



Ann Med Res

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Annals of Medical Research

journal page: www.annalsmedres.org

Cytotoxicity of myrtenal on different human cancer cell lines

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ARTICLE INFO

Keywords:

Myrtenal
Cancer
Cytotoxicity

Received: Apr 01, 2024

Accepted: May 13, 2024

Available Online: 29.05.2024

DOI:

[10.5455/annalsmedres.2024.04.065](https://doi.org/10.5455/annalsmedres.2024.04.065)

Abstract

Aim: Myrtenal (Myrt), a monoterpene found in essential oils of various plant species, such as *Citrus aurantium*, *Citrus limon*, *Mentha japonica* and *Zingiber officinale roscoe*. Preclinical studies have reported that Myrt induces apoptosis in various cancer models. This study aimed to investigate the effects of Myrt on cell viability in human prostate (LNCaP), colon (Caco-2), breast (MCF-7) and ovarian (A2780) cancer cell lines.

Materials and Methods: A2780, LNCaP, MCF-7 and Caco-2 cell lines were used in this study. All cell lines were treated with 1, 5, 25, 50 and 100 µM concentrations of Myrt for 24 hours. Changes in cell viability were determined by the MTT assay. The inhibitory concentration 50 (IC₅₀) and logIC₅₀ values of Myrt in cell lines was calculated based on the cytotoxicity results.

Results: Myrt concentrations applied to Caco-2, A2780, MCF-7 and LNCaP cancer cell lines for 24 hours significantly decreased cell viability (%) (p<0.05).

Conclusion: In conclusion, this study shows that Myrt has potent cytotoxic and antiproliferative properties against human A2780, LNCaP, MCF-7 and Caco-2 cancer cell lines.

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Introduction

According to the World Health Organisation, there were nearly 20 million cancer cases and 10 million deaths worldwide in 2020. It is widely acknowledged that cancer is a major contributor to the global burden of disease and projections suggest that this burden will continue to increase in the future [1]. Alongside increasing morbidity and mortality, the economic strain on countries is escalating [2]. Current research is focused on discovering new strategies or compounds for cancer treatment, given the side effects of most therapies and the unequal response of tumors to treatment. Anti-cancer drugs such as vinblastine, etoposide, vincristine and teniposide are derived from natural sources [3], suggesting that natural ingredients hold promise for effective cancer treatment [4].

Essential oils are concentrated and volatile liquids obtained from various parts of plants. Monoterpenes are the dominant compounds in the essential oils of various plants. Monoterpenes exhibit diverse pharmacological activities and show promise in cancer research [5, 6]. Research indicates that monoterpenes have potential for both chemoprevention and chemotherapy [7, 8]. These effects in preclinical studies are attributed to their ability to en-

hance endogenous antioxidant capacity and induce apoptosis by the caspase-3 activation [5]. Additionally, various monoterpenes have shown cytotoxic effects on different cancer cell lines [9, 10].

Myrtenal (Myrt), a monoterpene found in essential oils of various plant species, such as *Citrus aurantium*, *Citrus limon*, *Mentha japonica* and *Zingiber officinale roscoe* [11], exhibits bronchodilator, antiaggregant, antihemolytic, hypotensive and antibacterial properties [12]. Further studies have confirmed its anti-inflammatory, antioxidant [13], antihyperglycaemic [14] and neuroprotective properties [15]. Myrt has also demonstrated cytotoxic activity in human colon and melanoma cancer lines [16, 17]. Limited *in vivo* studies suggest that Myrt may promote apoptosis of cancer cells. For instance, in rats with hepatocellular carcinoma and bladder cancer, Myrt increased the Bax/Bcl2 ratio and induced caspase-3 activation [18, 19]. Additionally, it prevented tumor progression by reducing levels of inflammatory cytokines (TNF-α and IL-6) as well as oxidative stress biomarkers (MDA and NO) [18]. The aim of this study was to investigate the cytotoxic and antiproliferative effects of Myrt on colon, ovarian, prostate and breast cancer cell lines.

