Experimental study

Antioxidant and Antiproliferative Properties of the Essential Oils of *Satureja thymbra* and *Satureja parnassica* and their Major Constituents

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Abstract. Aim: The biopotential of the essential oils of the Greek aromatic plants *Satureja thymbra* and *Satureja parnassica* were investigated, together with their major components carvacrol, thymol, γ-terpinene and *p*-cymene. Materials and Methods: Antioxidant and cancer cell cytotoxic properties were determined using 2,2-diphenyl-1-picrylhydrazyl and sulforhodamine B assays, respectively. The antiproliferative potential was studied against the cell lines, MCF-7, A549, HepG2 and Hep3B. Results: *S. thymbra* oil possessed stronger antioxidant and antiproliferative capacity when tested on MCF-7 cells compared to *S. parnassica* oil. Thymol exhibited two-fold greater antioxidant potency than carvacrol, whereas γ-terpinene and *p*-cymene had no significant effect. Carvacrol was the most potent antiproliferative agent against A549 cells, while Hep3B cells were the most sensitive to thymol. *p*-Cymene and γ-terpinene demonstrated negligible bioactivity. Conclusion: *S. thymbra* and *S. parnassica* essential oils exhibit significant but diverse antioxidant and antiproliferative activities, mainly attributed to their main components, carvacrol and thymol.

Aromatic plants have been used for their preservative and medicinal properties since ancient times. These properties are attributed to some extent to essential oils, which are formed as secondary metabolites. The term 'essential oils' was first used five centuries ago by Paracelsus von Hohenheim, who named the active constituent of a drug '*Quinta essential*' (1). Essential oils are extracted from plants mainly by distillation. They contain mostly terpenes and some other non-terpene components. Their physical role is to protect plants against bacteria, fungi, viruses, insects and even against herbivores, whose appetite they reduce for these plants (2). They have been used against microorganisms, as sedatives, for food preservation, and as food additives for aroma in cosmetics and perfumes (1, 2). Although their therapeutic potential had been put aside for decades, it has drawn increasing attention from the scientific community since the 1950s. This coincides with a general public trend away from chemical substances and towards using natural products for the treatment of many diseases. As a result, many scientific groups are investigating the antioxidant, antimicrobial, antifungal, antiviral, anticancer and other properties of essential oils and their main constituents.

Satureja thymbra (common names: thyme-leaved savory, pink savory, thrubi) is an aromatic plant endemic to the Mediterranean region and is the most common specimen of the *Satureja* genus. The essential oil obtained from the *S. thymbra* plant has been tested against several organisms and showed to possess potent antioxidant, antifungal, antibacterial, antiviral and insecticidal properties (3- 8). It has also been studied for its cytotoxic potential against human cancer cell lines (9). *S. parnassica* is endemic to central and southern Greece. The biological activities of *S. parnassica* have been studied to a lesser extent; however, the existing studies reveal it to have a potent antibacterial and insecticidal essential oil (3-5). Both plants have been used

since ancient times as local spices and herbal home remedies, due to their antiseptic and diuretic properties. The composition of their essential oils depends on the harvesting time (*e.g.* before or after flowering). However, it has been shown that two phenol monoterpenes, carvacrol and thymol, are the major components at all harvesting times. Moreover, thymol and carvacrol, together with their biosynthetic precursors, *p*-cymene and γ-terpinene, comprise more than 70% of the oil (3, 4).

The composition of an essential oil may play an important role in its biological activities. Therefore, the major constituents of the essential oils of *S. thymbra* and *S. parnassica*, carvacrol and thymol, have also been studied for their potential protective and therapeutic role against a wide variety of pathological conditions and organisms (1, 2, 10). In moderate concentrations, they do not affect the viability of normal cells and have been shown to protect cells from oxidative stress induced by agents such as H_2O_2 (11- 13). Their promising activities, together with the low toxicity they present, may allow for their use as the basis for drug development, alone or together with known synthetic drugs in order to enhance their efficacy.

