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Original Research Article

Cytotoxic and Antioxidant Activities of Clary Sage (*Salvia sclarea* L.) Essential Oil Against AMJ-13 Cancer Cell Line

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ABSTRACT

Clary sage (*Salvia sclarea* L.) is a valuable medicinal plant. It has a wide range of medicinal properties and is among the species used for essential oil production in several countries. The present study was conducted to investigate the cytotoxic and antioxidant activities of clary sage essential oil (CSEO) against cancer cells. AMJ-13 cancer cells were treated with different concentrations (5, 10, 50, 100, or 200 µg/mL) of CSEO, and the control cells were untreated. The cytotoxic effect of the CSEO was evaluated using the MTT assay, while the antioxidant activity was evaluated by determining the amounts of reactive oxygen species (ROS), superoxide dismutase (SOD), and catalase (CAT). The results indicated that the CSEO has high cytotoxicity against AMJ-13 cells. The lowest (at 200 µg/mL) and highest (at 5 µg/mL) viability percentages of the AMJ-13 cells were 19 and 95%, respectively. Also, the IC_{50} value of CSEO was 54.85 µg/mL. It was discovered that CSEO has antioxidant activity and that there was a significant decrease in ROS value (undetected) in the treated cells compared to the control of 0.1395 ng/mL. Also, a significant decrease in the value of CAT enzyme (111.8 nmoles/min/million cells) was observed in cells treated with CSEO compared to the control (1637nmoles/min/million cells). Meanwhile, there was no increase in the value of SOD in the CSEO-treated cells, which was equal to the control (undetected). The research findings show that CSEO has cytotoxic and antioxidant properties and that it can be used to target various cancer cell types.

Keywords: AMJ-13 cell line, Antioxidant, Clary sage, Cytotoxicity, *Salvia sclarea*.

Introduction

Medicinal plants and herbs have been regarded as one of the most important kinds of therapeutic substances since the beginning of human history. Several medicinal plants used in traditional medicine have been evaluated as alternative medications due to their numerous applications in the cosmetic, food, pharmaceutical, and other industries, which have received a lot of attention.[1] Many medicinal plants are natural sources of antioxidants, so they play an essential role in the prevention of aging and disease.[2] Over the past twenty years, numerous studies have investigated the role of various aromatic plants as antioxidants. Among these plants, essential oils, in particular, have received great attention for their use as alternative therapies for the treatment of many infectious diseases.[3] Clary sage (*Salvia sclarea* L.) is a heliophilous, xerophytic herbaceous plant that grows as a biennial or perennial and belongs to the Lamiaceae family. It is cultivated for its essential oil, which is used in industry. It is widely grown in temperate and semi-tropical regions, especially in the Mediterranean region and Central Europe,[4] as well as Central Asia and sections of North Africa.[5] In the Mediterranean countries, the clary sage plant is one of the most valuable medicinal plants. It has a wide range of applications in health and cooking, as well as commercial usage in the cosmetics, perfume industries, and pharmaceutical sectors.[6]

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Since ancient times, clary sage has been regarded for its aromatic and therapeutic characteristics, and it is one of the most significant species for essential oil production, alongside *Salvia officinalis* and *Salvia lavandulifolia*, with an estimated annual production of 50 to 100 tons. The essential oils have a fresh and floral fragrance, and the whole plant, particularly the inflorescences, is quite scented.[5] Clary sage is widely grown commercially in France, Bulgaria, Morocco, and Russia, with a yearly oil production of around 150 tons. Additionally, clary sage oil is used to treat stress, tension, depression, insomnia, and other medical conditions.[4] Clary sage also has antibacterial, antioxidant, antitumor, antidiabetic, antimicrobial, anxiolytic, sedative, and anti-inflammatory properties,[6,8,9] as well as neuroprotective, antifungal, and antiviral activities.[5] There is a paucity of research information on the effectiveness of clary sage oil against cancer cells.

Therefore, the current study was aimed at evaluating the cytotoxic and antioxidant effects of clary sage essential oil against the AMJ-13 cancer cell line.

Materials and Methods

Source and maintenance of the AMJ-13 cancer cell line

The AMJ-13 cancer cell line was obtained from the Cell Bank Unit of Iraq Biotech in Basra, and the cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, and 100 µg/ml streptomycin. Cells were passaged with Trypsin-EDTA twice a week, reseeded at 50% confluence, and incubated at 37°C with 5% CO_2.[10]

Source of clary sage essential oil

The clary sage essential oil (100% pure; 10 ml/bottle) was obtained commercially from PYRRLA, Iraq.

