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Original Research Article

Myrcene exerts anti-tumor effects on oral cancer cells *in vitro* via induction of apoptosis

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Abstract

Purpose: *To investigate the anticancer potential of myrcene against oral cancer cells.*
Methods: *The effect of myrcene on oral cancer cell growth were studied using CCK-8 proliferation and clonogenic assays, while Annexin V-FITC/PI and DAPI staining methods were used for the determination of its effect on cell apoptosis. Protein expression levels were assayed by western blotting technique. Wound healing and Transwell chamber assays were used to determine the effect of myrcene on oral cancer cell migration and invasion, respectively.*
Results: *Myrcene significantly and dose-dependently inhibited oral cancer cell growth ($p < 0.05$). The anti-proliferative effect of myrcene was comparatively lower on Hs27 human fibroblast cells than on SCC9 oral cancer cells. Myrcene stimulated apoptosis in human oral cancer cells via enhancement of Bax expression and repression of Bcl-2 expression. The percentage of early and late apoptotic cells increased from 4.03 in untreated control to 27.77 when treated with 20 µM myrcene. Moreover, myrcene in vitro significantly suppressed the migration and invasion of oral cancer cells ($p < 0.05$).*
Conclusion: *These results indicate that myrcene exerts potent anticancer effects on oral cancer cells via induction of apoptosis, and should be further investigated in vivo for this purpose.*

Keywords: *Monoterpene, Oral cancer, Proliferation, Myrcene, Apoptosis, Anticancer*

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INTRODUCTION

Oral cancer is one of the frequently reported human cancers [1]. This and is the sixth most common human cancer worldwide [2]. At the global level, more than 300, 000 cases of oral cancer and 150, 000 oral cancer-related deaths are reported each year throughout the globe [3]. Indeed, there is an alarming increase in the incidence of oral cancer in several countries [4]. Although the overall 5-year survival of oral cancer ranges from 85 to 90 % at the earlier stages, the 5-year survival at the advanced stages of the disease is just 20 % [5]. In these cases, oral cancer shows local lymph node metastasis and short-term disease recurrence. Researchers have actively and consistently investigated the anticancer potential of different natural products against human oral cancer so as to devise more promising chemotherapeutic anticancer procedures against this deadly malignancy.

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Terpenoids represent by far the most abundant and highly ubiquitous type of plant-based secondary metabolites [6]. Several terpenoid molecules have been shown to possess marked pharmacological and health-promoting effects [7]. Besides, the anticancer effects of several terpenoids have been reported [8]. Myrcene is a volatile monoterpene which is abundantly produced by plants such as hops, cannabis and lemon grass [9]. Studies have shown that myrcene exhibits antioxidant, anti-inflammatory, antibacterial and analgesic properties [10]. Moreover, recent reports have indicated that myrcene exhibited antiproliferative effects against different types of human cancer cells such as lung cancer and breast cancer [11,12]. However, not much is known about the effect of myrcene on oral cancer cells. Therefore, the present study was designed to investigate the anticancer effect of myrcene on human oral cancer cells, as well as the underlying molecular mechanism.

EXPERIMENTAL

Cell culture

The oral cancer cell line (SCC9) and Hs27 normal human fibroblast cells were obtained from the American Type Culture Collection (ATCC). Dulbecco's Modified Eagle's Medium (DMEM, HyClone) supplemented with 10 % fetal bovine serum (FBS, Gibco) was employed to culture the cell lines. The cells were maintained in humidified CO_2 incubator at 37 ºC in a 5 % CO_2 atmosphere.

CCK- 8 viability assay

The SCC9 oral cancer and Hs7 fibroblast cells were seeded in 96-well plates, each at an initial density of 2×10^5 cells/well. After inoculation, the cells were treated with graded concentrations of myrcene (up to 640 µM). After 24-h incubation at 37 ºC, cell viability was determined using Cell Counting Kit-8 (CCK-8; Beyotime, Beijing, China) as per the manufacturer guidelines. Cell viability was estimated by measuring optical density (OD) of different cell samples at 450 nm in a microplate reader (Hitachi, Tokyo, Japan).

Clonogenic assay

Cancer cells (SCC9) were treated with graded doses of myrcene (5, 10 and 20 µM) and cultured in 6-well plates at an initial density of 500 cells/well for 15 days at 37 ºC. The growth medium was replaced every 3 days with fresh medium. After 15 days, the colonies were fixed in 70 % ethanol, and subsequently stained with 0.2 % crystal violet. Then, the number of colonies was estimated as an index of the effect of myrcene on clonogenic potential of SCC9 oral cancer cells.

