the plants, subjecting the same to mechanical fractionation followed by extraction, introducing to the cancer cells the active ingredients resulting from the extraction and identifying the dose of these active ingredients that kills 50 percent of the cancer cells (Riss et al., 2014).

In the light of this information, this study is to determine the cytotoxic effect of the extracts obtained from the leaves and flower parts of the Sideritis ozturkii species (Fig. 1) which are endemic to the province of Konya endemic to the province of Konya on the DLD-1 cell line and to have fewer side effects in the treatment of the cancer of our time. In order to support the production of an economical drug, which is more effective, it has been researched. The cytotoxic effects of Sideritis species on the cancer cells began in 1981 and have continued until the present (Jeremic et al., 2013). The literature research on the Sideritis species reveals that the studies are generally focused on the species Sideritis scardia Griseb. In the present study, the cytotoxic effects of the species Sideritis ozturkii of the plant Sideritis, an endemic species of Konya Province that has not yet been studied, were investigated on the cancer cells (particularly on the colon cancer cells).

Sideritis ozturkii is an endemic plant in the province of Konya and this is the first study on colon cancer cell line with Sideritis ozturkii. Therefore, we believe that this study will contribute to the literature. With the present study, it was aimed to provide support for the manufacture of an economical drug with fewer side effects and with the possibility of being more effective in treating the cancer, the disease of our age. The distribution map of the species Sideritis ozturkii is given in Figure 2.
Materials and methods

Collection material

*Sideritis ozturkii* type used in this study is located 3 km north of Konya Derebucak district Çamlık village; Kızıldağ’s 1450-1700 m high mountainous area collected approximately 1 kg in flowering period in July 2016.

Drying of the plant

The plant was allowed to dry in a room away from the direct sunlight. When the plant was completely dried after two months, the flower and leaf parts were separated. Next, the leaf and flower parts were separately triturated with a plant grinder, subjected to sieving and then moved on to the extraction process. The reason for collecting the plant during flowering period. The extract of the flower part was also intended to be obtained.

Extraction

Of the triturated plants, first a quantity of 15 g was weighed from the leaf part by means of precision scales, placed into the soxhlet apparatus and subjected to the extraction process with 200 mL of methanol for 6-8 h. We have used methanol in our study because methanol is often preferred in studies with *Sideritis*. The extracts recovered from the extraction process were kept overnight in a refrigerator at +4 °C. On the following day, the extracts were evaporated in a rotary evaporator at 40 °C in order to remove the solvents thereof. The recovered raw extracts were kept at -20 °C until their use in order to prevent any loss of activity. The extraction process with the Soxhlet device is shown in Figure 3.

Preparation of cell culture

In order to ensure that DLD-1 cells proliferate in vitro such as in vivo, the appropriate medium was prepared. For this purpose; 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin RPMI-1640 (Sigma) cell medium were used. A few minutes were kept at 37 °C for fast dissolution of DLD-1 cells from the liquid nitrogen tank. The dissolved cells were added to a falcon tube and FBS was added to remove DMSO and pipetted several times. It was then centrifuged for 5 min. After centrifugation, the
supernatant was discarded and the remaining cell pellet was plated on the prepared medium and sown to 25 cm² flask. Cells were cultured in 25 cm² flasks with 95% humidity and 5% CO2 gas and 37 °C CO2 incubator. The density of DLD-1 cells in the flask maintained in the CO2 incubator was increased over time by invert microscopy, and the cells from the flask were observed to be propagated by passaging.

Figure 3. Soxhlet device extraction process

**Determination of cytotoxic effect**

The cytotoxic effect of the obtained extracts was tested on the DLD-1 cell line comprising the colorectal cancer cells where in said cell line was cultured in RPMI-1640 medium. The obtained extract was subjected to characterization via HPLC in order to reveal the phenolic substance composition. The extract was first dissolved in a solvent, was then passed through the respective chromatography column under high pressure and the phenolic substance content was checked.

**High performance liquid chromatography (HPLC)**

HPLC is the most widely used device for the purpose of analytical separation techniques. The reasons for widespread use are that it is easily adaptable to quantitative determinations, suitable for the separation of compounds that are not volatile or easily decomposed by temperature. Most importantly, it is the wide applicability of many disciplines of science to the subjects of primary interest of the society. Examples of such compounds include amino acids, proteins, nucleic acids, carbohydrates, drugs and pesticides. HPLC unit: Degasser, pump, autosampler, column and detector consist of four parts. The Degasser; It provides removal of dissolved gases present in mobile phases.
HPLC (High Performance Liquid Chromatography) High Performance Liquid Chromatography is briefly referred to as HPLC.

