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Cytotoxic Effects of Different Aromatic Plants Essential Oils on Oral Squamous Cell Carcinoma- an *in vitro* Study

SUMMARY

Background/Aim: *Current approaches in therapy of head and neck cancers are surgery, radiotherapy and chemotherapy. However, recurrence, development of multidrug resistance, side effects, and high costs of therapy are significant problems which point to the need for more efficient and less toxic drugs and interventions.* ***Material and Methods:*** *Eight essential oils obtained from Thymus serpyllum, Mentha piperita, Juniperus communis, Rosmarinus officinalis, Melissa officinalis, Achillea millefolium, Zingiber officinale, and Helichrysum arenarium were tested for their anti-proliferative on oral squamous cell carcinoma (OSCC) culture and SCC-25 cell line. Cytotoxicity assays (MTT and Neutral red) were used to detect the effect of the mentioned essential oils.* ***Results:*** *T. serpyllum, M. piperita, J. communis, and R. officinalis essential oils exhibited the best anti-proliferative effect, on both types of cells. M. piperita had the greatest effect on SCC-25 cell line (4,5% of viable cells) and OSCC cells (7,2% of viable cells). Overall, cytotoxicity was higher in OSCC than in SCC-25 cell line.* ***Conclusions:*** *This study showed a clear anti-proliferative effect of four essential oils, in vitro making them novel potential antineoplastic agents.*

Key words: Essential Oils, Cytotoxicity, Squamous Cell Carcinoma, Cell Cultures

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Introduction

Head and neck cancer (HNC) represents a great public health problem as the 6th most common cancer in developed countries with approximately 350,000 deaths every year[1]. The great percentage of HNC is oral cancer (85%), mostly oral squamous cell carcinoma (OSCC). Despite significant advances in a modern therapy, rates of survival for these patients have only moderately improved during the last two decades[2,3]. Current approaches in therapy of this type of cancer are surgery, radiotherapy, and chemotherapy. However, recurrence, development of multidrug resistance, side effects, and high costs of therapy are significant problems which point to the need for more efficient and less toxic drugs and interventions.

In the last 15 years cytotoxic and anti-proliferative potential of different essential oils has been explored in several types of cancer[4]. From the current literature they seem to have a great prospective as anticancer therapeutic agents or as adjuvant therapeutics.

Several herbal products derived from mint (*Lamiaceae*) family have shown anti-carcinogenic properties. One important genus of the mint family is Thymus with about 350 species worldwide[5] and it has been used in traditional medicine for thousands of years in countries of the Mediterranean basin. Thyme is usually administrated as expectorant in upper respiratory tract infection, and thymol is often the main antiseptic ingredient in mouth rinses. Essential oil of the most studied species- *Thyme vulgaris*, and its principal component thymol has been shown to have antifungal, antibacterial, antioxidant[6] and anticancer effect[7].

From the same plant family, *Mentha piperita* or peppermint has a long history of safe use, both in medicinal preparations and as a flavoring agent. The cytotoxic effect of *Mentha* has been tested on several

tumors (cervical, breast, colon adenocarcinoma and other) and it has proved to be a valid antitumor agent[8].

Several studies have been conducted to verify the effects of *Achillea millefolium*. Anti-inflammatory[9], antioxidant, antimicrobial[10], liver protective[11], anti-secretory and gastro-protective feautures have also been detected[12].

A recent study has found that the extract of *Rosmarinus officinalis*, which also belongs to mint family, contains polyphenols (carnosic acid and rosmarinic acid) possesses clear anticancer potential[13]. *Melissa officinalis* essential oil has been shown to have antibacterial, antifungal, and spasmolytic effects[14]. Also, studies on lung, colon and breast cancer, as well as on leukemia and glioblastoma multiforme cancer cell lines, have shown that *M. officinalis* inhibited tumor cell viability and induced apoptosis in resistant tumor cells, which all suggests their antitumor potential[15]. *Juniperus communis* belongs to the *Cupressaceae* family and has been used as therapeutic agent in traditional medicine. Recent studies have reported strong antioxidant[16], antibacterial[17], anti-inflammatory, as well as anticancer effects in colorectal and cervical cancer[18].

