ORIGINAL ARTICLE

The anticancer activity of visnagin, isolated from *Ammi visnaga* L., against the human malignant melanoma cell lines, HT 144

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Abstract

Melanoma is a cancer of melanocyte cells and has the highest global incidence. There is a need to develop new drugs for the treatment of this deadly cancer, which is resistant to currently used treatment modalities. We investigated the anticancer activity of visnagin, a natural furanochromone derivative, isolated from *Ammi visnaga* L., against malignant melanoma (HT 144) cell lines. The singlet oxygen production capacity of visnagin was determined by the RNO bleaching method while cytotoxic activity by the MTT assay. Further, HT 144 cells treated with visnagin were also exposed to visible light ($\lambda \geq 400$ nm) for 25 min to examine the illumination cytotoxic activity. The apoptosis was measured by flow cytometry with annexin V/PI dual staining technique. The effect of TNF-α secretion on apoptosis was also investigated. In standard MTT assay, visnagin (100 µg/mL) exhibited 80.93% inhibitory activity against HT 144 cancer cell lines, while in illuminated MTT assay at same concentration it showed lesser inhibitory activity (63.19%). Visnagin was induced apoptosis due to the intracellular generation of reactive oxygen species (ROS) and showed an apoptotic effect against HT 144 cell lines by 25.88%. However, it has no effect on TNF-α secretion. Our study indicates that visnagin can inhibit the proliferation of malignant melanoma, apparently by inducing the intracellular oxidative stress.

Keywords Visnagin · *Ammi visnaga* L. · Malignant melanoma · Apoptosis

Introduction

Melanoma is a malignant type of cancer of the melanocytes [1, 2]. The skin cancer is a common disease among the cancer types diagnosed in the world [3, 4]. Melanoma accounts for about 4–5% among the skin cancers. But it causes 75% of skin cancer related deaths [5]. Unfortunately, the incidence and mortality rate of malignant melanoma has been increasing worldwide [6, 7]. Melanoma is the most aggressive form of skin cancer. Much of the genetic, functional, and biochemical studies revealed that melanoma cells are highly resistant to chemotherapeutic drugs. This resistance is due to the opposition to apoptosis and reprogramming proliferation, and survival pathways along the course of melanoma progression [8]. Therefore, it is necessary to develop new drugs, which can overcome the resistance against chemotherapy, and destroy the tumor cells.

Plant-derived secondary metabolites are relatively less toxic and possess lead compounds for modern drug development. In this context, *Ammi visnaga* L. is one of the most promising plants because of its chemical composition

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consisting of mainly furanochromone derivatives [9]. Furanochromones are well known oxygen-containing heterocyclic compounds which perform critical biological functions in nature. *Ammi visnaga* L. is an annual or perennial plant native to the Mediterranean flora, and is a species of flowering plant belongs to the Umbelliferae family. Its fruits have been used for centuries in Egyptian and Middle Eastern folk medicine as diuretic infusions, and against the kidney and bladder stones. In addition to its folk uses, it is currently utilized by pharmaceutical companies as a source of furanochromones (khellin, and visnagin). The active biological entities of *A. visnaga*, khellin, and visnagin have been widely employed for their vasodilating and antispasmodic activities [10]. Apart from this, several studies have also been conducted to evaluate the photo-biological properties of khellin and visnagin. Furanochromones (khellin, and visnagin) have an isomeric backbone with that of the psoralens exhibiting broad range of photo-biological activities [11]. The structural resemblance between furanochromones and psoralens (furanocoumarins) has led to many studies in which the photo-biological, photosensitizing, and phototoxic properties of khellin and visnagin were investigated [11–15].

The current study aims to evaluate the anticancer activity of visnagin against HT 144 cell lines for the possible treatment of melanoma. In this study, we determined the singlet oxygen ($^1O_2$) production capacity and the cytotoxic activity of visnagin against HT 144 cancerous and 3T3 mouse fibroblast cell lines. The apoptosis, the impact on the generation of reactive oxygen species (ROS), and the release of tumor necrosis factor-alpha (TNF)-α in an HT 144 human melanoma cell model *in vitro* was also investigated. This study on visnagin will lead to further studies against malignant melanoma.

Materials and methods

Plant material

Ammi visnaga L. was harvested in September 2014 near Hatay, Turkey, and a voucher specimen have been deposited in the Herbarium of the Faculty of Pharmacy of Istanbul University (Herbarium number: ISTE83916).

Isolation of visnagin

Visnagin was isolated from the fruits of *Ammi visnaga* L. by a rapid and easy technique. Briefly, the dry, ripe, and grounded fruits (1 kg) were extracted with 2 L of hot water for 48 h, and the extract was concentrated to 1/8 volume by rotary evaporator. It was then extracted many times with 150 mL *n*-hexane. The collected hexane fraction was dried by using Na_2SO_4, filtered, and the solvent was removed under reduced pressure to afford the crude product, which was subjected to silica gel 60 column chromatography. The elution with dichloromethane/ethyl acetate (9:1) yielded 1.1 g of visnagin (m.p. 142–143 °C) [9].