The aim of this study was to determine the antioxidant and potential antiproliferative activity of the Greek *S. thymbra* and *S. parnassica* essential oils. In addition, we wanted to examine the biological potency of their main components *in vitro*; therefore the antioxidant and antiproliferative properties of carvacrol, thymol, *p*-cymene and γ-terpinene against a panel of human cancer cell lines were also investigated.

Materials and Methods

Chemicals and reagents. Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), trypsin, and penicillin/streptomycin were purchased from Biochrom AG,

F12K medium was obtained from Gibco (Gaithersburg, MD, USA). Phosphate-buffered saline (PBS) was purchased from Biosera (Boussens, France). Ascorbic acid, dimethyl sulfoxide (DMSO), sulforhodamine B (SRB), Trizma base, carvacrol, thymol, *p*-cymene and γ-terpinene were purchased from Sigma-Aldrich (St Louis, MO, USA). Trichloroacetic acid (TCA) was purchased was from Applichem (Darmstadt, Germany), acetic acid from Scharlau (Barcelona, Spain), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Calbiochem (Darmstadt, Germany).

Essential oils. Essential oils of *S. thymbra* and *S. parnassica* originated from plants that were harvested after their flowering season and were extracted as described in the study by Chorianopoulos *et al.* (4). The main components of the oils is presented in Table I.

Cell lines and cell cultures. The human cancer cell lines MCF-7 (mammary adenocarcinoma, A549 (non-small cell lung adenocarcinoma), HepG2 and Hep3B (hepatocellular carcinomas) were obtained from the American Type Culture Collection (Rockville, MD, USA). HepG2, Hep3B, and MCF-7 cells were grown and maintained in DMEM, whereas the F12K medium was used for the A549 cell line. All media were supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) and were incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. Stock cultures were passaged at 2- to 3-day intervals. Cells were seeded at a density of 5.0×10^3cells per well in 96-well plates for the SRB assay.

Antioxidant activity (DPPH assay). The radical-scavenging activity of the essential oils and their major components was estimated using the free radical DPPH. Increasing

concentrations of the oils [*S. parnassica* 2-100% (v/v) and *S. thymbra* 0.4-100%(v/v)] and their major components (0.1-10 mM) were prepared using DMSO as a solvent. Using a final volume of 10 μl, each concentration was placed in a 96-well plate in triplicates then 190 μl of an 800 μM methanolic solution of DPPH were added. Ten microliters of DMSO with 190 μl DPPH were used as a control. The plate was left in the dark for 30 min, and then the absorbance was measured at 517 nm using an ELISA plate reader (Sunrise, Tecan, Männedorf, Switzerland). Ascorbic acid at concentrations of 10-640 μM was used as a standard as it is a known potent antioxidant.

The percentage inhibition of the DPPH radical for each concentration was determined according to the following formula:

$$\text{DPPH radical-scavenging activity (\%)} = [(OD_{control} - OD_{sample}) \div OD_{control}] \times 100$$

SRB assay. The viability of human cancer cells after treatment with essential oils and their components was determined using the SRB assay. SRB is a dye that binds to basic amino acids of cellular proteins, and the number of viable cells is then estimated with colorimetric evaluation. Cells were plated in 96-well plates and treated with different concentrations of each of the test substances for 72 h. The concentrations were 0.00000025-0.25% (v/v) for the oils, 1-10 mM for *p*-cymene and γ-terpinene for the MCF-7 cell line, and 1-1000 μM for carvacrol and thymol for the remaining cancer cell lines. After treatment, cells were fixed with the addition of 25 μl of 50% (w/v) cold TCA to the growth medium and the plates were incubated at 4°C for 1 h. The cells were washed five times with tap water and then stained with 50 μl of 0.4% (w/v) SRB in 1% (v/v) acetic acid for 30 min at room temperature. Five rinses with 1% (v/v) acetic acid followed to remove unbound dye. The fixed, stained plates were allowed to air-

dry and the bound dye was then solubilized by adding 100 µl of 10 mM Trizma base for at least 5 min. Absorbance was measured at 570 nm using an ELISA plate reader and the percentage cellular survival was calculated using the formula:

$$[(OD_{Sample} - OD_{medium\ blank}) \div (mean\ OD_{control} - OD_{medium\ blank})] \times 100$$

Data analysis. All experiments were performed at least in triplicate. The concentration of test sample required to cause a 50% reduction in the DPPH free radical (IC_{50}) and required to reduce cancer cell viability by 50% (EC_{50}) were calculated from the respective dose–response curves by regression analysis using a four-parameter logistic curve through Sigma Plot Software (v.10) (Systat Software Inc., San Jose, California, USA).

