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Journal of Bioscience and Applied Research
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Determination of ex vivo chemical and in vivo biological antioxidant activities of clary sage essential oil

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DOI: 10.21608/jbaar.2024.259473.1030

Abstract:

Several studies have emphasized the substantial medicinal properties of clary sage (scientifically: *Salvia sclarea*) oils or extracts in preventing and treating a wide variety of health issues and disorders. GC-Mass investigated the chemical components of sage oil. Examining the free radicals scavenging potency of the oil was performed via assay of DPPH, for the biological effect of sage oil on the development and progression of cancer cells. The oil's antiproliferative capacity on cancer cells (toxicology test) was assessed on the hepatocellular carcinoma (HCAM) cell line using the MTT viability test. According to the results of the GS-MS analysis, the procedure successfully yielded 100% total identification of the chemical composition of Clary sage oil, of those are Gamma-terpinene (17.847%), heptane (16.759%), D-Limonene (12.654%), isopropyl myristate (10.179%), and linalool (8.189%). The examined antioxidant activity showed an increase in the level of catalase enzyme at 1.5h followed by complete reduction at 72h post-treatment. SOD was also reduced significantly after 72 h incubation and was not observed to be affected by exposure to oil. The cytotoxic activity of the oil was evident at (IC50=17.32µg/ml) when compared to the control, and the genomic DNA integrity of the HCAM cell line was degraded as presented on a gel. These findings indicate the potential use of Clary sage oil as an antioxidant and anti-cancer agent. The current investigation highlights the importance of setting up shorter exposure intervals with the sage oil to achieve the best findings concerning the pathophysiological cues against hepatocellular carcinoma progression.

Keywords: Clary sage, Essential oil, Hepatocellular carcinoma (HCAM), antioxidants, anti-cancer, Radical scavenging activity

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Introduction:

Several reports highlighted the significant therapeutic characteristics of clary sage (scientifically: *Salvia sclarea*) oils or extracts for preventing and treating many health conditions and diseases (1). Clary sage is also widely utilized in commercial industries which involve the production of food, pharmaceuticals, and cosmetic products (2). This plant is commonly used as a flavoring spice which was traditionally applied for the management of high blood pressure (3), pain and discomfort (4), mood disorders (5), oral hygiene (6), arthritis (7),

Received: December 30, 2023. Accepted: February 20, 2024. Published: August 19, 2024

gastrointestinal diseases (8), and tissue oxidation and oxidative stress accumulation (9). Clary sage was also reported with potent pharmacological properties for numerous applications that have been widely employed both in clinical and research fields such as anti-inflammatory, antimicrobial, antioxidant, antidepressant, and anti-cancerous agents (10,11). Given the utilized chrematistics as an effective anti-oxidative stress and tissue oxidation, antioxidant compounds in Clary sage were found to play fundamental roles in sustaining normal physiological cell functions and preventing the development and progression of the wide range of diseases that their route of pathogenesis dependent upon tissue oxidation and free radicals formation (8). The antioxidant property of clary sage was linked to its main phytochemical compounds, the flavonoids, and phenolic glycosides, as both have been extensively reported in the herbal literature as potent antioxidants (12,13). The phenolic compounds were described with the ability to trigger a mechanism that ensures scavenging reactive oxygen species from tissues and activates the cellular antioxidant defense systems against oxidative stress and free radicals accumulation (14). This documented observation was not limited to the flavonoids and phenolic compounds, salvianolic acid was also found effective as both an antioxidant and as a scavenger of free radicals (1), in vitro analysis reported a much greater scavenging ability of the salvianolic acid over the other phenolic compounds of Salvia sp. (15). Additionally, it has been demonstrated that the aqueous extract of *Salvia sp.* possesses both antiviral and antioxidant properties. A study showed that the antioxidant status of the liver was found to be improved after individuals consumed sage tea for two weeks (16). Due to the abundance of chemicals with cytotoxic qualities that can kill cancer cells while sparing normal tissues, Salvia sp. where reported possesses a notable anti-cancer effect that stands out clearly in the literature (10). The proposed mechanism by which Salvia sp. to fight cancer development and progression was proposed through inhibiting oxidative stress and free radicals' buildups in tissues (8), which the latter were found to trigger cancer mutagenesis of the DNA materials (17), cell immortalization via inhibiting the physiological necrosis and apoptosis (18), and higher angiogenic activity which is required by most types of solid tumors for creating new blood vessels (19). Interestingly, plant extract showed anticancer activity (20). The potential free radical scavenging ability of any herbal extract can be assessed in vitro by following the chemical method of DPPH assay (2), whereas the antioxidant property can be measured in cells by examining levels of catalase and SOD (21).