The aim of this study was to investigate anti-proliferative effects of *Thymus serpyllum, Mentha piperita, Juniperus communis, Rosmarinus officinalis, Melissa officinalis, Achillea millefolium, Zingiber officinale, and Helichrysum arenarium* on primary OSCC cells culture and an oral cancer cell line (SCC-25), as control.

Material and Methods

Plant material and method of extraction

All herbs originated from Serbia. Essential oils were isolated from dried plant material by hydro-distillation process in semi-industrial distillation device SP-130, which works on the principle of distillation water and steam. The temperature during the hydro-distillation in device SP-130 ranged from 100°C to 102°C at atmospheric pressure. All the extracted essential oils were liquids of light yellow, green or brown color and their odor was characteristic of the distilled aromatic herbs. Semi-industrial distillation device SP-130 is a discontinuous device with the volume of 130l. The process of steam distillation with the device SP-130 has many advantages over the existing ones[19–21]. In distillers with large volume, it is almost impossible to control the behavior of the steam and preserve essential properties of the treated oil feedstock (especially in the lower layers). The device SP - 130 performs the separation of oil and water simultaneously by the condensation process, without any additional devices. The hydrostatic pressure of the charge did not exceed 101,325 KPa.

Cell isolation and cell line

The SCC-25 cancer cell line, originating from OSCC (ATCC® CRL- 16 28™ Rockville, MD), was maintained at 5% CO_2 and 37°C in complete growth medium containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin solution (AB), and 400 ng/ml hydrocortisone (Sigma-Aldrich, St. Louis, USA).

OSCC cells were isolated from tumor tissue immediately after surgical removal from patients who were hospitalized at the Clinic of Maxillofacial Surgery of the School of Dental Medicine, University of Belgrade. Patient was informed of the study and signed an informed consent. The study was approved by the institutional Ethical Committee (36/30) University of Belgrade, Republic of Serbia and in accordance with the Declaration of Helsinki. The isolation method was done as previously described[22,23]. After the surgery, the samples were transported to the laboratory in DMEM supplemented with 20% FBS and AB (Sigma-Aldrich). The tissue samples were cut with blades into small pieces and seeded onto T75 cell culture flasks in complete growth medium (DMEM supplemented with 10% FBS and AB). The cells were cultivated in humidified atmosphere under standard conditions, 5% CO_2 and 37°C. Medium was discarded every 2 to 3 days and after reaching 80% of confluence, passage of the cells was done.

MTT assay

SCC-25 and primary OSCC cells were seeded at density of 10^4 per well in 96-well culture plate and grown in complete growth medium under standard culturing conditions for 24 hours. Cells were then treated with 1500, 750 and 375 µg/ml of essential oil dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich) with complete growth medium, and cisplatin (Pfizer, New York, USA) in different dosages (10, 5 and 1 µg/ml). Negative (no cells), solvent (0,02% DMSO), and positive controls (cells in complete growth medium) were also included. After 24h of incubation, cells were washed with 100µl phosphate buffered saline (PBS) and 100µl of MTT solution was added and incubated for 3 hours[24]. Finally, after the dissolution in isopropanol the absorbance at 560 nm was measured using a microplate reader (RT- 2100C, China) and the obtained values were presented as percentages of viability. The experiment was done in triplicate, repeated three times.