Results

Antioxidant activity. DPPH assay is widely used for the evaluation of the properties of various plant constituents for scavenging free radicals (14). The results show that *S. thymbra* oil exhibited stronger antioxidant capacity [IC_{50}=0.22±0.25% (v/v)] compared with *S. parnassica* essential oil [IC_{50}=0.4±0.3% (v/v)]. The scavenging activity of the oils is presented in Figure 1. The IC_{50} value for ascorbic acid was estimated to be 0.032±0.0034 mM (data not shown), similar to a previous report (15). The IC_{50} values for carvacrol and thymol were estimated to be 2.28±0.45 mM and 1.14±0.18 mM, respectively (Figure 2). Thymol is a stronger antioxidant, exhibiting 35 times lower activity than ascorbic acid. Carvacrol, on the other hand, was found to have 70 times less scavenging activity than ascorbic acid. On the other hand, γ-terpinene and *p*-cymene did not show any significant scavenging activity in the range of concentrations tested (Table II).

Antiproliferative activity. The antiproliferative potential of the essential oils of the selected plants and their components was tested using the SRB cell viability assay. Essential oils of the plants *S. thymbra* and *S. parnassica* were tested for their anticancer potential against mammary adenocarcinoma MCF-7 cells. *S. thymbra* oil exhibited higher cytotoxicity [EC_{50}=0.002±0.00038% (v/v)] than *S. parnassica* oil [EC_{50}= 0.08±0.03% (v/v)] after incubation with cells for 72 h (Figure 3).

The antiproliferative potential of γ-terpinene and *p*-cymene was also tested against the MCF-7 cancer cell line. The viability of cancer cells was not significantly affected (EC_{50} values were 4.6±0.11 mM and 5.11±0.5 5mM for γ-terpinene and *p*-cymene, respectively) and, therefore, they were not further tested (Table III).

Carvacrol and thymol, on the other hand, exhibited noteworthy cytotoxicity and were examined against the A549, Hep3B and HepG2 cancer cell lines (Figure 4, Table III). Carvacrol had the most potent cytotoxicity against A549 cells (EC_{50}=0.118±0.0012 mM). Hep3B cells were found to be more resistant (EC_{50}=0.234±0.017 mM), while HepG2 cells were the least sensitive to treatment with carvacrol (EC_{50}=0.344±0.0035 mM).

Thymol, on the other hand, was most effective against Hep3B cells (EC_{50}=0.181±0.016 mM). A549 cells were less sensitive to thymol (EC_{50}=0.187±0.061 mM), similar to treatment with carvacrol. HepG2 cells were once again the most resistant, with an EC_{50} value greater than 0.35 mM (EC_{50}=0.390±0.01 mM).

Discussion

Antioxidant activity. The essential oil of *S. thymbra* was found to possess approximately two-fold stronger antioxidant capacity than the oil of *S. parnassica* using

the DPPH-scavenging method. This may be due to the fact that the oil of *S. parnassica* contains a higher percentage of γ-terpinene and *p*-cymene, which we showed to possess no antioxidant activity. To our knowledge, this is the first time that the antioxidant capacity of *S. parnassica* has been determined. The essential oil of *S. thymbra*, on the other hand, showed strong antioxidant activity in a study where the oil was derived from plants collected during their flowering stage in Libya (8).