To the best of the current understanding, however, there have been very limited studies conducted to date that have studied the possibility of exploiting the proposed antioxidant and radical scavenging capabilities of clary sage oils as an anti-cancer agent in tissues or neoplastic cell lines, specifically in hepatocellular carcinoma. Therefore, the main aim of the current study was to examine the antioxidant properties of clary sage on the progression of a murine cell line of hepatocellular carcinoma (HCAM) via measuring the in vitro cellular levels of SOD, catalase, and ROS before and after treatment with clary sage oil at different durations of incubation. The study will also include a comparison of free radicals scavenging capacity between the sage oil and ascorbic acid as a control via following the DPPH assay.

Methods:

1. HCAM cell line maintenance

HCAM cancer cell line was obtained from the Cell Bank Unit at the University of Basrah. Generation and characterization of the HCAM cell line was performed by (22). The cell line was maintained in RPMI-1640 (Gibco, USA) supplemented with 10% Fetal bovine (Gibco, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin. Routine cell passage was performed by using Trypsin-EDTA (Capricorn, USA) followed by reseeding at 50% confluence

twice a week and kept at 37 °C and 5% CO_2 incubator (Cypress Diagnostics, Belgium) (23).

2. GC-MS analysis

Clary sage essential oil was purchased from purely Black Australia Co. To evaluate the chemical components of clary sage essential oil, Gas chromatography-mass spectrometry (GC-Mass) was used for the analysis process according to (11). The identified chemical composition of Clary sage oil is shown in Table 1.

3. Scavenging activity of Clary sage oil

The DPPH solution was prepared as described previously by (24). Fresh preparation of 0.1 Mm DPPH stock solution (Sigma-Aldrich, USA) was suspended in 96% ethanol and mixed vigorously before being kept at room temperature in the dark before use. The working solution was prepared in a test tube by taking 1 ml of the stock and diluted by the same solvent. Serial concentrations of the working solution and the oil were prepared in different test tubes to a final concentration of 100, 200, 300, and 400 µg/mL, which were incubated in the dark for 30 minutes at room temperature. Data were acquired at 517nm by utilizing a spectrophotometer (Varian Cary 50 Bio UV-Vis, USA) under a dim light. Samples absorbance was measured against blank. The DPPH radical scavenging effect results in colorimetric changes which were calculated by following the equation from (25,26).

$$\% \text{ DPPH reduction rate} = ((A0 - As) / A0)) \times 100$$

Where (A0) represents the absorbance of the control and (As) is the absorbance of the sample. The (%) of DPPH reduction values were normalized per sampled air volume (m^3, DPPHV) or Per PM mass amount (mg; %DPPHM). Ascorbic acid (400µg/ml) was used as a positive control.

4. Antioxidant activity of clary sage essential oil

HCAM cells test samples were incubated with 400 µg/mL of clary sage essential oil for periods of, 0, 1, 1.5, 4.5, and 72 hours, respectively. Cellular protein was then extracted from cell lysates by incubating collected cells with RIPS lysis solution (Merck, USA) for 10 minutes at 37°C. Protein concentration was evaluated by following the bicinchoninic acid (BCA) assay protocol as described previously by (27), and according to the manufacturer's instructions (Merck, USA). Protein absorbance was then measured on a microplate reader at 562 nm (Bio-rad, France).

To measure levels of the enzymatic antioxidants catalase (CAT) and superoxide dismutase (SOD in the collected cellular proteins, the ELISA technique was applied, kits were purchased from Elabscience-USA and the methodology steps were followed according to the manufacturer's instructions. A microplate reader set at 450 nm was utilized to determine the optical density of the samples against the standard set of standard proteins.