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Materials and Methods

Chemicals

A stock solution was prepared by dissolving (1R)-(-)-Myrt (Sigma Aldrich-#MKCQ7460) in dimethyl sulfoxide (DMSO). Myrt concentrations of 1, 5, 25, 50 and 100 μM were prepared from this stock solution using culture medium.

Cell culture

The cytotoxicity of Myrt was investigated in four human cancer cell lines: LNCaP (prostate), MCF-7 (breast), A2780 (ovarian) and Caco-2 (colon) [20]. All cell lines were obtained from the American Type Culture Collection (ATCC). Cells were first removed from the liquid nitrogen tank and cultured in flasks. For the LNCaP and A2780 cell lines, RPMI1640 medium supplemented with penicillin (100 U/mL), streptomycin (10 $\mu\text{g}/\text{mL}$), fetal bovine serum (FBS, 10%), minimum essential medium (MEM, 1%) containing non-essential amino acids (NEAA) was used. MCF-7 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose and Caco-2 cells were cultured in DMEM F-12 medium. The media used for both lines contained penicillin (100 U/mL), FBS (10%), streptomycin (10 $\mu\text{g}/\text{mL}$), insulin (10 $\mu\text{g}/\text{mL}$) and MEM with NEAA (1%).

Cells were maintained in a sterile environment within an incubator (Esco CelCulture, Singapore) at 37°C with 5% CO_2 . To facilitate cell growth, the growth medium was replenished twice weekly. Upon reaching an appropriate density, the cells were detached from the flasks using trypsin-EDTA. Cell counting was performed using 0.4% Trypan Blue stain and a hemocytometer. Experimental procedures commenced once cell viability exceeded 90%.

For the cytotoxicity experiments, 96-well plates were utilized, with 15,000 cells seeded in each well [21]. After 24 hours of incubation, the cells were treated with varying concentrations of Myrt.

Determination of cell viability

The cytotoxic effect of Myrt was evaluated using the 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In this assay, MTT is reduced to a blue-violet colored, water-insoluble formazan inside the cell. This reduction is catalyzed by the mitochondrial enzyme succinate dehydrogenase and occurs only in active mitochondria. The occurrence of this reaction is directly linked to the number of active mitochondria in the living cell. Higher absorbance values indicate greater formazan production and consequently, more active mitochondria. The MTT assay is commonly employed to determine cell viability and measure cell number. The cytotoxicity of Myrt was assessed in Caco-2, A2780, LNCaP and MCF-7 cell lines using the MTT assay at concentrations of 1, 5, 25, 50 and 100 μM [22].

After 24 hours of treatment with Myrt concentrations ranging from 1 to 100 μM , the culture media was aspirated from the cells in the 96-well plate. Subsequently, 50 μL of MTT solution (0.5 mg/mL in phosphate buffer) was added to each well and incubated for 3 hours. Following the incubation period, the wells were emptied and 100

μL of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystals. The absorbance of the formazan was then measured at a wavelength of 570 nm using a microplate reader (BioTek Synergy-HTX, USA) [23].

Absorbance values obtained from the control wells were averaged to establish a baseline representing 100% cell viability. Subsequently, a comparative analysis was conducted between the absorbance readings of wells treated with Myrt and those of the control group. Percentage viability values were computed to evaluate the impact of Myrt treatment on cellular viability relative to the control condition. MTT experiments were repeated 10 times in triplicate on different days to ensure the robustness and reliability of the results.

Inhibitory concentration 50 (IC_{50}) and LogIC_{50} values of Myrt were determined using GraphPad Prism 8 software based on the MTT results to ascertain its cytotoxic potency.

Statistical analysis

The statistical analysis was executed through the utilization of the IBM SPSS Statistics 24 software package. To assess the normality distribution of the data, the Shapiro-Wilk test was employed. Subsequently, group-wise comparisons of variables were undertaken using the Kruskal-Wallis H test, followed by the Mann-Whitney U test with Bonferroni correction to account for multiple comparisons. Descriptive statistics were articulated as mean \pm standard deviation. A predetermined significance threshold of $p < 0.05$ was adhered to throughout the analysis.