Annexin V-FITC/PI and DAPI staining

The SCC9 oral cancer cells were seeded in 12-well plates at an initial density of 2.5×10^4 cells per well, followed by treatment with various concentrations of myrcene (5, 10 and 20 µM) for 24 h at 37 ºC. Then, the cells were harvested through centrifugation, washed with PBS, and resuspended in binding buffer of the Annexin V-FITC/PI staining kit (7 Sea Biotech, Shanghai, China). Thereafter, the cells were stained using 22.5 µL Annexin V-FITC and PI (1:2 v:v) for 20 min in the dark. Finally, the cells were subjected to flow cytometric analysis using FACS/Calibur flow cytometer (Becton Dickin-son, Franklin Lakes, NJ, USA).

Approximately 5×10^3 SCC9 cancer cells were placed in each well of a 12-well plate and administered different doses of myrcene (0 – 20 µM) at 37 ºC for 24 h. Then, the cells were harvested, washed with PBS, fixed with 70 % ethanol, and stained in the dark with 0.05 % 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 15 min. Then, nuclear staining of the cells was examined under a fluorescent microscope (Olympus, Tokyo, Japan).

Western blotting

Protein lysates were obtained from SCC9 oral cancer cells differentially treated with myrcene, using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) as per the manufacturer's method. The protein samples were resolved through SDS-polyacrylamide gel electrophoresis and electrically transferred onto nitrocellulose membranes. The membranes were blocked using 5 % fat-free milk at room temperature for 2 h, followed by incubation with specific primary antibodies overnight at 4 °C. Next, the membranes were incubated with horse radish peroxidase-linked secondary antibodies (Boster, Wuhan, China) at room temperature for 2 h. Finally, enhanced chemiluminescence substrate (ECS) was employed to detect the protein bands using a Bio-Rad ChemiDoc XRS Imaging system (Hercules, CA, USA). β-Actin was used as internal reference protein.

Cell migration and invasion assays

The migration and invasion of SCC9 oral cancer cells treated with 10 µM myrcene for 24 h or control untreated cells, were determined with

wound healing and Transwell chamber assays, respectively. For migration analysis, the cells were cultured at 37 ºC in 6-well plate until saturation. Then, a 200-µL pipette tip was used to scratch the cell surface, and the scratch was photographed under a light microscope (Olympus). Thereafter, the plate was incubated at 37 ºC for 24 h, after which the scratch area was again photographed.

For assessment of cell invasion *in vitro*, the myrcene-treated or untreated SCC9 cells were plated in the upper chamber of Transwell plate, while 10 % FBS supplemented DMEM was put in the lower chamber. After 24 h incubation at 37 °C, cells which migrated through the polycarbonate membrane were fixed with ethanol and subsequently stained with 0.2 % crystal violet. Finally, the cells were examined under a light microscope (Olympus).

Statistical analysis

Each experiment was carried in at least three replicates, and results are presented as mean ± standard deviation (SD). All statistical analyses were performed with GraphPad prism 7.0 software (La Jolla, CA, USA). Student's *t*-test was used to analyse differences between two treatment groups. Values of $p < 0.05$ were considered as indicative of statistically significant differences.

RESULTS

Myrcene selectively inhibited the growth of SCC9 oral cancer cells

In order to determine the anti-proliferative potential of myrcene, SCC9 oral cancer cells were treated with different concentrations of myrcene ranging from 1.25 to 640 µM for 24 h. It was shown that myrcene decreased the proliferation of SCC9 cancer cells in a concentration-dependent manner, with an estimated IC_{50} value of 10 µM (Figure 1 A). The Hs27 human fibroblast cells were also administered the same doses of myrcene for 24 h. However, the growth Hs27 cells was less severely affected by myrcene, with IC_{50} of 130 µM against Hs27 cells (Figure 1 B). These results are indicative of selective anti-proliferative effect of myrcene against the oral cancer cells studied. The effect of myrcene on oral cancer cells was also assessed in terms of clonogenicity. Treatment of SCC9 cancer cells with different doses of myrcene *in vitro* resulted in a dose-dependent reduction in colony formation, as shown in Figure 1 C. The relative percent colony numbers were 55, 37 and 19.5 at myrcene concentrations of 5, 10 and 20 µM, respectively.