5. Evaluation of the Clary sage essential oil's anti-proliferative activity

The anti-cancer activity (in vitro toxicology assay) for Clary sage was evaluated on the HCAM cell line. The antiproliferative activity of Clary sage was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma, USA). HCAM cell lines were treated with trypsin and resuspended in RPMI 1640 culture media with 10% (v/v) fetal bovine serum, before being reseeded on 96-well plates at a density of 1×10^4 cells per 100µl. After forming the confluent monolayer, the cells were then treated with clary sage essential oil at the known six concentrations of 1,5,20,50,100 and 750 µg/ml, respectively, in sets of triplicates. Plates were then incubated for 72 hours at 37 °C in a 5% CO_2 atmosphere. After 72 hours of treatment, cell viability was assessed by removing the medium, adding 28 µL of an MTT (Merck, USA) solution containing 2 mg/mL of solution, and incubating the cells for 2 hours at 37 °C, which was then further

incubated at 37 °C for 15 min while being shaken (28). The assay absorbency was measured on a microplate reader at 590 nm. The following equation was used to calculate the percentage of cytotoxicity or the rate at which cell growth was inhibited:

Proliferation rate as (PR)= B/A*100 where A is the mean optical density of untreated wells and B is the optical density of treated wells and IR=100- PR (29). The inhibition concentration of Clary sage oil that kills 50% (IC_{50}) of HCAM cells was calculated by extrapolating the samples' cell numbers against the standard numbers.

6. Evaluating DNA integrity in HCAM cells

The Geneaid DNA extraction kit-USA was used to extract the genomic DNA from HCAM cells. The procedure was carried out following the manufacturer's instructions after cells were pre-incubated with clary sage oil at the IC_{50} concentration. The concentration of the eluted DNA was determined by using NanoDrop-UK. The freshly prepared DNA samples were then loaded into microwells of the TAE agarose gel (1.5%) which was stained with 2 µl ethidium bromide dye for the electrophoresis step. The agarose gel was run at 85 volts for 50 minutes, and the DNA integrity was then assessed on the agarose gel by using a UV transilluminator.

7. Statistical analysis

Data were collected on Microsoft Excel 365 and processed for statistical analysis on SPSS v.28 (IBM, USA). Graphs were created by plotting data on Graphpad Prism version 9 for Windows 10 (California, USA). Significant differences between the studied and control groups were obtained by performing an independent sample t-test. Differences were considered significant at values of $p \leq 0.05$ (30).

Results:

GC-Mass findings successfully yielded 100% total identification of the chemical composition of Clary sage. In this sense, thirty-seven elements were identified in the oil, and percentages of these elements are presented in Table 1 and Figure 1. The main important components that constitute above 5% of the total are Gamma-terpinene (17.847%), heptane (16.759%,), D-Limonene (12.654%), isopropyl myristate (10.179%), and linalool (8.189%).

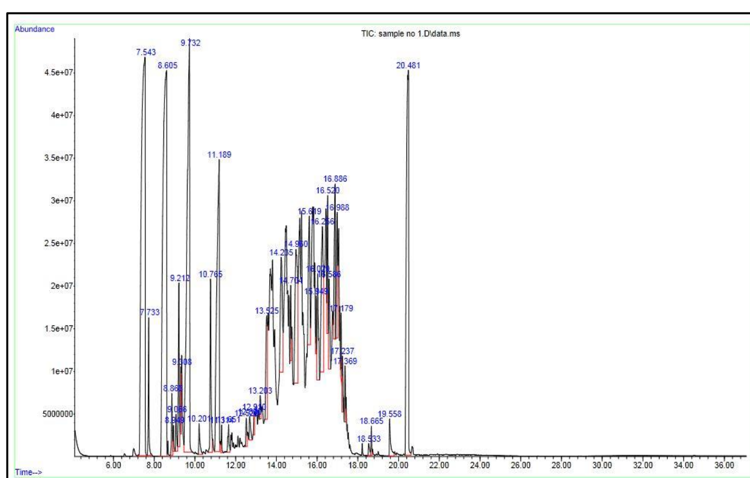

Figure 1: GC-Mass findings and composition of *Salvia sclarea* essential oil investigated by GC-Mass.

