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ORIGINAL ARTICLE

Inhibition of cancer cell growth in cisplatin-resistant human oral cancer cells by withaferin-A is mediated via both apoptosis and autophagic cell death, endogenous ROS production, G2/M phase cell cycle arrest and by targeting MAPK/RAS/RAF signalling pathway

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Summary

Purpose: *Oral cancer is one of the common oral cavity and pharynx cancers and its treatment is limited by early metastasis, late diagnosis and emergence of drug resistance. Herein we investigated the anticancer effects of Withaferin-A against the cisplatin-resistant SCC-4 human oral cancer cells.*

Methods: *The proliferation rate of the human oral cancer cells was evaluated by CCK-8 assay. Apoptotic cell death was studied by acridine orange (AO) / ethidium bromide (EB) staining as well as by flow cytometry using annexin V/propidium iodide (PI) staining. Cell cycle analysis was performed by flow cytometry. Reactive oxygen species (ROS) examination was carried out by flow cytometry. Protein expressions were determined by immunoblotting.*

Results: *The results of the MTT assay showed that Withaferin-A caused decrease in the proliferation of SCC-4 cells and exhibited an IC_{50} of 14 μM. The anticancer effects of Withaferin-A were mainly due to the induction of apoptosis which was linked with upsurge of Bax and depletion of BCl-2. Annexin V/PI assay showed that the apoptotic cells were 0.75, 5.8, 12.4 and 22.66% at 0,7,14 and 28 μM concentrations of Withaferin-A. Withaferin-A also caused increase in the ROS and decrease in the MMP levels of the SCC-4 cells. Western blot analysis showed that the expression of LC3B II increased while of p62 decreased remarkably upon treatment with Withaferin-A, suggestive of autophagic cell death. Cell cycle analysis by flow cytometry showed that Withaferin-A caused increase in the G2/M phase cells triggering arrest of cancer cells at the G2/M checkpoint of the cell cycle. Finally, western blot analysis showed that Withaferin-A blocked the MAPK/RAS/RAF signalling pathway in the SCC-4 cells.*

Conclusions: *These results suggest that Withaferin-A may prove a potent lead molecule in oral cancer treatment and warrants further investigations.*

Key words: *oral cancer, withaferin-A, apoptosis, autophagy, cell cycle arrest*

Introduction

Plant-derived molecules have been used in the treatment of different diseases [1]. Over the last few decades, the utilisation of plant-extracted edibles and consumption of highly active naturally or synthesized highly specific drugs, the inclination towards the use of natural products has increased [2]. The natural plant-derived molecules have comparatively lower side effects and are easily available. The potency of the natural drugs or toxicity, if any, can be overcome by semi-synthetic approaches

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[3]. *Withania somnifera* is an important medicinal plant with impressive pharmacological potential. Its bioactivities, such as neuroprotective, anticancer and antimicrobial have been attributed to different extracts of this plant [4]. *Withania somnifera* is a rich source of Withaferin-A which has been reported to exhibit potent anticancer activities [5] like suppression of the growth of pancreatic cancer by targeting heat shock proteins [6]. It has also been shown to cause apoptosis of prostate cancer cells [7]. In yet another study this molecule has been reported to suppress breast cancer growth by inducing apoptotic cell death [8]. Nonetheless, the anticancer effects of Withaferin-A have not been examined against cisplatin-resistant oral cancer cells. This study was designed to investigate the anticancer effects of Withaferin-A against the drug-resistant human oral cancer cells and attempts were made to explore the underlying mechanisms for its anticancer properties. The MAPK/RAS/RAF signalling pathway is an important pathway that has been shown to be activated in cancer and has been reported to have a role in the progression and development of several malignancies [9]. Therefore, we also examined the effects of Withaferin-A on MAPK/RAS/RAF signalling pathway in drug-resistant SCC-4 oral cancer cells. The findings of the study showed that Withaferin-A may exert potent anticancer effects on the drug-resistant oral cancer cells via induction of apoptosis and autophagy. Furthermore, Withaferin-A also induced G2/M cell cycle arrest of the SCC-4 cancer cells. Taken together these results suggest that Withaferin-A may prove beneficial in the treatment of oral cancer and warrants further investigation.