Results

The impact of Myrt on the viability rates of Caco-2, MCF-7, A2780 and LNCaP cell lines was investigated by incubating them with different concentrations of Myrt (1, 5, 25, 50 and 100 μM) for 24 hours. The resulting changes in cell viability rates were determined as percentages and are presented in Figures 1-4.

Figure 1 illustrates the impact of Myrt concentrations on Caco-2 cell viability. The antiproliferative effect of all

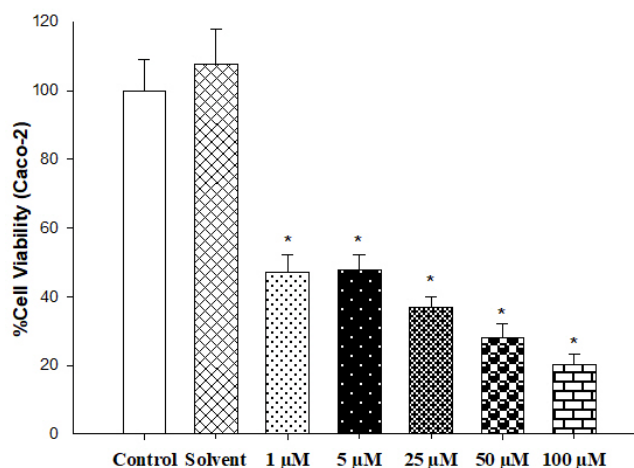


Figure 1. Cell viability results in Caco-2 cell line after 24 hours incubation of Myrt (* $p < 0.05$).

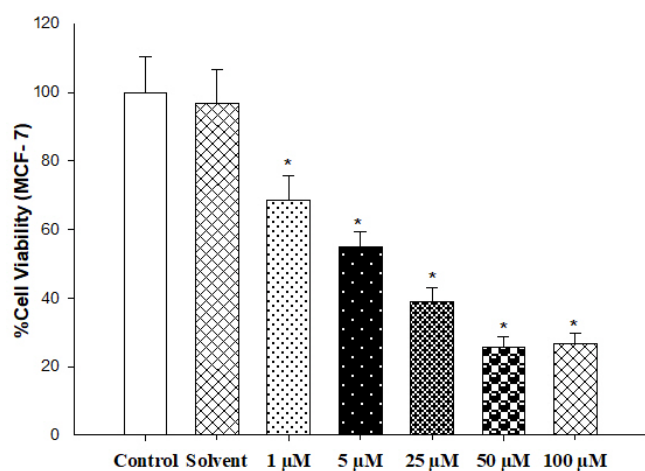


Figure 2. Cell viability results in MCF-7 cell line after 24 hours incubation of Myrt (* $p < 0.05$).

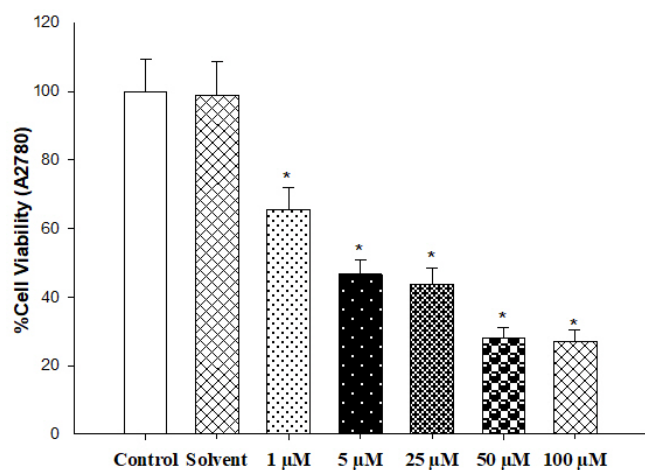


Figure 3. Cell viability results in A2780 cell line after 24 hours incubation of Myrt (* $p < 0.05$).