Neutral red (NR) assay

SCC-25 and primary OSCC cells were seeded in 96 well plate and incubated as described in previous assay. After 24h of incubation period the solutions were removed and replaced with 150µl of Neutral red solution (3-amino-7dimethyl- 1-2-methylphenazine hydrochloride) (Sigma-Aldrich) and incubated for 4 hours at 5% CO_2 and

37 °C. Next, the wells were washed with PBS and 150µl of Neutral red eluent (96% of ethanol:dH_2O: CH_3COOH 50:49:1) was added to each well and incubated at room temperature for 15 minutes[25]. The absorbance was measured at 560 nm using a microplate reader (RT-2100C, China) and the obtained values were presented as percentages of viability. The experiment was done in triplicate, repeated three times.

Statistical Analysis

Student's T tests were performed in the study. Statistical significance was set at $p<0,05$. Software package SPSS ver. 20 was used for the analyses (SPSS inc, Chicago, USA).

Results

MTT assay

T. serpyllum, Mentha piperita, J. communis, and R. officinalis essential oils exhibited the best anti-proliferative effect, in both types of head and neck cancer cells (Figure 1). *Mentha piperita* had the greatest effect on SCC-25 cell line (4,5% of viable cells at the highest dose) (Figure 1a) and OSCC primary cells (7,2% of viable cells at the highest dose) (Figure 1c and 2a). Overall, the cytotoxicity of 8 essential oils was higher in OSCC cells compared to SCC-25 cell line. The effect was dose-dependent. Cisplatin, a commonly used chemotherapeutic for head and neck cancers, showed lower toxicity compared to the first group of essential oils (effective oils): *T. serpyllum, M. piperita, J. communis, and R. officinalis* (Figure 3a). The highest dose of cisplatin (10 µg/ml) was the most effective in OSCC cells (63% of viable cells).

Neutral red assay

To confirm the results of MTT assay, complementary NR assay was also done. NR assay showed similar cytotoxicy of essential oils compared to MTT assay. *M. piperita* had the greatest effect on SCC-25 cell line (14,4% of viable cells in the highest dose) and OSCC cells (Figure 2b and 4) (15,2% of viable cells of the highest dose). The highest, statistically significant anti-proliferative effect against OSCC and SCC-25 cells showed *T. serpyllum, M. piperita, J. communis, and R. officinalis* essential oils (Figure 4). Similary to MTT assay, cisplatin showed lower toxicity compared to some essential oils. The highest dose of cisplatin (10 µg/ml) was the most effective in OSCC cells (65% of viable cells) (Figure 3b). The cytotoxic effect of cisplatin was dose-dependent.