Methods

Cell viability determination

Briefly, the cisplatin-resistant SCC-4 oral cancer cells were seeded in 96-well plates and treated with varied concentrations of Withaferin-A at 37°C for 24 h. Thereafter, 10 µL of CCK-8 solution were added to the cell culture and incubated for 2 h at 37°C in an incubator with 5% CO_2 and 95% O_2. Optical density (OD_{450}) was taken with a microplate reader to determine the cell viability.

AO/EB staining

The cisplatin-resistant SCC-4 cells (0.6×10^6) were seeded in 6-well plates and incubated with varied concentration of Withaferin-A for 24 h at 37°C. Ten µl cell culture were put onto a glass slide and stained with acridine orange (AO)/ethidium bromide (EB). The slides were cover-slipped and examined with a fluorescent microscope. Annexin V/PI staining was performed as described previously [10].

Cell cycle analysis

The cisplatin-resistant SCC-4 cells were treated with varied concentrations of Withaferin-A and incubated for 24 h at 37°C and were then washed with phosphate buffered saline (PBS). Afterwards, the Withaferin-A treated SCC-4 cells were stained with PI and the cell distribution in cell cycle phases was assessed by FACS flow cytometer.

Western blot analysis

Protein expression estimation was carried out by western blotting. The Withaferin-A treated SCC-4 cells were harvested with centrifugation and were then lysed in lysis buffer containing the protease inhibitor. Around 45 µg of proteins from each sample were separated on 10% SDS-PAGE, followed by transferring it to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Afterwards, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, the protein signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalisation.

Statistics

Data are shown as mean ± SD. Statistical analyses were done using Student's *t*-test with GraphPad prism 7 software. Values of $p<0.05$ were considered as statistically significant.

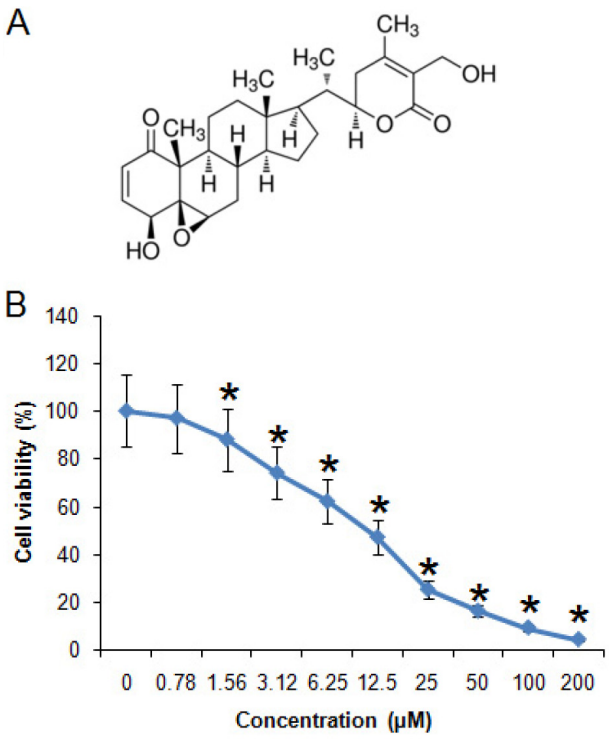

Figure 1. A: Structure of Withaferin-A. **B:** Effect of Withaferin-A on the viability of cisplatin-resistant SCC-4 cells as depicted by CCK-8 assay. The values are the mean of three experiments and shown as mean ± SD (*$p<0.05$).

Results

Suppression of oral cancer cell growth by Withaferin-A

The determination of antiproliferative effects of Withaferin-A (Figure 1A) on the SCC-4 cells treated with 0-200 μM concentrations of Withaferin-A was carried out by CCK8 assay. The determination of the CCK8 cell viability assay revealed that Withaferin-A causes dose-dependent decrease in the viability of the SCC-4 cells (Figure 1B). It was further revealed Withaferin-A exhibited an IC_{50} of 14 μM against the SCC-4 oral cancer cells.

