JBUON 2019; 24(6): 2426-2431
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
Email: editorial_office@jbuon.com

ORIGINAL ARTICLE

Murrayanine exerts antiproliferative effects on human oral cancer cells through inhibition of AKT/mTOR and Raf/MEK/ERK signalling pathways *in vitro* and inhibits tumor growth *in vivo*

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Summary

Purpose: *Oral cancer ranks as the 6th most prevalent type of cancer accounting for significant mortality around the world and studies are being directed to develop efficient chemotherapy for oral cancer. In this study the anticancer effects of a carbazole alkaloid Murrayanine were investigated in vitro and in vivo.*

Methods: *Cell counting assay and colony formation assay were used to examine cell viability. DAPI and propidium iodide (PI) staining were used to detect apoptosis. Western blotting was used to examine protein expression. Xenografted mice were used for in vivo study.*

Results: *The results showed that Murrayanine decreased the viability of the oral cancer SCC-25 cells and exhibited an IC_{50} of 15 μM. The cytotoxicity of Murrayanine was also investigated on the normal hTERT-OME cells and it was found that this molecule exerted very low toxic effects on these cells exhibiting an IC_{50} of 92 μM. Murrayanine also caused considerable changes in the morphology of the SCC-25 cells and inhibited their colony forming potential. PI and DAPI staining revealed that Murrayanine prompted apoptosis of the SCC-25 cells. The apoptotic cells from 2.2% in the control increased to around 35% at 30 μM concentration. Moreover, Murrayanine caused increase in the Bax/Bcl-2 ratio and also increased the expression of Caspase-3. Murrayanine also deactivated the AKT/mTOR and Raf/MEK/ERK signalling pathways and suppressed the growth of the xenografted tumors in vivo.*

Conclusion: *The findings of the present investigation suggest that Murrayanine may prove essential in the development of systemic therapy for oral cancers.*

Key words: *murrayanine, apoptosis, xenografted tumors, cell viability*

Introduction

Being one of the commonly diagnosed oral cavity neoplasias, oral cancer causes high morbidity and mortality around the world [1]. Among all oral cancers, oral squamous cell carcinoma (OSCC) is the most prevalent type accounting for almost 90% of all oral cancers [2]. The OSCC-related mortality is due to rapid metastasis to distant parts of the body [3]. The unavailability of appropriate and reliable biomarkers and therapeutic targets, late diagnosis and inefficient drugs form bottleneck in the treatment of oral cancers [4]. Over the years a lot of research has been directed at the discovery and development of chemotherapy for deadly diseases such as cancer [5]. However, the clinical applicability of most of the anticancer drugs is restricted by their adverse effects [6]. Consistently, naturally occurring compounds with anticancer effects have attained attention due to their negligible/low tox-

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Received: 18/12/2018; Accepted: 05/01/2019

icity [7]. Murrayanine is an important carbazole alkaloid, mainly isolated from *Murraya koenigii* [8]. Carbazole alkaloids have been shown to exhibit anticancer effects against a wide range of cancers and Murrayanine is no exception [9,10]. However, the anticancer effects of Murrayanine have not been investigated against oral cancer. This study was designed to explore the anticancer effects of Murrayanine against the SCC-25 oral cancer cells and to decipher the underlying mechanisms. AKT/mTOR and Raf/MEK/ERK signalling pathways are considered of utmost importance in cancer research owing to their potential as therapeutic targets for anticancer drugs and it is believed that drugs able to target these pathways may prove beneficial in the development of systemic therapy for the treatment of several types of cancers [11,12]. This study also investigated the effects of Murrayanine on AKT/mTOR and Raf/MEK/ERK.

Methods

Cell viability and colony formation assays

The cell counting assay was employed to determine the viability of the SCC-25 oral cancer cells. In brief, 5×10^4 cells/well were seeded in 12-well plates and incubated for 24 h with different concentrations of Murrayanine. The aliquots of cells were then removed and counted in triplicate following Trypan blue staining. The morphological analysis of the Murrayanine-treated SCC-25 cells was carried out by phase contrast microscopy as described previously [13]. The effect of Murrayanine on the formation of SCC-25 colonies was investigated as described earlier [14].