Table 1: Chemical components of *Salvia sclarea* essential oil obtained by GC-Mass.

No.	Compounds	Percentage
1	gamma.-Terpinene	17.8474%
2	Camphene	1.1156%
3	Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-	16.7591%
4	beta.-Myrcene	0.5764%
5	Cyclohexene, 1-methyl-4-(1-methylethyl)-	0.2257%
6	Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	0.3984%
7	3-Carene	1.5935%
8	7-Oxabicyclo[2.2.1]heptane, 1-methyl-4-(1-methylethyl)-	0.6068%
9	D-Limonene	12.6543%
10	gamma.-Terpinene	0.4084%
11	Cyclohexene, 1-methyl-4-(1-methylethylidene)-	2.1028%
12	Linalool	8.189%
13	Fenchol	0.2036%
14	Bicyclo[3.1.0]hexan-3-ol,4-methylene-1-(1-methylethyl)-	0.363%
15	.alpha.-Terpineol	0.3377%
16	Dodecane	0.372%
17	Tetradecane	0.247%
18	Dodecane, 3-methyl-	0.2785%
19	Dodecane	1.6922%
20	Tridecane	2.7196%
21	Tetradecane, 2-methyl-	0.7109%
22	Carbonic acid, decyl tridecyl ester	4.162%
23	Tetradecane	2.3109%
24	Heneicosane	0.3329%
25	Pentadecane, 2-methyl-	1.5117%
26	Pentadecane	3.6002%
27	Pentadecane, 2-methyl-	1.3152%
28	Dodecane, 2,6,11-trimethyl-	1.1509%
29	Pentadecane	2.0101%
30	Pentadecane	1.6726%
31	Pentadecane, 4-methyl-	0.3813%
32	Pentadecane, 3-methyl-	0.4778%
33	Pentadecane, 7-methyl-	0.5413%
34	Cyclopentaneacetic acid, 3-oxo-2-pentyl-, methyl ester	0.1787%
35	Anthracene, 9-dodecyltetradecahydro-	0.2147%
36	Octanal, 2-(phenylmethylene)-	0.5595%
37	Isopropyl myristate	10.1786%
	Total	**100%**

The free radicals scavenging activity of Clary sage was assessed with DPPH assay and results showed that Clary Sage oil has a moderate to weak dose-dependent activity when compared to positive control 400µg/ml ascorbic acid. The maximum reported scavenging activity of the oil was found at 17.7 % at 400µg/ml, figure 2.

Regarding the antioxidant activity of Clary sage oil on the HCAM cell line, results showed a significant reduction in SOD at 72 hours post incubation ($P=0.002$). However, shorter incubation periods did not present any substantial differences, table 2. Catalase presented a significant decrease in concentration at 1, 4.5, and 72 hours when compared to control, being ($P=0.010$, $P=0.011$, and $P<0.001$, respectively). Interestingly. At 1.5 hours incubation, catalase highly increased than control, yet no significant variance was reported in Table 2.

The anti-proliferative activity of Clary sage essential oil was determined by the MTT viability test, in which the assays present a cytotoxic effect on the oil on the HCAM cell line. Higher concentrations of the oil showed a damaging effect on the viability of the investigated cancer. This effect was dose-dependent, therefore the effects enhanced as the concentrations increased. It was found that the inhibitory concentration (IC_{50}) required to kill 50% of the tumor cell was verified at 17.32 µg/ml. Investigating DNA integrity and level of fragmentation of the treated HCAM cells with 17.32 µg/ml of the oil on gel showed that the treated group with Clary sage essential oil suffered genomic DNA degradation and smearing on gel indicating an anti-cancerous activity, figure 3.