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Treatment and cytotoxicity assay on the AMJ-13 cancer cells

The cytotoxic effect of clary sage pure essential oil was estimated using the MTT test. The AMJ-13 cell line was seeded at 1×10^4 cells/well. After obtaining a confluent monolayer or after 24 hours, the cells were treated with 5, 10, 50, 100, or 200 µg/ml clary sage oil, and the control cells were untreated. The cells were then incubated at 37°C with 5% CO_2. The media was removed after 72 hours and replaced with 28 µL of 2 mg/ml MTT stain solution, and the cells were incubated for 2 hours at 37°C with 5% CO_2. After removing the MTT stain solution, the crystals in the wells were solubilized by adding 100 µl of DMSO (Dimethyl sulphoxide), followed by a 15-min incubation at 37°C with 5% CO_2.[11] The absorbency was evaluated at 620 nm using a microplate reader, and the assay was carried out in triplicate. The percentage of viable cells was calculated using the following equation:[12]

$$\text{Cell viability percent} = \frac{\text{Optical density of treated wells}}{\text{Optical density of untreated wells}} \text{X } 100$$

Detection of the amount of reactive oxygen species

The amount of reactive oxygen species (ROS) was determined from the cancer cell lines following the Elabscience kit manufacturer's instructions. The standard working solution was added to the first two columns of the micro-ELISA plate: each concentration of the solution was added in duplicate side by side to one well each (100 µL). The remaining wells were filled with 100 µL of samples for each well. The plate was then sealed with the sealer contained in the package and incubated for 90 min at 37°C. Following the removal of the liquid from each well, 100 µL of Biotinylated Detection Ab working solution was added to each well, which was then sealed using the plate sealer. After that, it was carefully mixed and incubated for 1 hour at 37°C. Following incubation, the solution was carefully withdrawn from each well, and 350 µL of wash buffer was added, which was then soaked for 1-2 min before being decanted and patted dry against clean absorbent paper. This process was repeated three times. In each well, 100 µL of HRP conjugate working solution was added, covered with the plate sealer, and then incubated for 30 min at 37°C. The solution was carefully removed from each well, and the wash procedure was repeated five times. Each well was filled with 90 µL of substrate reagent and sealed with a new plate sealer. The plate was then incubated for 15 min at 37°C with the plate protected from light. Fifty microliters of stop solution were added to each well. The optical density value of each well was determined simultaneously using a microplate reader set to 450 nm.

Detection of the amount of SOD

The superoxide dismutase (SOD) quantity was determined according to the instructions of the Elabscience kit's manufacturer. A standard working solution was added to the first two columns of a micro-ELISA plate in duplicate side by side to one well each (50 µL). The other wells were filled with samples for each well (50 µL). Then, after gently mixing, 50 µL of biotinylated detection Ab working solution was immediately added to each well. The plate was sealed with the sealer included in the kit and incubated for 45 min at 37°C. The other steps, previously described in the detection of the amount of ROS were, followed.

Detection of the amount of catalase

The amount of catalase (CAT) was determined following the manufacturer's instructions for the Elabscience kit. The microplate reader was preheated for 30 min, and then the wavelength was adjusted to 240 nm and set to zero with distilled water. Before detection, the CAT assay working solution was incubated at 37ºC for more than 10 min. Then 10 µL of sample and 190 µL of CAT assay working solution were added to the 96 wells of the UV microplate and was thoroughly mixed. The absorbance was measured at 240 nm at initial absorbance (A1) and 1 min (A2), respectively. The value of ΔA was calculated by ΔA=Al-A2. The CAT activity was calculated based on the density of cells as shown in the following equation:

$$\text{CAT activity (nmol/min/10}^4\text{ cell)} = (\Delta A \text{ X } \frac{\text{V total}}{\text{c x d}} \text{ X } 10^9) \frac{500\text{xV sample}}{\text{V total sample}} \div T = 1.836\text{X } \Delta A$$

Where V total: the whole volume of the reaction system ($2\text{x}10^{-4}$ L); c: molar extinction coefficient of H_2O_2 ($4.36\text{x}10^4$ L/mol/cm); d: the optical path of the Microplate well (0.5 cm); V sample: volume of sample added into the reaction system (0.01 mL); V total sample: volume of the added extract solution (1 mL); T: reaction time (1 min); 500: the total amount of cells ($5\text{x}10^6$). The amount of CAT in 1 X 10^4 cells that catalyze 1 nmol of H_2O_2 per minute is defined as 1 unit.

Statistical analysis

The IC_{50} value was calculated using the GraphPad Prism software (version 6.04) and the data were statistically analyzed using the chi-square and t-test. The analyses were conducted with the Statistical Package for the Social Sciences (SPSS) program (version 19). $P \leq 0.05$ was considered to be statistically significant.