In our study, thymol had the strongest antioxidant activity among the other components of theseessential oils (IC_{50}=1.14±0.18 mM), at a value that was 35 times lower than that of ascorbic acid. Carvacrol was found to possess two-fold lower activity compared to thymol and 70-fold lower compared to ascorbic acid (IC_{50}=2.28±0.45 mM) whereas *p*-cymene and γ-terpinene did not exhibit any antioxidant capacity *in vitro*. These results are in agreement with a study in 2012 where carvacrol and thymol were found to be the most potent antioxidants using the 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid method; however, the IC_{50} values for the terpenes were significantly lower (16).

Carvacrol and thymol are considered strong antioxidant agents, and this is proven by studies that deal not only with the isolated phenols but also with oils that contain these compounds at a high percentage. In a study of 2012, for example, the essential oils of seven *Thymus* species were divided according to their content and tested for their antioxidant capacity. The group of oils that contained carvacrol as their main component demonstrated the highest activity (17). Carvacrol has also shown strong antioxidant capacity using different *in vitro* methods (18). In addition, both phenols have been examined individually for their properties, showing significant antioxidant properties *in vitro* (19).

Carvacrol and thymol were the components considered responsible for the antioxidant activity of the essential oil of *Thymus pectinatus*, *Ocimum basilicum* L. *Origanum vulgare* L. and *Thymus vulgaris* L (20, 21). Finally, in a study where different extracts and essential oil chemotypes of *Thymus vulgaris* were screened, the highest activity was shown by the oils that were abundant in thymol (22).

p-Cymene has demonstrated only weak antioxidant activity whenever it has been studied and regardless of the assay used (19, 23, 24). γ-Terpinene, on the other hand, was found to possess strong antioxidant capacity using the DPPH method (23). Similarly, in another study, γ-terpinene had significant antioxidant activity, comparable with that of α-tocopherol, a known antioxidant (19). Conversely, more recently it was considered to possess moderate antioxidant capacity, but the concentrations that were used in that study were quite high (0-180 mM) (25). Finally, its IC_{50} value was found to be 30 mM using the DPPH assay after an incubation period of 26 h (26).

Antiproliferative activity. This is the first time as far as we are aware that the antiproliferative activity of the oil of *S. parnassica* has been studied. Our results show that the oil of *S. parnassica* is a weaker antiproliferative agent compared to that of *S. thymbra* against MCF-7 cells. From the EC_{50} values, *S. thymbra* oil possesses 40-fold times stronger activity than that of *S. parnassica* [EC_{50}=0.002±0.00038% (v/v)] and EC_{50}=0.08±0.03% (v/v), respectively]. The essential oils of *S. thymbra* has been studied again for its effect on MCF-7 cells together with C32 (human amelanotic melanoma cell line), ACHN (renal cell adenocarcinoma) and LNCaP (hormone-dependent prostate carcinoma) cells (9). The oil derived from plants harvested in Lebanon was unable to affect the viability of MCF-7 and LNCaP cells. Nevertheless, it exhibited moderate cytotoxicity against C32 and ACHN cells.

In our study, *p*-cymene and γ-terpinene were tested on MCF-7 cancer cells and did not appear to affect significantly their viability (EC_{50} > 4 mM). Conversely, carvacrol and thymol were approximately 15-25 times more potent than the other two agents. More specifically, thymol affected the viability of Hep3B and A549 cells (EC_{50}=0.181±0.016 mM and EC_{50}=0.187±0.061 mM, respectively) to a greater extent compared with HepG2 cells, which were the most resistant not only to thymol (EC_{50}=0.390±0.010 mM) but also to carvacrol (EC_{50}=0.344±0.0035 mM). In the case of carvacrol, the most sensitive cell line was the lung adenocarcinoma A549 cell line (EC_{50}=0.118±0.0012 mM), while Hep3B cells exhibited an intermediate effect (EC_{50}=0.234±0.017 mM). In a study in 2003, carvacrol affected the viability of A549 cells in concentrations greater than 0.250 mM when treated for 24 h (27). The activity of carvacrol against A549 cells was also described in another study, where it was considered a weakly cytotoxic agent (EC_{50}>0.1 mM), not only against these cells but also against the DLD-1 colon adenocarcinoma cell line (28). The sensitivity of HepG2 cells to carvacrol was also determined in a study in 2011 (29). Cells were treated with different concentrations of carvacrol for 24, 48 and 72h. The EC_{50} value was lower after the 24 h treatment (0.4 mM) compared with that of the 48 and 72 h treatments (0.5 mM). This was verified in more recent work where HepG2 cells were treated with carvacrol for 24 h (30). Carvacrol has been tested against MDA-MB 231 (human metastatic breast cancer cells) (31), P-815 (murine mastocytoma), K-562 (human chronic myelolytic leukemia), CEM (acute T-lymphoblastoid leukemia), MCF-7 (human breast adenocarcinoma) and its counterpart resistant to gemcitabine (MCF-7 gem) (32, 33), human cervical cancer HeLa and SiHa cells (34), CEM leiomyosarcoma cells (35), B16-F10 murine melanoma cells and rat brain N2a neuroblastoma cells (36), exhibiting moderate to strong antiproliferative activity.