Propidium iodide and DAPI staining for apoptosis

The detection of apoptosis in the SCC-25 oral cancer cells was performed by acridine orange (AO)/ethidium bromide (EB) staining. In brief, the oral cancer SCC-25 cells (0.6×10^6) were grown in 6-well plates. Following an incubation period of around 12 h, the SCC-25 cells were subjected to Murrayanine [treatment for 24 h at 37°C. Around 25 µl cell culture were put onto glass slides and stained with PI or DAPI. The slides were cover-slipped and examined with a fluorescent microscope.

In vivo xenograft study

Male mice C57 BL/6 were obtained from the animal holding capacity of China-Japan Union Hospital of Jilin University and maintained in the animal facility following the National Institutes of Health standards for the care and use of laboratory animals. The animals received free access to standard diet in well ventilated rooms with a 12:12 dark/light cycle and a temperature of around

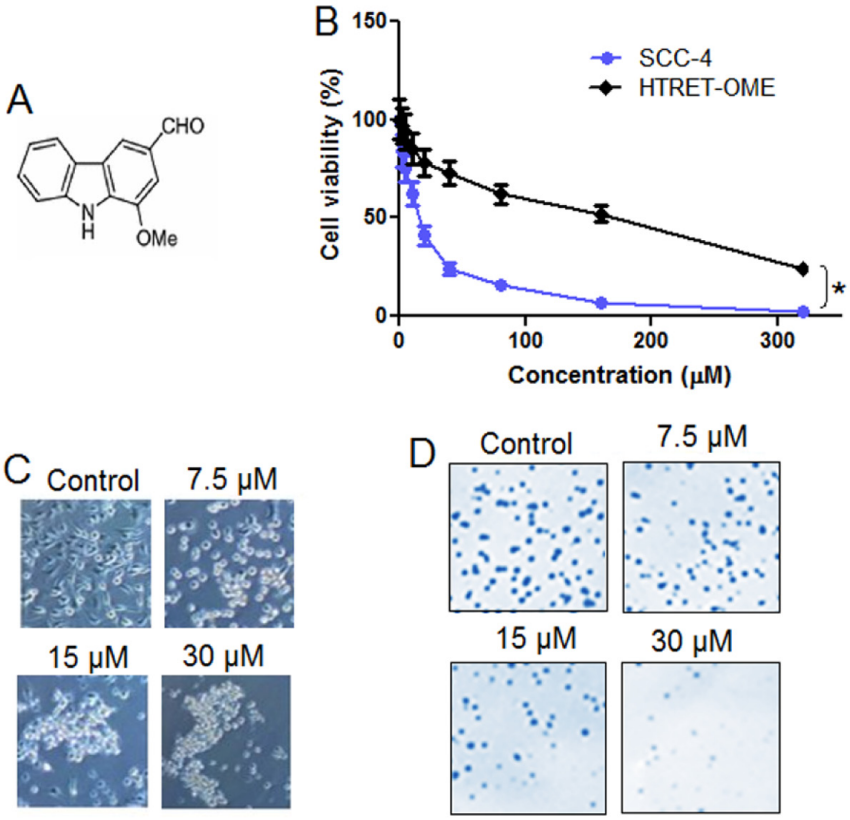

Figure 1. (A: Chemical structure of Murrayanine. **(B:** Cell counting assays showing the effects of Murrayanine on the viability of SCC-4 oral cancer cells and HTRET-OME normal cells. **(C:** Morphology of Murrayanine-treated SCC-4 cells. **(D:** Colony formation of Murrayanine-treated SCC-4 cell. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

27°C. The mice were randomly divided into five groups and the SCC-25 cells were injected subcutaneously into the left flanks of the mice. The mice were monitored and as the growth of the tumors was apparent, the four groups received 0.1% DMSO-dissolved Murrayanine at 10, 20, 30 and 40 mg/kg concentrations, while the fifth group was kept as control and received 0.1% DMSO in normal saline thrice weekly. The mice were euthanized at the end of the study (5 weeks) and tumors were harvested and used for further experiments.

Western blotting

Briefly, the SCC-25 cells were washed with ice-cold phosphate buffered saline (PBS) and suspended in a lysis buffer at 4°C which then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford assay. About 40 µg of protein were loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then treated with tris buffered saline (TBS) and exposed to primary antibodies at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent.

Statistics

Data are shown as mean ± SD. Statistical analyses were done using Students *t*-test with SPSS software. Values of $p<0.05$ were taken as indicative of significant difference.