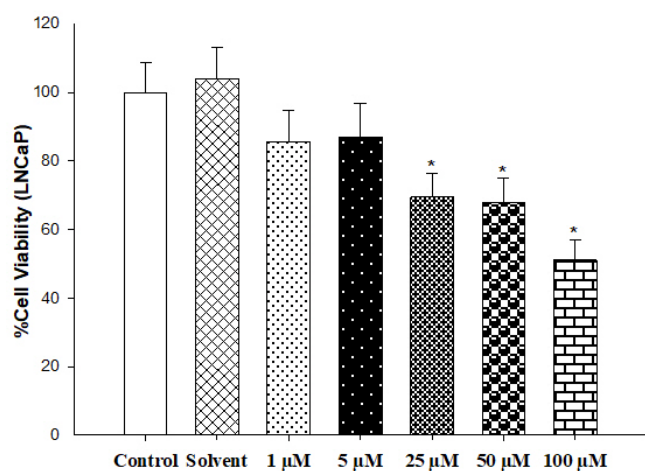


Figure 4. Cell viability results in LNCaP cell line after 24 hours incubation of Myrt (* $p < 0.05$).

Myrt concentrations on Caco-2 cells was statistically significant ($p < 0.05$). Figure 2 shows the impact of Myrt on

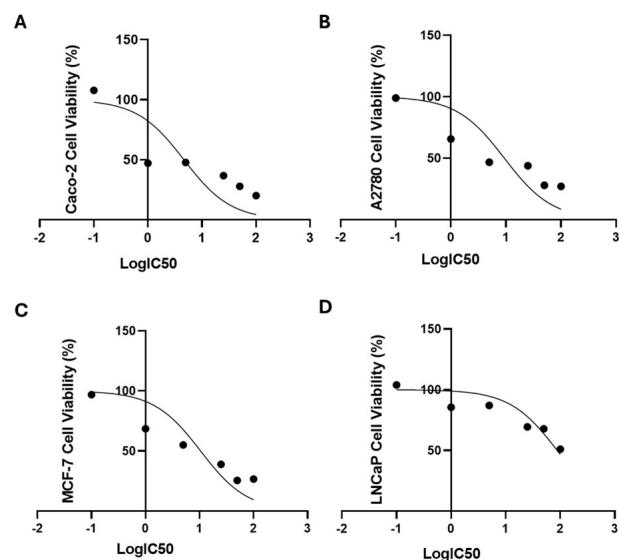


Figure 5. The LogIC_{50} values against Caco-2, LNCaP, A2780 and MCF-7 cell viability (%) of Myrt.

Table 1. LogIC_{50} and IC_{50} (μM) values after 24 h effect of Myrt on Caco-2, LNCaP, MCF-7 and A2780 cancer cell lines

$\text{LogIC}_{50}/\text{IC}_{50}$ (μM)			
Caco-2	LNCaP	MCF-7	A2780
0.6672/4.647	1.945/88.14	1.021/10.50	0.9718/9.37

the viability rate of the MCF-7 cell line after incubation. The results indicate that all concentrations of Myrt significantly reduced cell viability ($p < 0.05$).

The effects of Myrt on the A2780 cell line are shown in Figure 3, where all concentrations significantly decreased cell viability ($p < 0.05$). Figure 4 displays the effects of Myrt on the LNCaP cell line. The study revealed that Myrt had a cytotoxic effect on the LNCaP cell line at concentrations of 25, 50 and 100 μM ($p < 0.05$), but not at 1 and 5 μM ($p > 0.05$).

Table 1 and Figure 5A-D present the IC_{50} and LogIC_{50} values calculated for the Caco-2, A2780, MCF-7 and LNCaP cells based on the results of the 24-hour cytotoxicity experiments using Myrt. Among the cell lines, Caco-2 exhibited the most significant decrease in cell viability, followed by A2780, MCF-7 and LNCaP cell lines, respectively.

Discussion

Cancer chemoprevention has emerged as a vital area of research aimed at identifying novel inhibitors of cancer development and elucidating the underlying mechanisms of carcinogenesis [24]. This field seeks to devise strategies for preventing or repairing cellular and molecular damage induced by carcinogens. Current efforts are largely directed towards exploring the potential of natural compounds derived from herbal sources to impede cancer cell proliferation or induce apoptosis [24]. Natural herbal compounds are being rigorously assessed as potential chemopreventive

agents, with their clinical utility under thorough examination [25, 26]. Notably, many clinically used chemopreventive drugs have originated from plants or plant-derived substances [27]. Monoterpenes, prevalent in plant essential oils, have demonstrated promising chemopreventive and therapeutic effects in clinical trials involving cancer patients [28, 29].