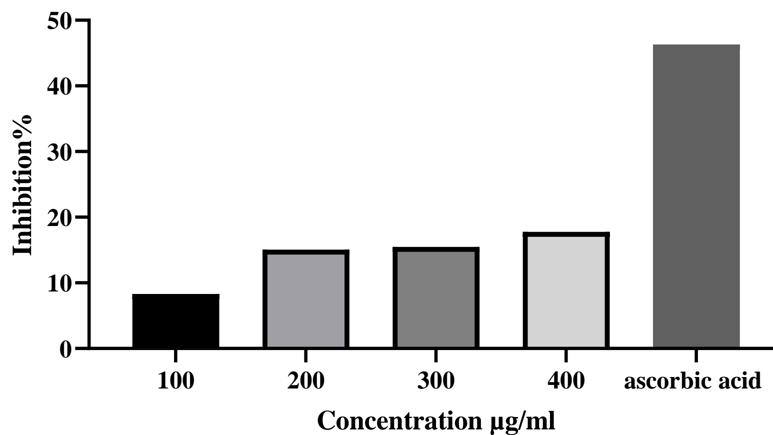

Figure 2. Shows radical scavenging activity (RSA) of the extracted clary sage essential oil. Ascorbic acid (400µg/ml) was used as a positive control. $P<0.01$.

Table 2. Shows HCAM cell line responses to different incubation periods with 400µg/ml of the Clary sage essential oil.

Characteristics	Control (Mean±SD)	Study (Mean±SD)	P value
SOD µg/ml			
0 hour	874±49.8	874±49.8	-
1 hour	874±49.8	852.5±21.6	0.605
1.5 hour	874±49.8	866.5±7.8	0.843
4.5 hour	874±49.8	811±4.1	0.149
72 hour	874±49.8	0.0±0.0	0.002
Catalase nmol/ml/min			
0 hour	3045±879.4	3045±879.4	-
1 hour	3045±879.4	195.5±15.9	0.010
1.5 hour	3045±879.4	5326.5±935.3	0.066
4.5 hour	3045±879.4	291±77.6	0.011
72 hour	3045±879.4	111.8±1.4	<0.001

Independent sample t-test

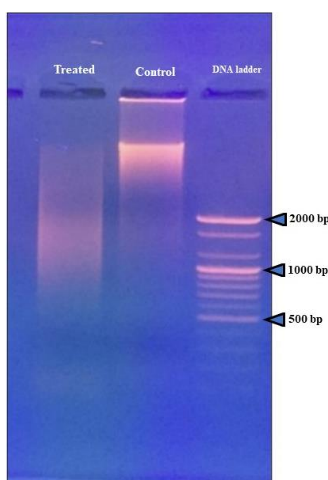

Figure 3. Effect of 17.32 µg/ml of Clary sage essential oil on HCAM cell line genomic DNA integrity (Control vs Treated).

Discussion:

Salvia sp. displays a noteworthy anti-cancer effect that stands out clearly in the literature, and this is due to the richness of compounds with cytotoxic properties that can destroy cancer cells while sparing normal tissues (10,31,32). Cancer is characterized by aberrant cell development that has a propensity to multiply uncontrollably and, in certain situations, spread to other body areas (33). Clary sage is one of the most commonly utilized sage species in applications of traditional medicine, and that has been employed for many years to treat illnesses like indigestion, stress, migraines, oxidation stress, and heart conditions (1,34,35). However, there is a lack of research indicating whether or not the plant can effectively combat hepatocellular carcinoma through the proposed anti-cancerous properties.

In general, researchers have highlighted the application of Salvia species preparations as a possible adjuvant reagent which can be utilized for boosting anticancer immune reaction in the treatment of hepatocellular carcinoma. In earlier

studies, it was discovered that aqueous extract from *S. miltiorrhiza* caused apoptotic cell death and inhibited the proliferation of hepatocellular carcinoma in vitro investigation. This phenomenon was related to the depletion of intracellular GSH and the reduction of mitochondrial membrane potential (36). Moreover, *Salvia chinensis* alcohol extract was found to inhibit tumor development and angiogenesis in Hepatoma H22-bearing mice while drastically lowering VEGF expression and tumor microvessel density (37). In vitro analysis of the sage oil showed that polysaccharides (SMP-W1) which were isolated from *S. miltiorrhiza* suppressed the proliferation of hepatocellular carcinoma H22 cells and enhanced the immune response to cancer development in animal models carrying tumors (38).