Results and Discussion

Cytotoxic effect of clary sage essential oil on AMJ-13 cancer cells

The results (Figure 1) of the cytotoxicity assay indicate that clary sage oil has a high cytotoxicity action against the AMJ-13 cancer cell line. Each of the five different concentrations of clary sage oil was employed in this study, each of which had a different effect on the cell line. The lowest viability percentage of the AMJ-13 cells was 19% at 200 g/ml, while the maximum viability percentage was 95% at 5 g/ml. Clary sage oil has an IC_{50} value of 54.85 µg/ml on the AMJ-13 cells. The statistical analysis revealed that there were significant differences (P=0.000) in the viability of the AMJ-13 cell line at all concentrations. Anticancer medications have been developed in several classes, but their use in cancer treatment has been limited due to negative side effects.[13,14] As a result, plant-based drugs are receiving more attention in cancer treatment.[15] To screen for cytotoxicity in natural or chemical compounds, cytotoxicity tests are commonly employed in pharmaceutical research.[16] The present study results showed that clary sage oil has a high cytotoxic effect against the AMJ-13 cancer cell line, with an LC_{50} value of 54.85 g/ml. Many *Salvia* species, including *Salvia sclarea* (clary sage), have been shown to exhibit toxic and apoptotic effects against a variety of human malignancy cells.[7] The oil of clary sage exhibited a cytotoxic effect on promyelocytic leukemia HL-60 and acute lymphoblastic leukemia NALM-6 cell lines with LC_{50} values of 6.4-6.5 g/ml and 8.1-20.1 g/ml, respectively.[4] Another study reported that clary sage oil caused apoptosis and cytotoxic effects in the HeLa cell line with an LC_{50} of 80.69±0.01 g/ml. Essential oils are aromatic and volatile liquids derived from plant components with biological characteristics.[17]

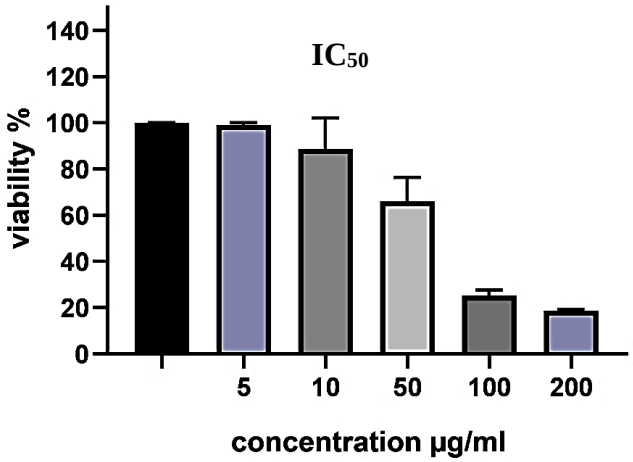

Figure 1: Cytotoxic effect of different concentrations of clary sage essential oil against AMJ-13 cells.

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The active components in clary sage oil are responsible for its cytotoxicity. According to several studies, linalyl acetate (monoterpenoid acetate ester of linalool) and linalool (monoterpenoid) are the two most important components in the essential oil of clary sage.[4] It has been shown that there were notable variances in the linalool/ linalyl acetate percentage amongst clary sage oil samples from diverse geographic areas. However, in clary sage essential oil, the average linalyl acetate content was 31.07, while the average linalool content was 23.11. Increased membrane fluidity, ion, cytoplasmic content leakage, a decrease in ATP production, change in pH gradients, loss in mitochondrial potential,[7] and cell cycle arrest,[18,19] are some of the mechanisms that explain clary sage oil's cytotoxicity. The antitumor effectiveness of several oil chemicals was evaluated to see which compound would respond to the oil's anticancer action or if the oil's anticancer action was due to the synergistic effects of its constituents. According to a study conducted by Zhao *et al.*,[19] linalool and linalyl acetate had a strong inhibitory effect on human prostate cancer PC-3 cells, and linalool significantly slowed tumor growth in a xenograft model with PC-3 cell transplantation. Apoptotic cell populations in PC-3 cells treated with linalool and linalyl acetate were 67.11% and 56.14%, respectively, and PC-3 cells were mostly arrested in the G2/M phase. Also, linalool has been demonstrated to stop the cell cycle of U937 acute myeloid leukemia cells in the G0/G1 phase and HeLa cells in the G2/M phase. The mechanism of action involves the enhancement of the expression of cyclin-dependent kinase inhibitors (CDKIs) p53, p21, p27, p16, and p18, as well as the non-expression of the activity of cyclin-dependent kinases (CDKs) by linalool.[18]

Effect of clary sage essential oil on ROS in AMJ-13 cancer cells

As shown in Figure 2, clary sage oil has antioxidant activity, with a significant decrease in ROS value in cells treated with 54.85 µg/ml clary sage oil compared to the control (0.1395 ng/ml). This observation indicated that the decrease in ROS value is an indicator of clary sage oil's efficiency as a scavenging antioxidant in controlling free radicals, thereby reducing their level.