Results

Murrayanine inhibits the growth of oral cancer cells

The growth inhibitory effects of Murrayanine (Figure 1A) were assessed on the SCC-25 oral cancer cell line and hTERT-OME non-cancer cell line. The results of the cell counting assay showed that Murrayanine decreased the viability of the SCC-25 oral cancer cells dose-dependently. The IC_{50} of Murrayanine against the SCC-25 oral cancer cell was 15 µM (Figure 1B). Nonetheless, the cytotoxicity of Murrayanine on the hTERT-OME non-cancer cells was comparatively negligible (IC_{50} 92 µM). Moreover, Murrayanine also induced some morphological changes such as shrinkage and membrane blebbing of the SCC-25 cells suggestive of apoptosis (Figure 1C). The effects of Murrayanine treatment were also assessed on the colony formation potential of the SCC-25 cells and it was revealed that this molecule exerted dose-dependent inhibition on the colony formation of SCC-25 cells (Figure 1D).

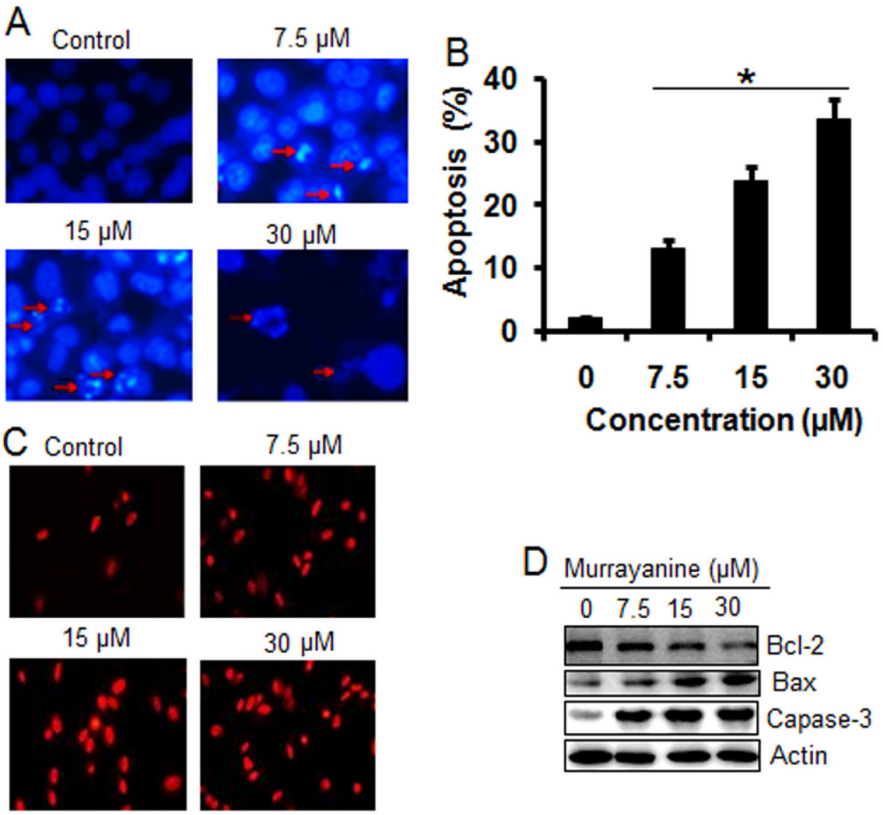

Figure 2. (A): DAPI staining of Murrayanine-treated SCC-4 cells showing that this molecule induces apoptosis dose-dependently (arrows show apoptotic cells). **(B):** percentage of apoptotic cells at indicated concentrations of murrayanine. **(C):** PI staining showing induction of apoptosis. **(D):** expression of apoptosis-related proteins. The experiments were performed in triplicate and expressed as mean±SD (*$p<0.05$).

Murrayanine activates apoptotic cell death of oral cancer cells

The PI and DAPI staining were performed to ascertain if Murrayanine exerts antiproliferative effects on the SSC-25 cells. DAPI staining showed remarkable changes in the nuclear morphology and membrane blebbing of SCC-25 cells (Figure 2A). The apoptotic cells increased from 2.2% in the control to around 35% at 30 µM concentration (Figure 2B). PI staining also showed that Murrayanine increased the PI-positive cells, indicative of apoptosis (Figure 2C). The results of western blot analysis showed that Murrayanine also caused considerable increase in the expression of Caspase 3 and Bax and decrease in the expression of the Bcl-2 in SCC-25 cells (Figure 2D).