Of the cell lines we examined, thymol has only previously been tested again against HepG2 cells, where its EC_{50} value was estimated to be >0.1 mM (37). It has been studied for its activity against several cancer lines, including HL-60 acute promyelotic leukemia cells, B16-F10 cells and finally P-815, K-562, CEM and MCF-7 cell lines. Thymol affected the viability of HL-60 cells after 24-h incubation, causing concentration-dependent apoptosis (38). B16-F10 melanoma cells exhibited the same sensitivity to thymol in two separate studies (39). This sensitivity was higher than a third study dealing with the same cell line (37), perhaps due to different incubation times and cell viability assays. Finally, its effect on P-815, K-562, CEM and MCF-7 cell lines was diverse, with EC_{50} values ranging from 0.15-0.48 μM (33).

p-Cymene and γ-terpinene are the least studied substances among the four terpenes for their antiproliferative activity. They were both found to be weak anticancer agents against A549 and DLD-1 cells (EC_{50}>0.1 μmM) (28). Their antiproliferative potential was also tested against K562 and murine B16-F10 cells, showing stronger activity against the B16-F10 cells, with γ-terpinene being more potent (37). The potential of *p*-cymene against B16-F10 cells has been examined before, but it did not have any significant anticancer activity (39). γ-Terpinene was tested against Jurkat (T-leukemia cells), J774A.1 (murine macrophage tumor), and HeLa cells, where it showed weak cytotoxicity against Jurkat cells but had no effect on the other two cell lines (40).

Taking these data into consideration, the higher potency of *S.thymbra* compared to *S.parnassica* oil may be attributed to its composition (Table I). *S. thymbra* contains a higher percentage of carvacrol, which is a proven strong natural agent. In addition, *p*-cymene and γ-terpinene account for 25% of *S. parnassica* essential oil, whereas they comprise <20% in the oil of *S. thymbra*.

In conclusion, these data suggest that the essential oils of *S. thymbra* and *S. parnassica* may have potential as health-promoting agents. Their major components, carvacrol and thymol, exhibited significant antioxidant capacity and also diverse antiproliferative activity against A549 non-small cell lung adenocarcinoma and HepG2 and Hep3B liver hepatocellular carcinoma cells, with the first two being the most sensitive to their action. *p*-Cymene and γ-terpinene did not exhibit equivalent antioxidant and antiproliferative potency. Some of these results are reported for the first time, while others are in agreement with previous studies, supporting the need for further investigation of the molecular mode of action of these substances in order to enable their future exploitation as potential effective nutraceutical compounds.

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Table I. Content of the main components of the essential oils of the plants *Satureja thymbra* and *S. parnassica* harvested after their flowering season from Greek mountains [4].

	S. thymbra	*S. parnassica*
Component	Content (%)	
Carvacrol	39.10	33.72
Thymol	12.59	17.82
p-Cymene	8.83	10.32
γ-Terpinene	10.61	15.47

Table II. Antioxidant capacity of the terpenes γ-terpinene and *p*-cymene expressed as inhibition of the free radical 2,2-diphenyl-1-picrylhydrazyl. Data are given as the mean of at least three independent experiments ± standard deviation.