This result was in agreement with the finding of Gülçin *et al.*,[20] who discovered that the clary sage plant contains antioxidants. According to research on ten *Salvia* species, the plants and their components exhibit excellent antioxidant activities. Antioxidant activity was identified in all the species, as measured by oxygen free radical absorption efficacy, total phenolic content, and free radical scavenging efficacy. *Salvia miltiorrhiza* has been demonstrated to lower ROS production by suppressing oxidases, decreasing superoxide formation, inhibiting low-density lipoproteins from oxidative modification, as well as alleviating oxidative stress in mitochondria.[21] The antioxidant activity of clary sage oil can be attributed to its high fatty acid content. The seeds of clary sage have a high fatty acid content ranging from 23.83 to 29.34%, with polyunsaturated fatty acids being the most common and having high antioxidant activity, according to Aćimović *et al*.[4]

Effects of clary sage essential oil on CAT and SOD activities in AMJ-13 cancer cells

The CAT and SOD enzyme activities in cells treated with 54.85 g/ml clary sage oil and control cells are displayed in Figures 3 and 4. The catalase value in cells treated with clary sage oil (111.8 nmoles/min/million cells) was significantly lower than the control (1637.6 nmoles/min/million cells) as shown in Figure 3. However, the value of the SOD enzyme in cells treated with clary sage oil did not increase and was equivalent to the value of the control cells that were not detected, indicating that the cells did not produce the enzyme.

SOD and CAT are antioxidant enzymes that decompose superoxide radicals, hydrogen peroxides, and hydroperoxides into harmless molecules.

These enzymes prevent the formation of reactive species or free radicals in cells by neutralizing any component that can transform into a free radical or any free radical that can trigger the generation of other radicals. SOD enzyme deficiency is common as SOD levels decline with age, but CAT is a common antioxidant enzyme found in abundance in cells.[22]

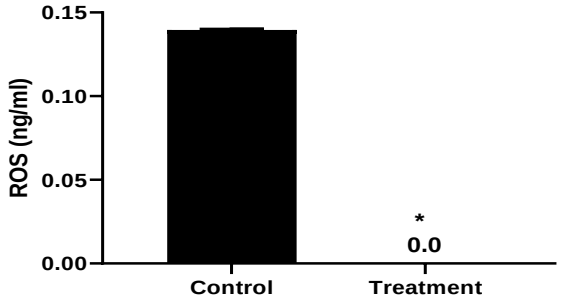

Figure 2: The level of reactive oxygen species in the AMJ-13 cells.

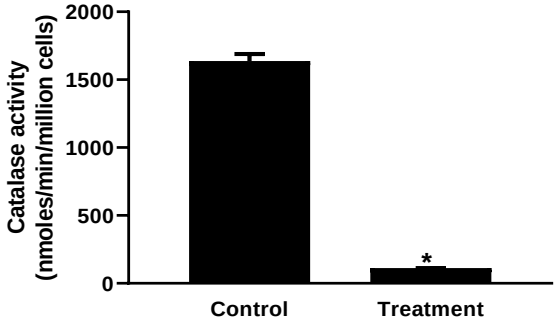

Figure 3: Catalase activity in the AMJ-13 cells.

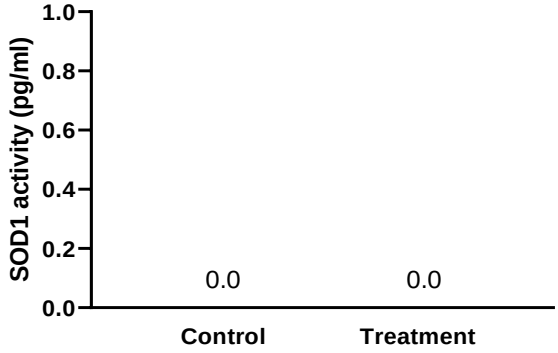

Figure 4: Superoxide dismutase (SOD)-1 activity in the AMJ-13 cells.

It is possible that the significant decrease in the value of catalase enzyme, and the equal level of SOD enzyme in clary sage oil-treated cells compared to control, is related to a significant decrease in the value of ROS in clary sage oil-treated cells. The oil serves as an antioxidant by blocking free radicals and lowering their levels, decreasing the need for the cell to increase the synthesis of both enzymes.

Conclusion

The findings from the present research show that clary sage oil has cytotoxic and antioxidant properties. Therefore, it can be utilized as a nutritional enhancer for cancer prevention and disease reduction, and it may be employed to inhibit other types of cancer cells after further research. Taking into consideration that the clary sage is one of the plants that are used in the field of health to treat some diseases, and in the field of culinary as a source of income, it is unlikely that it is harmful. As a result, it is recommended as a preferred choice as an

enhanced treatment over other chemical treatments that have adverse effects.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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