Induction of apoptosis by Withaferin-A in SCC-4 cells

The induction of apoptosis by Withaferin-A was determined by AO/EB staining and the results showed that this molecule triggered blebbing and nuclear fragmentation of apoptosis as can be seen from the AO/EB staining. The increase in the orange color fluorescence was also indicative of apoptosis (Figure 2). The annexin V/PI staining assay showed Withaferin-A increased the percentage of the apoptotic cells. The apoptotic cells were found to be 0.75, 5.8, 12.4 and 22.66% at 0,7,14 and 28 μM concentrations of Withaferin-A (Figure 3). The effects of Withaferin-A were also examined on the expression of Bax and Bcl-2 and the results showed that the expression of Bax increased while Bcl-2 decreased in a concentration-dependent manner (Figure 4).

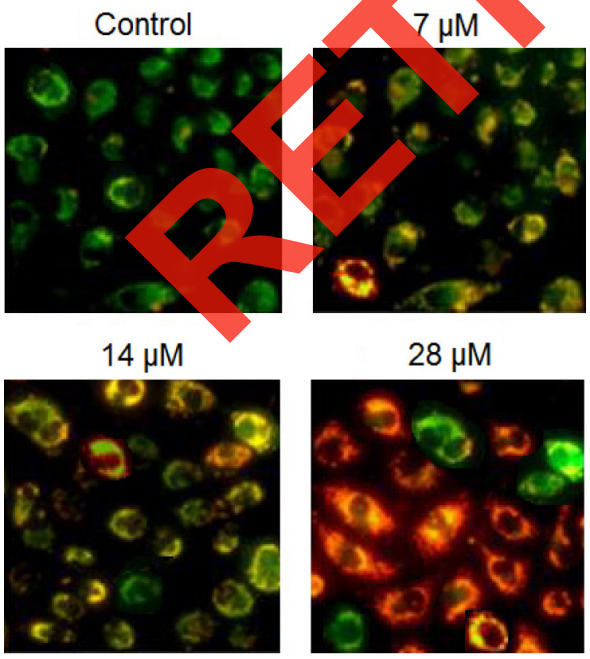

Figure 2. AO/EB staining showing that Withaferin-A at indicated concentrations induces apoptosis in SCC-4 cells. Green color depicts normal cells, orange color depicts early apoptotic cells and red color depicts late apoptotic cells. The experiments were performed in triplicate.

Withaferin-A caused increase in the ROS levels of SCC-4 cells

The effects of Withaferin-A were examined on the reactive oxygen species (ROS) levels in the SCC-4 cells at 0, 7, 14 and 28 μM concentrations of Withaferin-A. The results showed that the increase in the ROS levels were 100, 120, 170 and 230% at 0, 7, 14 and 28 μM concentrations of Withaferin-A, respectively (Figure 5A). The results also showed that Withaferin-A caused decrease in the MMP levels in the SCC-4 cells. The MMP levels were 100, 75, 45 and 35% at 7, 14 and 28 μM concentrations of Withaferin-A (Figure 5B).

Withaferin-A caused autophagy in SCC-4 cells

The expression of autophagy-related proteins was determined to investigate if Withaferin-A trig-

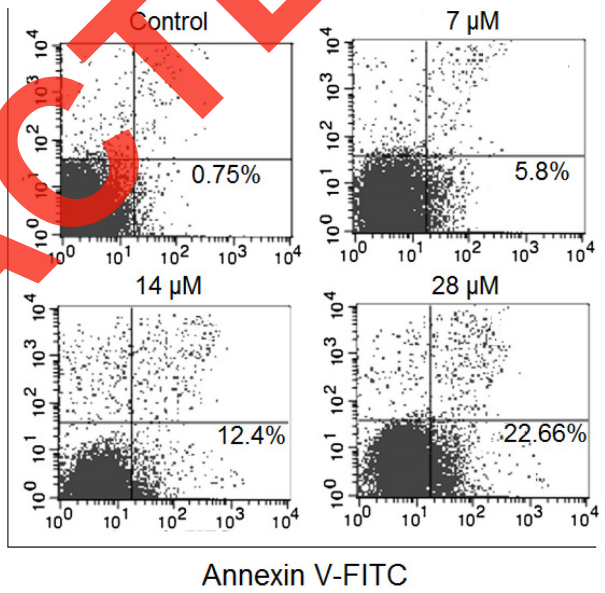

Figure 3. Annexin V/PI staining assay showed that the percentage of the apoptotic cells increased upon treatment of the SCC-4 cells with Withaferin-A. The experiments were performed in triplicate.