Concentration (mM)	Inhibition (%)	
	γ-Terpinene	*p*-Cymene
0.1	0.4 ± 0.007	0.7 ± 0.04
0.2	0.4 ± 0.005	0.5 ± 0.075
0.3	0.6 ± 0.014	0.5 ± 0.022
0.6	0.3 ± 0.01	0.6 ± 0.007
0.8	0.4 ± 0.0083	0.8 ± 0.023
1	0.2 ± 0.0038	0.4 ± 0.002
2	1.8 ± 0.015	1.6 ± 0.02
3	1.2 ± 0.017	1 ± 0.031
4	2.5 ± 0.022	1 ± 0.031
6	3.4 ± 0.008	1.3 ± 0.009
8	4.7 ± 0.014	1.8 ± 0.036
10	3.6 ± 0.045	1.1 ± 0.03

Table III. EC_{50} (50% efficient concentration) values for the studied terpenes against different cancer cell lines. Data are given as the mean of at least three independent experiments ± standard deviation.

	EC_{50} values (mM)			
Cell line	Carvacrol	Thymol	γ-Terpinene	*p*-Cymene
MCF-7	-	-	4.6±0.11	5.11±0.55
A549	0.118±0.0012	0.187±0.061	-	-
Hep3B	0.234±0.017	0.181±0.016	-	-
HepG2	0.344±0.0035	0.390±0.01	-	-

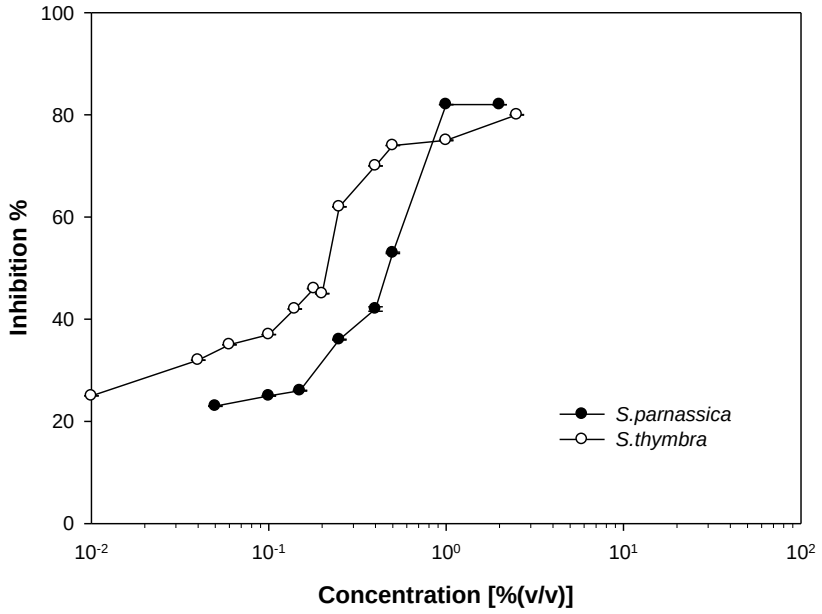

Figure 1. Antioxidant capacity of the essential oils of *Satureja thymbra* and *S. parnassica* using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging method. Increasing concentrations of the essential oils of *S. thymbra* [0.01-2.5% (v/v)] and *S. parnassica* [0.05-2% (v/v)] were incubated in the presence of DPPH for 30 min. The results are shown as mean ± S.D. of three independent experiments. IC_{50} (50% inhibitory concentration) for *S. parnassica* = 0.4±0.3% v/v, and IC_{50} for *S. thymbra* = 0.22±0.25 % v/v.