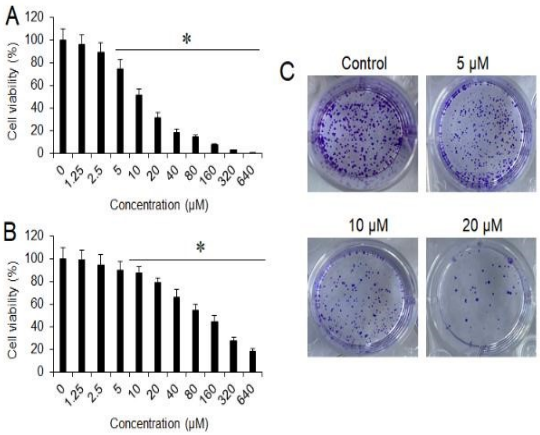

Figure 1: Effect of myrcene on oral cancer cells. Myrcene exhibited selective anti-proliferative potential against oral cancer cells. A: Treatment of SCC9 oral cancer cells with increasing concentrations of myrcene (1.25 - 640 µM) resulted in dose-dependent loss of viability, with IC_{50} of 10 µM. B: Myrcene inhibited the proliferation of Hs27 human fibroblast cells less effectively, with IC_{50} of 130 µM. C: Myrcene decreased colony formation in SCC9 oral cancer cells in a dose-dependent manner, with relative colony formation values of 55, 37 and 19.5 at 5, 10 and 20 µM myrcene, respectively, relative to corresponding untreated cells. Three independent replicates were used in each experiment. Values are expressed as mean ± SD

Myrcene induced apoptosis in SCC9 oral cancer cells

In order to determine the mechanism underlying the growth-inhibitory effect of myrcene on oral cancer cells, apoptosis was measured in myrcene-treated SCC9 cells using Annexin V-FITC/PI double staining. Flow cytometric analysis of SCC9 cells treated with varying concentrations of myrcene showed that the percentage of cell apoptosis increased with increasing doses of myrcene (Figure 2).

The percentages of early and late apoptotic cells were increased from 4.03 in untreated control to 27.77 by 20 µM myrcene. Moreover, DAPI staining and nuclear fluorescence of SCC9 cells treated with myrcene at doses of 5, 10 and 20 µM revealed that myrcene decreased the viability of the cells dose-dependently, indicating induction of apoptotic cell death (Figure 3 A). Results from western blot assay revealed that myrcene increased the protein expression of Bax, and down-regulated the protein expression of Bl-2 in oral cancer cells (Figure 3 B).

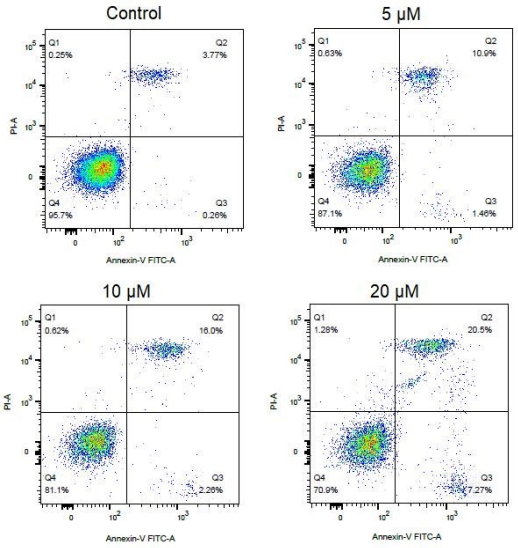

Figure 2: Myrcene induced apoptosis in oral cancer cells. Flow cytometry revealed that myrcene induced apoptosis in SCC9 oral cancer cells in a dose-dependent fashion. Data are presented as mean ± SD