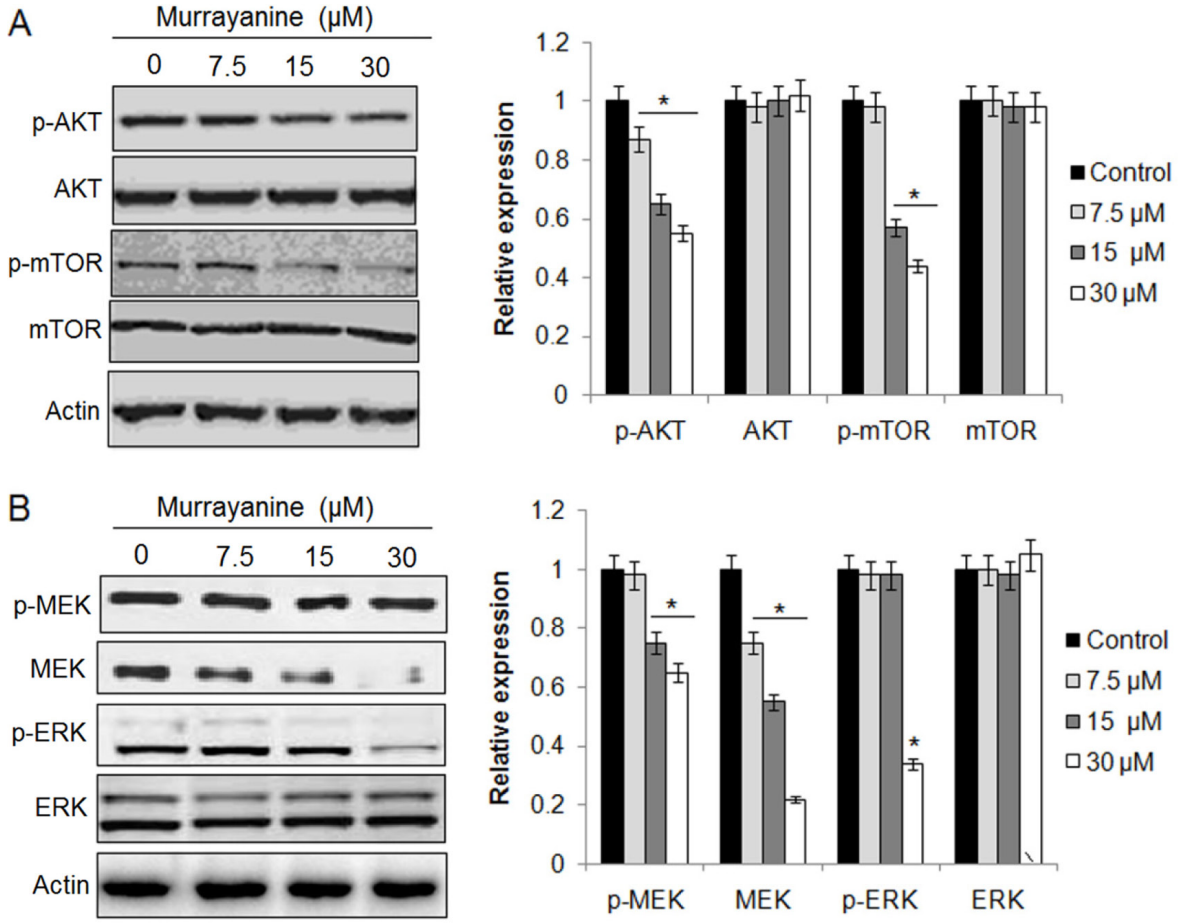

Figure 3. Effect of Murrayanine on **(A)** AKT/mTOR and **(B)** Raf MEK/ERK signalling pathways as depicted by western blot analysis. The experiments were performed in triplicate and expressed as mean±SD (*p<0.05).