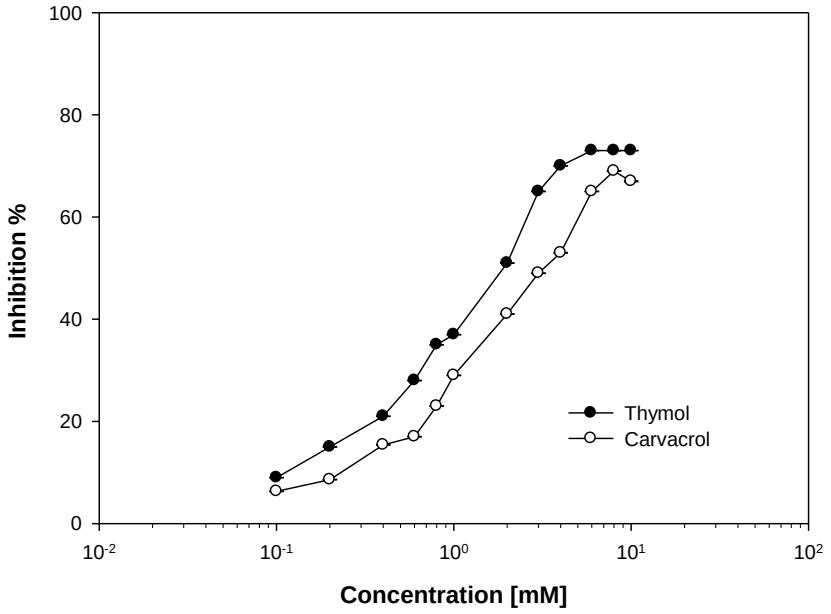

Figure 2. DPPH-free radical scavenging activity of carvacrol and thymol. DPPH was incubated in the presence of increasing concentrations of carvacrol and thymol (1-10 mM) for 30 min. The results are shown as the mean ± S.D. of three independent experiments. IC_{50} (50% inhibitory concentration) for thymol = 1.14±0.18 mM, and IC_{50} for carvacrol = 2.28±0.45 mM.

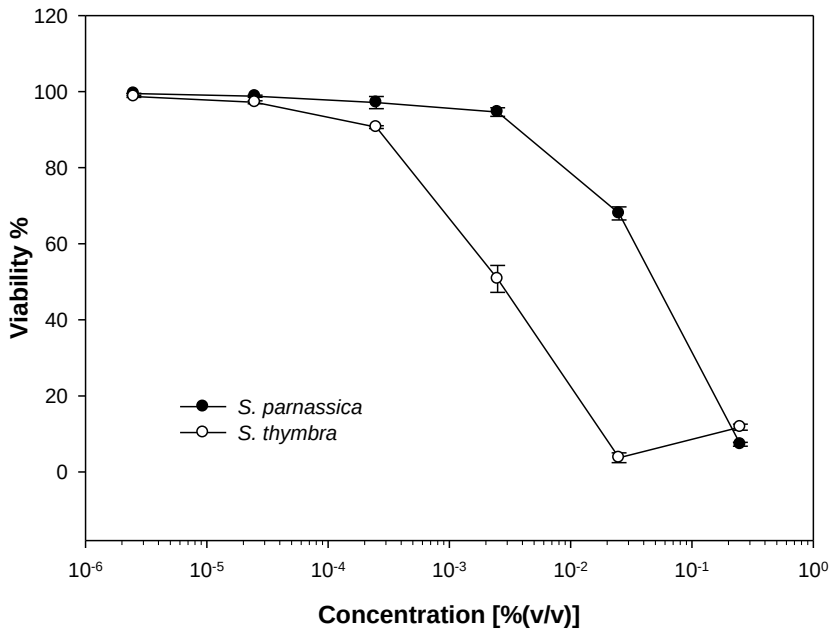

Figure 3. Antiproliferative activity of *Satureja thymbra* and *S. parnassica* essential oils. MCF-7 cells were incubated with increasing concentrations of *S. thymbra* and *S. parnassica* essential oils [0.00000025-0.25% (v/v)] for 72 h. Estimation of cell viability was determined by the SRB assay. The results are shown as the mean ± S.D. of three independent experiments. EC_{50} (50% efficient concentration) for *S. parnassica* = 0.08±0.03% v/v, EC_{50} for *S. thymbra* = 0.002±0.0038% v/v.