Myrt, a monoterpene variety, has garnered attention for its antioxidant, anti-inflammatory [13, 30] and anti-hyperglycemic properties in various tissues [31]. Moreover, studies have indicated that Myrt does not induce genotoxicity in normal cells [32]. *In vivo* investigations have revealed Myrt's potential to exert chemoprotective effects against various cancers. Its ability to inhibit tumor cell development is attributed to its antioxidant properties, which help modulate dysregulation within the tumor microenvironment [33]. Long-term administration of Myrt in rodent models of colon cancer has been shown to inhibit colon carcinogenesis by neutralizing free radicals through the activation of endogenous antioxidant enzymes [34]. Additionally, Myrt has been observed to regulate the activity of lysosomal and mitochondrial enzymes and activate pro-apoptotic pathways such as Bax and caspase-3 in liver cancer models. Furthermore, Myrt prevents tumor progression by modulating inflammatory processes in the liver and enhancing antioxidant enzyme activity [19, 35]. *In vitro* studies have also demonstrated Myrt's cytotoxicity by inhibiting the V-type ATPase on the surface of melanoma cells, subsequently reducing the spread of melanoma metastasis in mice [17].

Given the aforementioned properties, our study aimed to investigate the cytotoxic effects of Myrt on various cancer cell lines. The results revealed a significant reduction in the survival of all tested cancer cell lines following Myrt treatment. Caco-2 exhibited the most pronounced decrease in cell viability, followed by A2780, MCF-7 and LNCaP cell lines, respectively, based on IC₅₀ levels. These findings corroborate previous research demonstrating Myrt's potent anticarcinogenic activity, particularly against human colon carcinoma cells [16]. While no cytotoxicity studies of Myrt on Caco-2, LNCaP, MCF-7 and A2780 cell lines have been conducted to our knowledge, prior investigations have reported the cytotoxic effects of various monoterpenes on similar cell lines, including LNCaP, MCF-7 and A2780.

Several studies have highlighted the cytotoxic effects of monoterpenes on various cancer cell lines. For instance, *Achillea membranacea*, rich in monoterpene content, and cineole have been reported to exhibit cytotoxicity against the A2780 cell line [36]. Additionally, α -pinene a bicyclic derivative of Myrt [37], was found to induce cytotoxicity in HepG2 human hepatoma cells [38]. Furthermore, several monoterpenes such as thymol, carvacrol, carveol, eugenol and carvone have demonstrated antiproliferative effects in the MCF-7 cell line [39]. Similarly, Bayala et al. reported cytotoxic effects of monoterpene compounds on PC-3 and LNCaP cancer cell lines [40].

The study shows that Myrt has a cytotoxic effect on A2780, Caco-2, MCF-7 and LNCaP cell lines. This effect may be due to Myrt's ability to increase caspase-3 activity and induce cell apoptosis by modulating the Bax and Bcl-

2, which are involved in both the extrinsic and intrinsic pathways of apoptosis. However, based on this theoretical proposition further investigation into the specific interaction mechanisms and signaling pathways of Myrt in cancer cells is required.

Conclusion

In conclusion, our findings demonstrate the antiproliferative and cytotoxic activity of Myrt on tumor cells. Understanding the molecular mechanisms underlying this effect holds considerable promise for informing future medical applications and guiding drug development strategies. Further exploration of these mechanisms may lead to the development of novel therapeutic interventions targeting cancer.

Ethical approval

Ethical approval was not required as it was an *in vitro* study.

Declaration of competing interests/Conflict of interest

The authors declare no competing interests.

Author's contributions

E.K. performed the research, wrote the original draft, designed the research study; S.T. revised the manuscript, analyzed the data. The final version of the manuscript was approved by all authors.

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