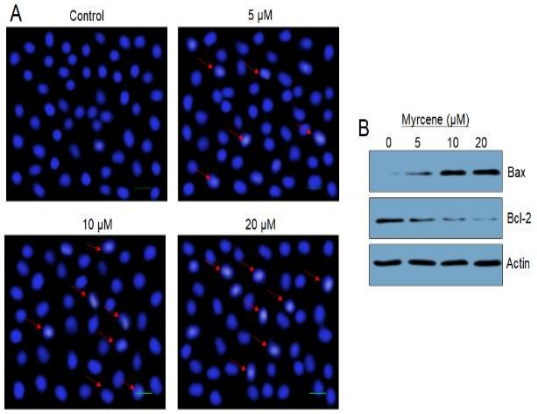

Figure 3: Myrcene induced apoptosis in oral cancer cells by modulating the expressions of apoptosis-related proteins. A: DAPI staining showing that apoptosis of SCC9 cancer cells increased with increasing doses of myrcene. B: Myrcene modulated the expressions of apoptosis-related proteins in SCC9 oral cancer cells in a dose-dependent fashion. Each experiment was performed in three independent replicates

Myrcene reduced migration and invasion SCC9 oral cancer cells

As shown in Figure 4, the migration and invasion of SCC9 oral cancer cells were significantly reduced by myrcene administration *in vitro* ($p < 0.05$). This is further evidence of the anticancer potential of myrcene against human oral cancer cells.

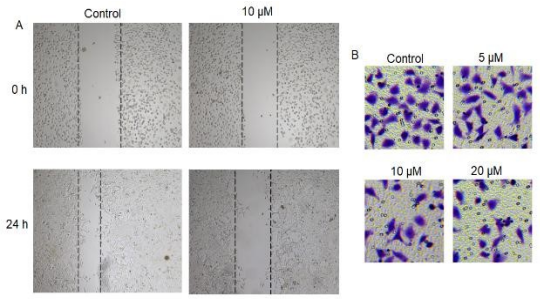

Figure 4: Myrcene inhibited the migration and invasion of oral cancer cells. Treatment of SCC9 oral cancer cells with 10 μM myrcene produced significantly lower migration (A) and lower invasion (B), when compared with control untreated cells. Each assay was done in triplicate

DISCUSSION

The natural environment is an immense reservoir for drug discovery [13]. The huge diversity of natural products enables researchers to evaluate their effectiveness against a wide array of human pathological disorders. In recent times, several research investigations have focused on the usefulness of plant-based natural compounds in the treatment of different types of human cancers. These studies have led to the identification of potent anticancer agents, as well as the specific signaling pathways targeted in malignant cells by these compounds [14,15]. Based on this background, the present study investigated the effects of myrcene, a plant-based volatile compound, on human oral cancer cells *in vitro*. Studies have shown that myrcene exhibits several beneficial effects on human health [10,16]. Moreover, recent studies reported that myrcene exerted anticancer potential [11,12]. Consistent with these findings, administration of myrcene to oral cancer cells resulted in significantly reduced cell proliferation *in vitro*. Furthermore, myrcene was less severe on the growth of normal human cells, suggesting that it was selective in its antiproliferative action against malignant oral cancer cells.

The anticancer effect of myrcene on oral cancer cells was shown to be due to induction of apoptosis. Myrcene-treated oral cancer cells had increased expression of the pro-apoptotic protein Bax, and decreased protein expression of Bcl-2. It is known that Bax/Bcl-2 ratio is a reliable indicator of the release of apoptotic mediators from mitochondria, and apoptotic cell death. Moreover, apoptosis is a tightly regulated and highly robust cellular program modulated by pro-apoptotic and anti-apoptotic proteins [17]. Cancer cells are characterized by aberrant apoptotic signaling which prolongs their survival.

Thus, the restoration of apoptosis in cancer cells has a significant potential in pharmacotherapy of human cancer [18]. The pro-apoptotic effect of myrcene indicates its potential effectiveness as a therapeutic agent for human oral cancer. In addition, the results of the current study indicate the anti-metastatic potential of myrcene against the human oral cancer cells. Myrcene treatment reduced the migration and invasion of oral cancer cells with myrcene in vitro; these effects are crucial for metastasis and tumor suppression [19]. Collectively, the results obtained in study indicate that myrcene induced apoptosis in human oral cancer cells via induction of apoptosis. These results are suggestive of the potential of myrcene as a lead molecule for management of oral cancer.

CONCLUSION

The results of the present study reveal that myrcene exerts selective anti-proliferative activity against oral cancer cells. Myrcene also induces apoptosis in oral cancer cells, thereby suppressing their growth and viability *in vitro*. Moreover, the *in vitro* anticancer effect of myrcene against oral cancer cells is evident from its restriction of the migration and migration of oral cancer cells. These results suggest that myrcene is a potential lead molecule for the development of chemotherapy for oral cancer.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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