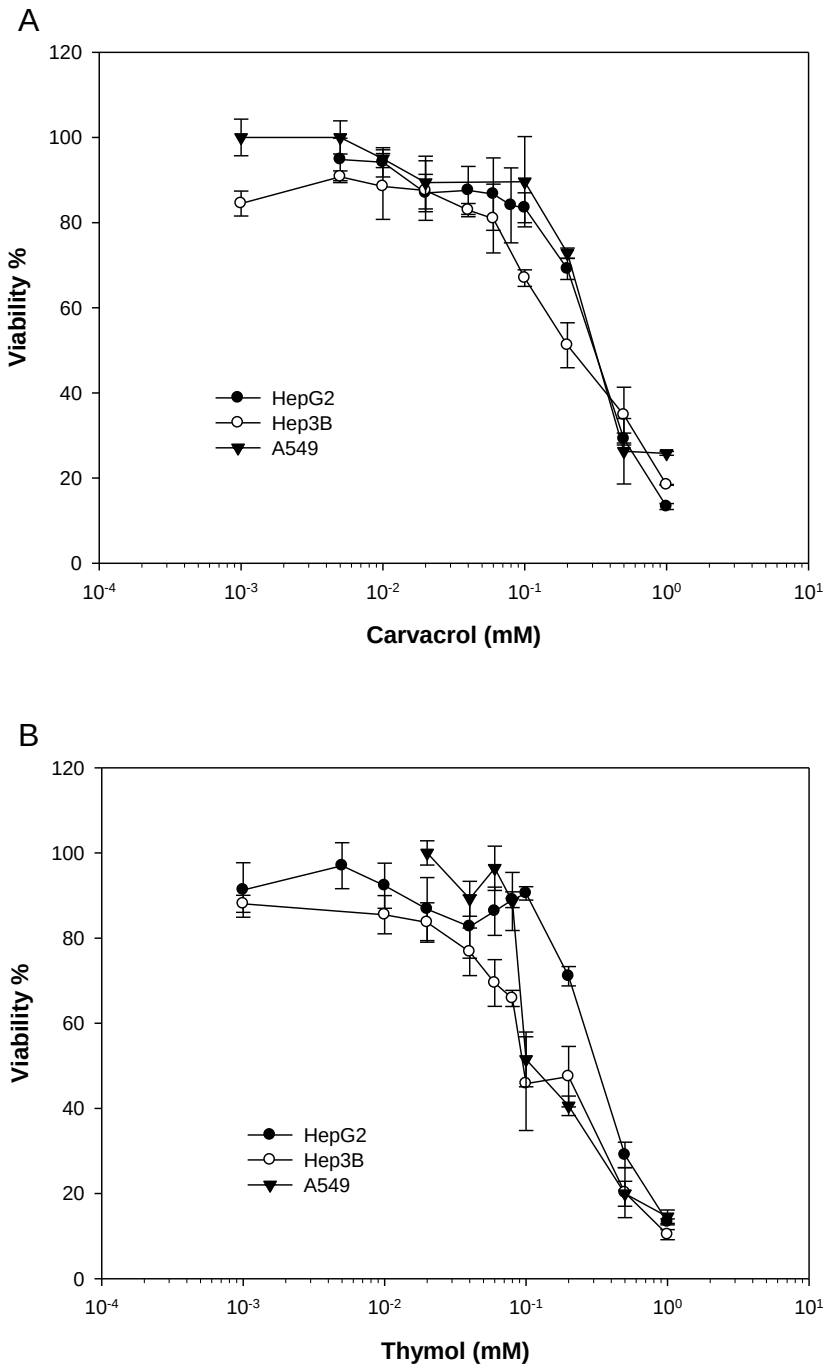

Figure 4. Antiproliferative activity of carvacrol (A) and thymol (B). Cells were incubated with increasing concentrations of carvacrol, and thymol (0.001-1mM) for 72 h. Estimation of cell viability was determined by the sulforhodamine B assay. The results are shown as the mean ± S.D. of three independent experiments.