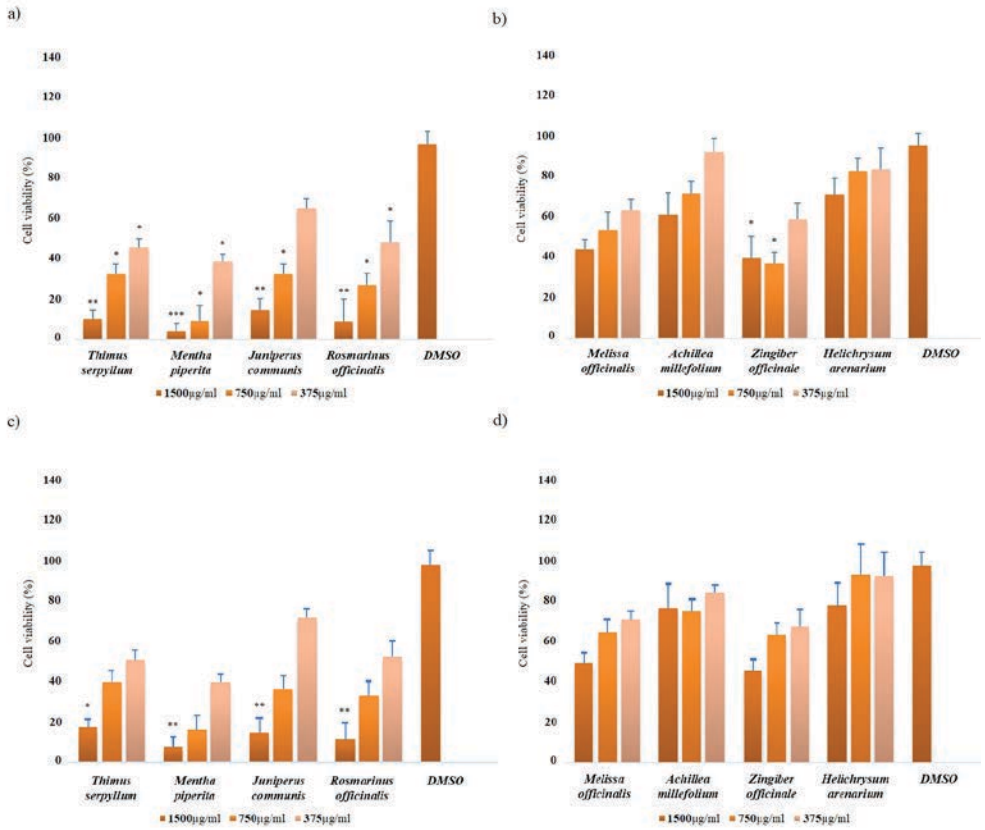

*Figure 1. MTT assay after 24h treatment with essential oils on SCC-25 cells (a, b) and oral squamous cell carcinoma cells (c, d) *p<0,05*

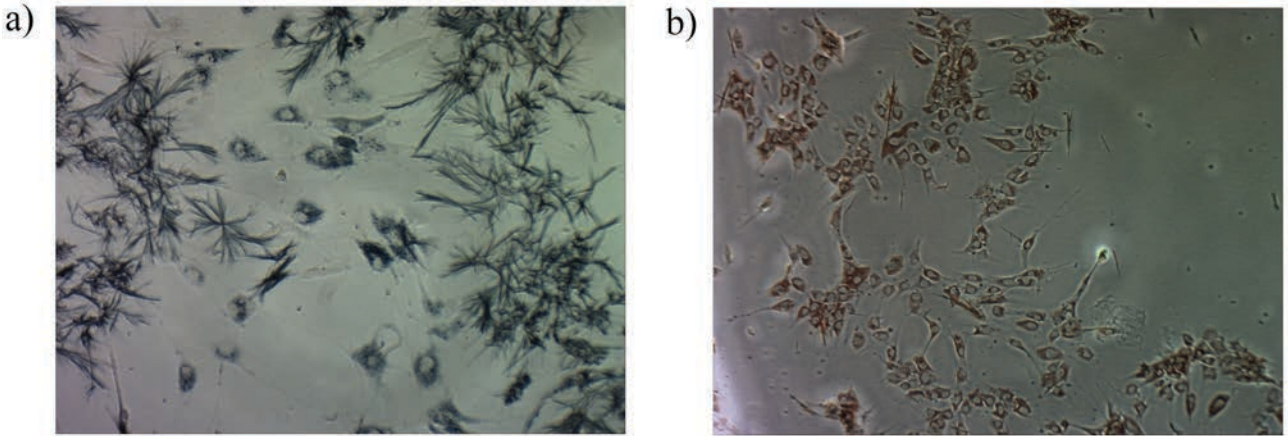

Figure 2. Native pictures of oral squamous cell carcinoma cells after 24h treatment with essential oils. MTT assay (a); Neutral red assay (b). Magnification 100x