The current GS/MS analysis of sage oil showed a variety of active compounds that were successfully identified in oil which seems to correspond with previous reports (11,33,39,40). However, the relative abundances might fluctuate quite slightly between studies. Climate or the conditions of the soil might be the reason for the variations in the composition of oils. The results highlighted major compounds identified by the current analysis including Gamma-Terpinene (17.847%), D-Limonene (12.654%), isopropyl myristate (10.179%), heptane (16.759%), and linalool (8.189 %).

In vitro, oil supplementation to cultured HCAM cells showed that the sage oil possesses radical scavenging activity that was witnessed chemically in DPPH analysis. However, when compared to ascorbic acid scavenging performance, the oil showed noticeably weaker scavenging activity. Similar observations have also been reported by several studies (33,41,42). Nevertheless, prolonged incubation with the oil (72h) showed significant reductions in SOD when compared to control. The drop in SOD can be considered a sign of clary sage oil's efficacy as an effective anti-cancer agent by limiting scavenging oxidation stress in cancer cells while sparing normal cells from free radicals. In contrast, a shorter duration of incubation seems to correspond with the proposed potency of the oil as an effective agent against tissue oxidation. Higher oxidative stress and free radical accumulation in tissues damage DNA integrity and induce cancer cell apoptosis (43,44). Similarly, levels of catalase were also significantly reduced by 72h incubation, however, an earlier rise by almost double to the control was witnessed at (1.5) hours. The fluctuations in catalase might indicate different pathological cues being stimulated at various time intervals. In general, the antioxidant activity of catalase was inhibited at 72h in HCAM cells. Suggests that shorter durations of incubation could play a key role in investigating extracted oils from plants in studies concerning tissue antioxidation markers.

This might indicate an alternative cue that has been stimulated the induce the significant reduction in HCAM cell line viability and genomic DNA smearing on gel post-treatment. Findings from Clary sage are consistent with studies that highlighted the anti-tumorigenic property of *Salvia sp.* (45). In a study by Raveau *et al.* (34) found that essential oil obtained from in vitro-grown Clary sage exhibited cytotoxic activity against the leukemia cell line that was nearly two times more potent than that of a control. However, Raveau *et al.* (34) reported a higher cytotoxic effect of the studied *Salvia sp.* at (IC50=8.1 µg/ml) compared to the study finding (IC50=17.32 µg/ml).

Indeed, the total flavonoid compounds from *S. chinensia* promoted apoptosis in HepG2 and Huh-7 in human hepatocellular carcinoma cells by inhibiting cellular NF-B signaling. In vivo setups, flavonoids were found capable of inducing H22 cell death without discernible harmful effects on healthy hepatocytes and essential organs of animals with tumors (46). Limonene, linalool, terpineol, and α-terpineol were reported with six times cytotoxic activity on cancer cell lines when compared to the control (47). The abundant amount of Heptane in the

current investigation showed in studies effectively suppressing impacts on the NFκ-B signaling pathway which is required in malignancy proliferation, cell cycle arrest, and induction of DNA damage (48,49).

These findings point to the possibility of utilizing oil from Clary sage as an antioxidant, and anti-cancerous agent. This is crucial when considering the biological activity of essential oil as well as its future applications in the clinical fields of battling cancer.

Conclusion:

Findings revealed that phytochemical constituents of Clary sage exhibited significant contributions in preventing cancer progression and initiating cancer cell death that in the future might result in extensive usage of the plant extracts in the clinical applications of fighting cancer or augmenting conventional anti-cancer treatments. Similarly, gathered evidence given by the current investigation supports the notion regarding the cancer-preventative effects of Clary sage that could also be applied against a wide variety of malignancies which are not limited to hepatocellular carcinoma. Care must be taken, however, in designating similar studies as the current study observed shorter duration of tissue supplementation with oil seems to uncover pathophysiological cues in favor of battling cancer that were not necessarily detectable in longer time intervals.

Conflict of interest:

The authors declare no conflict of interest.

Funding: None

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