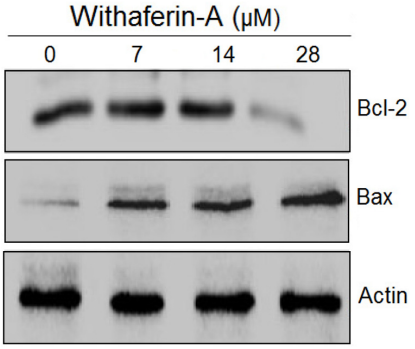

Figure 4. Western blot analysis showing that Withaferin-A increases the expression of Bax and decreases the expression of Bcl-2. The experiments were performed in triplicate.

gered autophagy in the SCC-4 cells. The results showed that Withaferin-A caused increase in the expression of LC3B-II concentration-dependently, while that of LC3B-I decreased. The expression of p62 was decreased concentration-dependently in SCC-4 cells, indicative of autophagy (Figure 6).

Withaferin-A caused G2/M arrest in SCC-4 cells

The effects of Withaferin-A were also investigated on the cell cycle distribution of the SCC-4 cells by flow cytometry. It was found that Withaferin-A caused significant increase in the percentage of the G2/M phase SCC-4 cells. The percentage of the G2/M phase cells increased to 68% at 60 µM as compared to 8% in the control (Figure 7).

Withaferin-A inhibited the MAPK/RAS/RAF signalling pathway in SCC-4 cells

The effects of Withaferin-A were also examined on the MAPK/RAS/RAF signalling pathway in 0, 7, 14 and 28 µM concentrations. The results showed that the phosphorylation of MAPK p38 decreased while the expression of MAPK p38 remained constant. Moreover, the expression of Ras and Raf decreased concentration-dependently upon treatment with Withaferin-A (Figure 8).

Discussion

Oral cancer causes significant mortality worldwide. Approximately 90% of the oral cancers are squamous cell carcinoma (SCC) [11]. The frequent metastasis of oral cancers to lymph nodes makes it one of the deadly malignancies [12]. The treatment of oral cancers is obstructed by limited treatment options and late diagnosis [13]. Herein, the

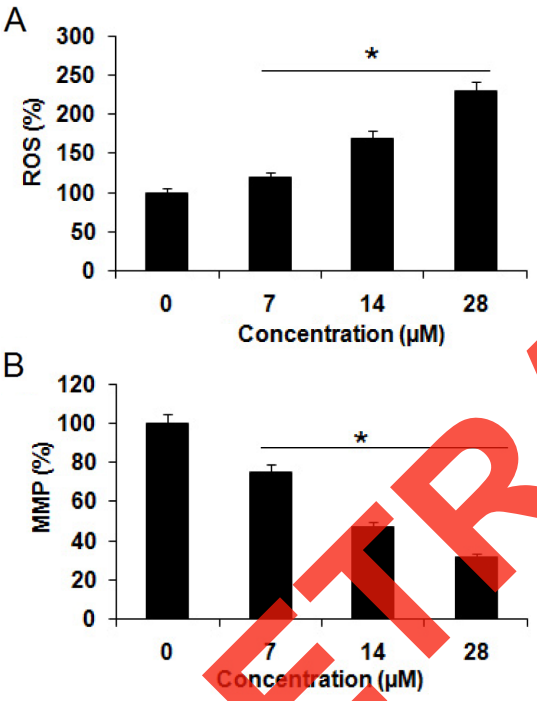

Figure 5. Effect of Withaferin-A on **A:** ROS levels and **B:** MMP levels in SCC-4 cells. The Figure shows that Withaferin-A causes increase in ROS and decrease in MMP levels dose-dependently. The values are the mean of three experiments and are shown as mean ± SD (*p<0.05).