High performance; expresses high resolution. It is also called High-pressure Liquid Chromatography since the mobile phase is progressed with high pressure (Lindsay, 1987).

**Calculation of cytotoxicity indexes**

The prepared extract was dissolved using DMSO at a nontoxic level and it was used on the cell line at different concentrations and for different time intervals. After different concentrations of the extract were added on the cell line, the measurement of cytotoxicity was performed through the use of MTT. Four hours after the addition of MTT on the cells, DMSO or isopropanol was added and the cells were incubated for four hours in an ambience not exposed to light. After the exposure of the cells to the extract for 24 and 48 h, the cytotoxicity indices based on absorbance were calculated by using an Elisa Reader. The cells allowed to proliferate under appropriate conditions were removed from the flask base by treatment with trypsin-EDTA when they covered 70% of the flask surface. The cells stained with Trypan blue dye were counted 3 times with the help of thoma slide and suspended in the number of cells required for MTT testing. 100 μl of cell suspension was then transferred to a 96-well cell culture plate. 24 h incubation at 37 °C was achieved to allow the cells to adhere and adapt to the new environment. After 24 h, fresh media containing (100 μg/ml, 250 μg/ml, 500 μg/ml, 750 μg/ml and 1000 μg/ml) the desired final concentrations of extracts were added to the cells to determine the cytotoxic effects of the extracts and incubated for 24 and 48 h at 37 °C in a CO2 incubator.

**Results**

The % viability graphs obtained in line with the MTT test data, which were measured at time points of 24 and 48 h after the application, on DLD-1 cell line, of the methanolic extracts obtained from the leaf and flower parts of the plant *Sideritis ozturkii* and of the extract obtained by way of brewing from the leaf thereof are in Figures 4, 5 and 6.

When the mitochondrial activity after 24 h of application was evaluated as the % viability in the plates that were started with equal numbers of cells, the extract obtained from the leaf led to the cell death rates of 1% at the dose of 100 µg/mL, 26% at the dose of 250 µg/mL, 54% at the dose of 500 µg/mL, 67% at the dose of 750 µg/mL and 74% at the dose of 1000 µg/mL, while the extract obtained from the flower part, though causing an increase in the number of cells at the dose of 100 µg/mL, resulted in the death of the cells at increasing doses; namely the cell death rates of 12% at the dose of 250 µg/mL, 32% at the dose of 500 µg/mL, 45% at the dose of 750 µg/mL and 80% at the dose of 1000 µg/mL. On the other hand, the extract obtained by the brewing method from the leaf resulted in cell death rates of 57% at the dilution of 1/10, 70% at the dilution of 1/5 and 79% at the dilution of 1/2. Taking the % viability values into consideration, it was observed that the extracts obtained from the leaf were more effective in causing the death of the DLD-1 cells as compared to the extract obtained from the flower. The extracts obtained from the flower were also effective in providing the cell death, but they exhibited lower effect than the leaf extracts.
When the results for 48 h of extract application were compared to those for the 24-h application, some increase was observed in the cell death in a time-dependent manner. The extract obtained from the leaf led to the cell death rates of 8% at the dose of...
100 µg/mL, 45% at the dose of 250 µg/mL, 61% at the dose of 500 µg/mL, 79% at the dose of 750 µg/mL and 89% at the dose of 1000 µg/mL; the extract obtained from the flower part led to the cell death rates of 24% at the dose of 100 µg/mL, 22% at the dose of 250 µg/mL, 47% at the dose of 500 µg/mL, 69% at the dose of 750 µg/mL and 80% at the dose of 1000 µg/mL; and the extract obtained by the brewing method from the leaf led to the cell death rates of 78% at the dilution of 1/10, 83% at the dilution of 1/5 and 85% at the dilution of 1/2.

The IC₅₀ dose for the extract obtained from the leaf was calculated as 0.46 mg/mL for 24 h and 0.33 mg/mL for 48 h. On the other hand, a higher IC₅₀ dose was found for the extract obtained from the flower, as compared to the extract obtained from the leaf. For the extract obtained from the flower, the values of 0.7 mg/mL and 0.53 mg/mL were calculated for 24 h and 48 h, respectively (Fig. 7).

![DLD-1](image)

**Figure 7.** 24- and 48-h effects of the obtained extracts on DLD-1 cell line

The graphs for the results of HPLC (high pressure liquid chromatography) we performed on two methanolic extracts obtained from the plant *Sideritis ozturkii* are in Figures 8 and 9.