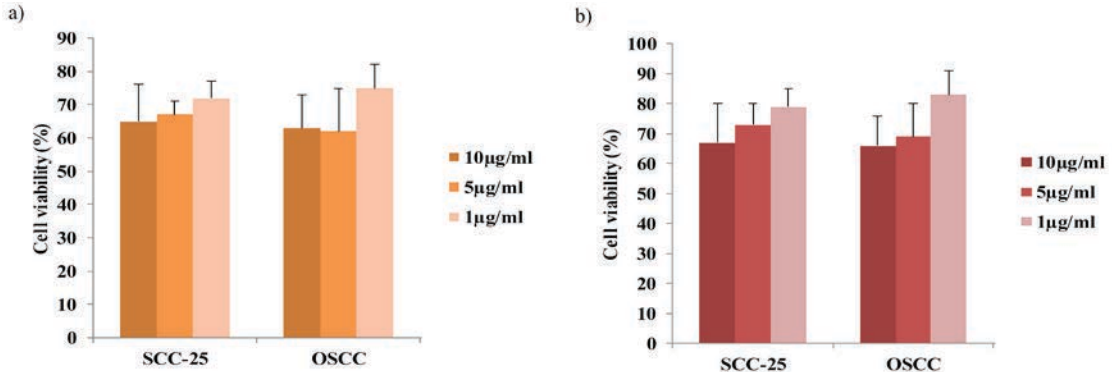

Figure 3. Cell viability after 24h treatment of cisplatin: MTT assay (a); Neutral red assay (b)

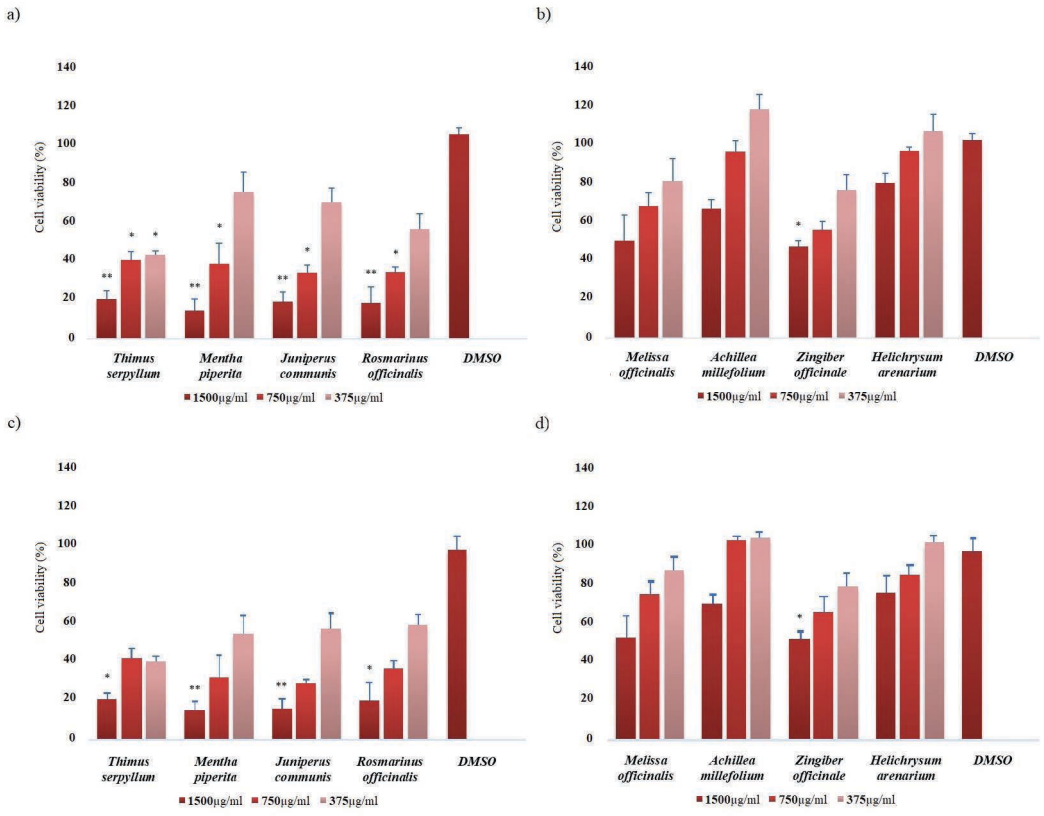

*Figure 4. Neutral red assay after 24h treatment with essential oils on SCC-25 cells (a, b) and oral squamous cell carcinoma cells (c, d) *p<0,05; ** p<0,01*

Discussion

Searching for new natural products with antineoplastic characteristics represent a fascinating field of research in oncology[26]. Plant-derived anticancer drugs such as vinblastine, vincristine, colchicine, etc. are widely used in clinical practice[27–29], but the introduction of novel compounds of plant origin, proven to be efficient in reducing tumor cell viability, should be taken into consideration as well, and potentially recommended.