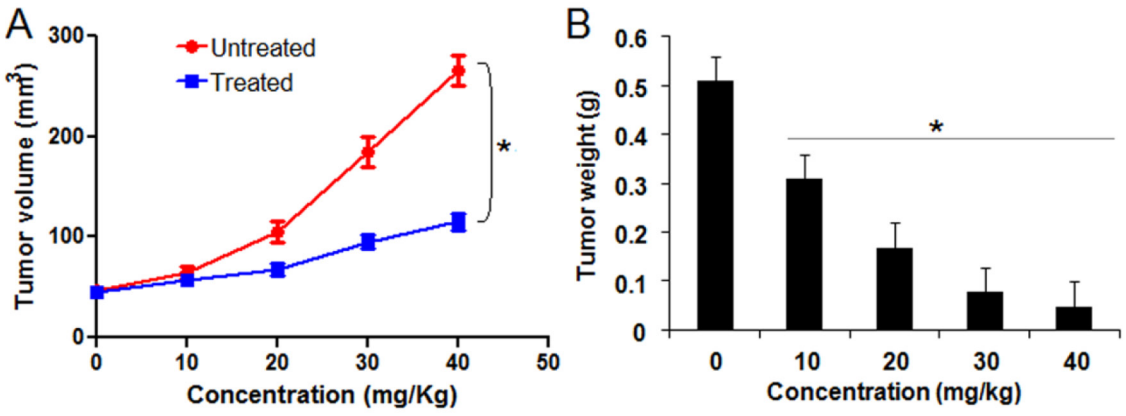

Figure 4. Effect of Murrayanine on **(A** tumor volume and **(B** tumor weight in xenografted mice models at indicated concentrations. The experiments were performed in triplicate and expressed as mean±SD (*p<0.05).

Murrayanine deactivates the AKT/mTOR and Raf/MEK/ERK pathways

The effects of Murrayanine were also examined on the therapeutically important AKT/mTOR and Raf/MEK/ERK signalling pathways. It was found that Murrayanine inhibited the phosphorylation of AKT and mTOR dose-dependently (Figure 4A). Moreover, the expression of p-ERK and p-MEK was also significantly downregulated dose-dependently (Figure 4B). These results suggest that Murrayanine deactivates both the AKT/mTOR and Raf/MEK/ERK signalling pathways.

Murrayanine suppresses xenografted tumor growth in vivo

Since Murrayanine showed potent anticancer effects *in vitro*, its anticancer effects were also examined in xenografted tumor mice models *in vivo*. The results showed that this molecule inhibited the growth of xenografted tumors as the tumor volume and weight were found to decrease with increasing concentrations of Murrayanine (Figure 4A and B).

Discussion

Ranking 6th among the most prevalent cancers, the oral cancers have a very poor 5-year survival rate in comparison to other cancers such as breast and prostate cancers to name a few [15]. There is urgent need to develop efficient systemic therapies to treat oral cancers and to improve the patient overall survival [16]. This study evaluated the anticancer effects of the carbazole alkaloid Murrayanine against the SCC-25 oral cancer cells, as well as the MRC5 normal oral cells. Murrayanine caused significant decrease in the viability of the SCC-25 cells with minimal effects on the viability of the normal oral cells suggesting selective anticancer effects of this molecule on oral cancer cells. Murrayanine also caused remarkable morphological changes in SCC-25 cells and inhibited their colony formation potential. Previous studies carried out on carbazole alkaloids have also shown similar results. Carbazole alkaloids, such as Mahanine, have been shown to halt the growth of leukemia cells [17]. Similarly Mahanimbine has been found to trigger arrest of pancreatic cancer cells in the G0/G1 checkpoint of the cell cycle [18]. Moreover, many of the carbazole alkaloids, such as Mukonal, have been shown to induce apoptotic cell death of cancer cells [19]. Apoptotic cell death is another imperative mechanism to eliminate cancer cells [20] and in this study Murrayanine was found to induce apoptotic cell death of the SCC-25 cells. Furthermore, Murrayanine also enhanced the expression of caspase 3 and increased the Bax/Bcl-2 ratio. Given the importance of the Akt/mTOR pathways in cancer treatment [10,11], the effects of Murrayanine were also examined on these pathways and it was found that this molecule deactivated both these pathways in SCC-25 oral cancer cells in a dose-dependent manner. Finally, the effects of Murrayanine were also examined in the xenografted mice models and the results showed that it could suppress both tumor volume and weight in xenografted mice, suggestive of the anticancer potential of Murrayanine.

Conclusion

It is concluded that Murrayanine is an important carbazole alkaloid with strong anticancer potential. Murrayanine inhibits the growth of oral cancer cells by induction of apoptosis and it also inhibits tumor growth *in vivo*. Hence, it may prove beneficial in treating oral cancer and therefore warrants further investigations.

Conflict of interests

The authors declare no conflict of interests.

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