![HPLC chromatogram](image)

**Figure 8.** View of the HPLC chromatogram for the methanolic extract obtained from the leaf of *Sideritis ozturkii*
The screening was performed on both extracts for 15 phenolic substances. The phenolic substances used as the standard were as follows: Gallic Acid, Protocatechuic Acid, Catechin, 4-Hydroxybenzoic Acid, Caffeic Acid, Syringic Acid, Rutin Trihydrate, Trans-P-Coumaric Acid, Trans-Ferulic Acid, Myricetin, Trans-Resveratrol, Quercetin, Trans-Cinnamic Acid, Naringenin and Kaempferol. The standards were prepared by way of dissolving the same in methanol. The phenolic substances found in each extract and their respective quantities are given in (Table 1). When the extracts were compared in terms of the phenolic substance content, the extract obtained from the leaf was found to contain the highest quantities of rutin trihydrate and Trans-P-Coumaric Acid.

**Table 1. Phenolic substances present in the extracts obtained from Sideritis ozturkii and their respective quantities**

| Phenolic substance          | Sideritis ozturkii leaf (mg/100 g) | Sideritis ozturkii flower (mg/100 g) |
|----------------------------|-----------------------------------|-------------------------------------|
| Gallic Acid                | 7.278                             | 11.067                              |
| Protocatechuic Acid        | 10.244                            | 4.972                               |
| Catechin                   | 70.056                            | 64.100                              |
| 4-Hydroxybenzoic Acid      | 11.178                            | 15.922                              |
| Caffeic Acid               | 8.844                             | 8.467                               |
| Syringic Acid              | 12.722                            | 13.472                              |
| Rutin Trihydrate           | 1805.756                          | 823.367                             |
| Trans-P-Coumaric Acid      | 49.811                            | 9.556                               |
| Trans-Ferulic Acid         | 24.289                            | 98.372                              |
| Myricetin                  | 37.811                            | 424.517                             |
| Trans-Resveratrol          | 6.089                             | 29.856                              |
| Quercetin                  | 19.811                            | 22.433                              |
| Trans-Cinnamic Acid        | 4.900                             | 4.506                               |
| Naringenin                 | 14.367                            | 12.328                              |
| Kaempferol                 | 7.900                             | 72.367                              |
Discussion

Cancer is one of the most important reasons of disease-caused deaths in the world. World Health Organization reports that approximately 84 million people lost their lives due to cancer between 2005 and 2012 (Behzad et al., 2014). Despite the frequent use of chemotherapy in the treatment of cancer, the toxic effects that occur over time limit the use of these drugs. Both the high mortality rate of cancer cases and the emergence of serious side effects of chemotherapy and radiotherapy lead cancer patients to alternative and/or complementary therapies (Shah et al., 2013). At this point, natural products are viewed as potential raw materials for new drug discoveries, and phenolic compounds found in natural products come to the forefront with their structures and activities. For this reason, the search for the anticancer effects of natural products, either as whole extracts or compounds isolated from them, has become one of the most popular study areas. In preliminary studies to determine the availability of natural products for humans, the use of in vitro methods is recommended. Clinical trials are recommended only if positive results are obtained from in vitro and in vivo studies (Demir et al., 2016, 2017). Some criteria have been established to demonstrate if a natural compound is effective and acceptable in terms of chemopreventive or chemotherapeutic efficacy. The most important of these criteria is that the natural product (that is cytotoxic to cancerous cells) used should not exhibit the cytotoxic effect in normal cells, and should be effective against multiple cancer cell lines (Demir et al., 2017).

In line with this information, in order to support the production of a herbal preparation that has less side effects in treating cancer - the disease of the century - or at least has the potential to reduce the side effects of chemotherapy; *Sideritis ozturkii* (which is endemic to Konya province and has not been studied before) extracts of leaf and flower parts (extracted with methanol) were isolated and their cytotoxic effects on colon cancer were examined through MTT method.

Tadić et al. (2012) evaluated the cytotoxic effects of plant extracts in PBMC, B16 and HL-60 cells. Cytotoxic effects were analyzed by dual staining of apoptosis cell death and neurotic cell death with annexin V and PI-coupled fluorescein isothiocyanate (FITC). All plant extracts were also analyzed phytochemically. Analysis by HPLC revealed that ferulic acid was the predominant active ingredient of the phenolic compounds. It has been determined that this phenolic compound has a higher cytotoxic effect in PBMC, B16 and HL-60 cells than that of the other active substances. In this study, the cytotoxic effects of the extracts of the flower and leaves of the *Sideritis ozturkii* plant on the DLD-1 cell line were investigated. Cytotoxicity was analyzed by MTT test. All phytochemical analysis of the plant was carried out by HPLC method. As a result of this analysis, it was determined that the dominant active substances of the plant were routine trihydrate, myricetin and catechin. It has been found that routine trihydrate, the predominant active ingredient in the leaf, has a higher cytotoxic effect on colon cancer cells than other active agents.