In the present study, we have demonstrated cytotoxic effects of a variety of essential plant oils on cancer cell cultures. Generally, cytotoxicity of all eight essential oils was higher in OSCC than in SCC-25 cell cultures, which could be explained by differences in cell proliferative rates and chemoresistance. It must also be emphasized that, under the experimental conditions in this study, phytotherapy proved to be more efficient than the most standard cytostatic therapy used in head and neck cancer treatment, i.e. cisplatin.

M. Piperita oil had the highest antineoplastic effect on oral cancer cells, and our results support some previous findings on peppermint anticancer properties achieved *in vitro* through different mechanisms such as mitochondria mediated apoptotic pathways, increase of p53 and p21 expression, perturbation of oxidative balance, etc.[8].

Thymus spp. is associated with the regulation of three pathways- the interferon signaling, N-glycan biosynthesis, and ERK5 signaling, all involved in cancer cell development[30]. Higher concentrations of *T. vulgaris* essential oil showed dose-dependent cytotoxic effects in a previous study on OSCC cell line UMSCC1[30]; the present study also observed dose-dependent anti-proliferative effect induced by *T. serpyllum* essential oil.

In accordance with the present study, *Juniperus communis* showed the capacity to inhibit cancer cell growth in two cancer cell lines-colorectal carcinoma cell line ($CaCo_2$) and cervical cancer cells line (HeLa)[18].

To the best of our knowledge, this is the first study on oral squamous cell carcinoma cells dealing with anti-proliferative potential of *Rosmarinus officinalis* essential oil. Several *in vitro* studies using colon cancer[31], rat RINm5F insulinoma[32], breast cancer[33], pancreatic[34], and HeLa (Cervical adenocarcinoma)[35] cells lines have shown that *R. officinalis* had anticancer capacity. However, underlying mechanisms are yet to be unveiled. Recently, Moore, et al.[36] found that *R. officinalis* can inhibit the activation of the Akt/mTOR/p70S6K signaling pathway, which in turn leads to reduced cell proliferation.

The antitumoral effect of *Melissa officinalis* was found in different types of cancers- colon (Caco-2), breast (MCF-7), leukemia (HL-60 and K562), lung (A549), human cancer cell lines and in a mouse melanoma cell line (B16F10)[15]. In this study, the antitumoral effect of *M. officinalis* was not confirmed, maybe in part due to specific conditions of essential oil production (15). Still, the dose-dependent effect was present.

Achillea millefolium had no significant effect on cell viability after 24 hour-long treatments, although its anti-proliferative potential has been reported in non-small cell lung cancer (NCI-H460) and human colorectal adenocarcinoma (HCT-15) cells[37].

A recent study examined the effect of two major pharmacologically active compounds of *Zingiber officinale*, 6-gingerol and 6-shogaol, on head and neck cancer cell lines[38] and found, similarly to this study, dose-dependent decrease of cell capacity to proliferate. However, the results of the present study did not show a significant reduction of cell number. A similar result regarding the effects on OSCC and SCC-25 cell cultures was obtained with *Helichrysum arenarium* essential oil.

Conclusions

This study showed clear anti-proliferative effects of *Thymus serpyllum, Mentha piperita, Juniperus communis* and *Rosmarinus officinalis* essential oils against OSCC primary cultures and SCC-25 cell line. However, further investigations are necessary in order to uncover the essential oil components responsible for the effect and the underlying molecular pathways.

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