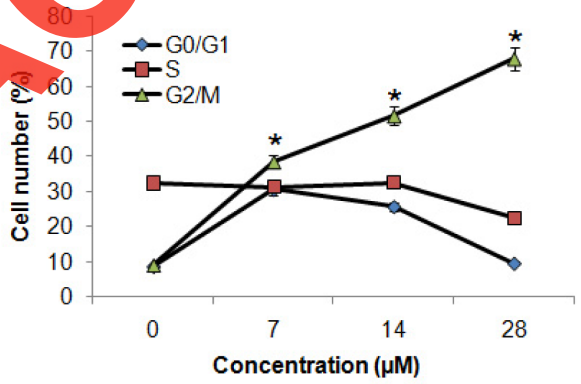

Figure 7. Flow cytometry shows that Withaferin-A increases the G2/M phase of SCC-4 cells dose-dependently. The values are the mean of three experiments and shown as mean ± SD (*p<0.05).

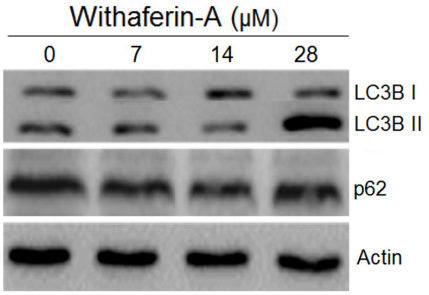

Figure 6. Western blot analysis showing the effect of Withaferin-A on the expression of LC3B I, LC3B II and p62. The Figure shows that the expression of LC3BII increases and of p62 decreases upon Withaferin-A treatment. The experiments were performed in triplicate.

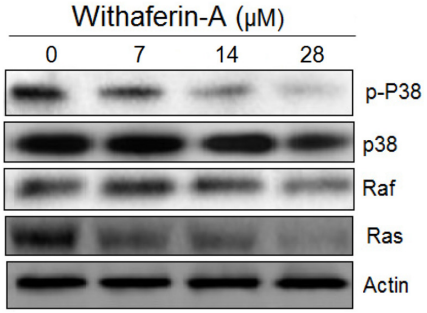

Figure 8. Effect of Withaferin-A on the MAPK/Raf/Ras signalling pathway as depicted by Western blot analysis. The Figure shows that the expression of p-P38, Raf and Ras increases, while that of p38 remains constant. The experiments were performed in triplicate.

anticancer effects of a plant-derived bioactive molecule - Withaferin-A - were examined against the human oral SCC-4 cells and the results showed that this molecule caused inhibition of the SCC-4 cell growth. These results are in concordance with previous investigations wherein Withaferin-A has been shown to suppress the growth of breast cancer cells by targeting FOXO3a [14]. Molecules of plant origin suppress the proliferation of cancer cells via multiple mechanisms which include but are not limited to the induction of apoptosis, autophagy and arrest of the cancer cells at different cell cycle phases [15]. Herein, we found that Withaferin-A triggered apoptosis in the SCC-4 oral cancer cells which was also accompanied by increase in the Bax and Bcl-2 ratio. A previous study also indicated that Withaferin-A induced apoptosis in cervical cancer cells [16].

Studies have shown that some plant-derived molecules induce apoptosis in cancer cells via generation of ROS [17]. Herein, we observed that Withaferin-A caused increase in the ROS levels which was also accompanied by decrease in the MMP levels of the SCC-4 cells. The results of Western blot analysis also showed that Withaferin-A causes upregulation of LC3B-II and downregulation of p62, suggestive of the autophagic cell death of the SCC-4 cells. These results are consistent with previous studies wherein Withaferin has been shown to induce ROS-mediated autophagy in ovarian cancer cells [18].

Cell cycle arrest is one of the important mechanisms by which anticancer agents suppress the growth of cancer cells [19]. The results showed that Withaferin-A caused increase in the G2/M phase cells in a concentration-dependent manner, triggering G2/M phase arrest.

MAPK/Ras/Raf pathway is one of the important pathways that have been implicated in the development and progression of different cancers [9] and the results in this study showed that Withaferin-A blocked the MAPK/Ras/Raf signalling pathway. Taken together these results indicate that Withaferin-A exerts potent anticancer effects on oral cancer cells.

Conclusion

The outcomes of the present study indicate that Withaferin-A exerts significant anticancer effects on human oral cancer cells. The anticancer effects of Withaferin-A are mainly due to the induction of apoptosis, autophagy and cell cycle arrest. Taken together, Withaferin-A may be utilised in the development of systemic therapy for oral cancer and deserves further studies.

Conflict of interests

The authors declare no conflict of interests.

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