Jeremic et al. (2015) have examined the mechanisms of cytotoxic action of flavonoids (from the active ingredients of *Sideritis scardia* L. extracts) on rat C6 glioma cells. As a result of their study, flavonoids (detected as the predominant active ingredient in *Sideritis scardia* L. extracts) were found to have elevated rates of apoptosis, necrosis and autophagic cancer cell death in C6 glioma cells, and thus have a high cytotoxic effect. In this study, cytotoxic effects of *Sideritis ozturkii* extracts on colon cancer cells were investigated. In the results, it was found that methanol extracts had a significant cytotoxic effect on the detected tumor line. As a result of the HPLC
analysis, it is thought that *Sideritis ozturkii* has an anti-tumor characteristic due to its rich flavonoid content and therefore it can be proposed as an anti-tumor agent with cancer prevention and an alternative cancer prevention food.

Radovaroic (2015) in this study, chemical components of plants with cytotoxic activity and plant extracts selected for producing chemotherapeutic drugs were evaluated. Various plant extracts (*Sideritis scardia* L. *S. linearifolia*) were studied and polyphenolic, diterpenoids, flavanoids, betulinic acid, triterpenoids, anthraquinone and berberine were determined as predominant active agents and their effect rates on cytotoxicity were compared. These active ingredients were measured on the effects of apoptosis to stop the cycle of cancer cells on viability. As a result of these studies, it has been determined that plant extracts have potential cytotoxic effects on cancer cells and especially flavonoids are effective anti-chemotherapeutic agents.

Zegerac (2015) investigated the cytotoxic, antioxidant and antimicrobial effects of the active substances in extracts extracted from *Sideritis lamiaceae* in in vitro studies on cancer cells in mice. However, *Sideritis lamiaceae* L. extracts were not as effective as *Sideritis scardia* L. in cancer cells. However, flavonoids, the dominant active ingredient in antioxidant properties, were found to be positive according to ORAC 5.0 assay. The in-vitro studies of the active ingredients in extracts from the leaves and flower parts of the endemic *Sideritis ozturkii* investigated the cytotoxic effects on the DLD-1 cell line (colon cancer cells). *Sideritis ozturkii* extracts have been found to have a higher cytotoxic effect than *Sideritis congesta* species in which Zegerac works in cancer cells. This has also shown us that *Sideritis ozturkii*’s cancer prevention may also be an alternative anti-cancer agent.

To our knowledge, no cytotoxic effect studies on *Sideritis ozturkii* have been reported so far. However, studies on similar species of *Sideritis* have shown cytotoxic effects on cancer cells.

In this study, cytotoxic effects of methanol extracts, obtained from endemic *Sideritis ozturkii* leaves and flowers, on DLD-1 cell line were investigated by MTT method and Elisa reading. As a result of this study, leaf and flower extracts obtained from *Sideritis ozturkii* displayed cytotoxic effects on DLD-1 cells, depending on the dose and time. The extracts obtained from the leaves were found to be more effective than the ones obtained from the flowers. The IC\textsubscript{50} doses for leaves and flowers were estimated to be approximately 0.3 μg/ml and 0.5 μg/ml, respectively. When extracts were compared to phenolic substance content, it was found that the extract obtained from leaf yielded the highest amount of routine trihydrate and Trans-P-Coumaric Acid. We think that this phenolic substance content of the leaves causes these results.

**Conclusions**

According to the results obtained from the MTT test, the extracts obtained from the leaf and flower parts of the species *Sideritis ozturkii* showed cytotoxic effects on DLD-1 cells in a dose- and time-dependent manner. The leaf extract was determined to be more effective than the flower extract. The IC\textsubscript{50} dose was calculated to be approximately 0.3 μg/mL for the leaf extract and 0.5 μg/mL for the flower extract. The data suggest that the extracts obtained from the species *Sideritis ozturkii* could be beneficial as a natural anti-proliferative agent.
Acknowledgements. We would like to thank Selçuk University BAP (Coordination Office for Scientific Research Projects) for supporting this study with the project no. 16201095.

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