Determination of singlet oxygen

The assay media was prepared by passing oxygen from demineralize water for 2 h. Sequentially, 20 μL of the 2 mg/mL visnagin dissolved in methanol (final concentration 200 μg/mL), 20 μL 0.05 M phosphate buffer (pH 7.4), and 80 μL 25 μM RNO was added to 96-well plate, and then the reaction was initiated by 80 μL 10 mM imidazole. The 12 cm away placed wells were illuminated with a 150 W halogen lamp by putting above 6 mm plexiglass filters. The measurements were taken every 2 min at 440 nm and terminated after 24 min. The resulting absorbance was plotted versus time. Methanol was used as a control. Rose Bengal (32 μM), singlet oxygen inducer, was used as a standard [16]. Methanol, RNO, imidazole, and Rose Bengal were purchased from Sigma (Saint Louis, Missouri, USA).

Cell lines and culture conditions

HT 144 (human malign melanoma) (ATCC; Manassas, VA, USA), and 3T3 (normal mouse fibroblast) cell lines were provided by the Biobank of PCMD, ICCBS (International Center for Chemical and Biological Science, University of Karachi, Pakistan). HT 144 cells were cultured in McCoy's 5A (Gibco, ThermoFisherScientific, USA), and 3T3 cells were cultured in DMEM(Gibco, ThermoFisherScientific, USA), supplemented with 10% fetal bovine serum (PAA; PAA Laboratory GmbH, Austria). The cultures were incubated in a 5% CO_2 humidified atmosphere at 37 °C.

MTT cytotoxicity assay

The cytotoxic activity of the visnagin was tested by MTT assay [17]. HT 144 (7×10^4 cells/well), and 3T3 (6×10^4 cells/well) cells were seeded in 96-well culture plates and grown overnight. The cells were treated with visnagin at different concentrations (12.5, 25, 50, 100 μg/mL) for 24 h. After incubation, media were replaced with 50 μL MTT (0.2 mg/mL) and incubated in a 5% CO_2 incubator at 37 °C for 3 h. HT 144 cells were also treated with visible light (λ ≥ 400 nm) in illuminated assay, and the illumination cytotoxic activity of visnagin was also tested by MTT assay. In illumination cytotoxicity assay, the cells were exposed to visible light under a 6 mm plexiglass filter with a 150 W halogen lamp for 30 min. At the end of the incubation, the media were removed from 96-well plates, and the formazan crystals were dissolved with 100 μL DMSO. Doxorubicin was used as a positive control. Absorbances were

measured at 570 and 540 nm, respectively, by using Spectra Max spectrophotometer (Applied Biosystems, CA, USA).

ROS assay

HT 144 cells were seeded in 96-well black fluorescent cell culture treated plate at a density of 1×10^5 cells/mL and incubated for 24 h. HT 144 cell lines seeded on 96-well plate was pre-treated with the 2′,7′-dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe at 37 °C for 45 min. After removal of the probe, the cells were washed with 1X PBS and treated with 100 μg/mL of visnagin for 24 h. 0.5% H_2O_2 was used as a positive control. At the end of incubation, fluorescence measurements were made by using Spectra Max spectrophotometer (Applied Biosystems, CA, USA) at the wavelengths of 495 (excitation) and 520 nm (emission) [18, 19].

Apoptosis assay

Apoptosis analysis was performed by using the FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen, BD Biosciences, San Jose, CA, USA). HT 144 cell lines were seeded in a 24-well plate with a density of 5×10^4 cells/mL and incubated for 24 h. 100 μL of visnagin (100 μg/mL) was added to a final volume of 1000 μL. Incubated for 24 h at 37 °C. After incubation, 10×100 μL binding buffer were added. 1 μL propidium iodide (PI), and 2 μL FITC Annexin V were added and incubated for 15 min in the dark. As a positive control untreated cells and as a negative control DMSO was used. The data were analyzed by CellQuest Pro software, using a BD FACSCalibur instrument (BD Pharmingen, BD Biosciences, San Jose, CA, USA) [20].

Measurement of TNF-α secretion

The TNF-α concentration in the supernatant was determined by performing DuoSet ELISA Human TNF-α DY 210 (RandD Systems, Minneapolis, MN, USA). Briefly, HT 144 cells were seeded in a 48-well cell culture plate at a density of 2×10^6 cells/mL. At the end of incubation, 50 μL stop solution was added, and measured at 450 nm, using Spectra Max spectrophotometer (Molecular Devices, San Jose, CA, USA) [21]. Untreated cells were used as a positive control while doxorubicin in four concentrations as a negative control.

Statistical analyses

All data on singlet oxygen production, cytotoxicity, illumination cytotoxicity, ROS, apoptosis, and TNF-α secretion assays are the average of three independent experiments. The data were analyzed *via* One-way ANOVA and were expressed as mean $\pm$ SEM, *p*-values < 0.05 were considered significant.

Results and discussion

The decreasing in the absorbance indicates higher singlet oxygen production. Visnagin produced singlet oxygen by the RNO (*p*-Nitrosodimethylaniline) bleaching method. The singlet oxygen production of visnagin increased time dependently. After 24 min, the absorbance of visnagin was 0.404 ± 0.001 while the absorbance of Rose Bengal was almost 0.209 ± 0.001 ($p < 0.05$) (Fig. 1).

Doxorubicin has a broad-spectrum and widely used in the treatment of different types of cancer since the 1960s

Fig. 1 The singlet oxygen generation of visnagin and rose bengal by the RNO bleaching assay. Values are mean $\pm$ SEM, $n = 3$, $p < 0.05$
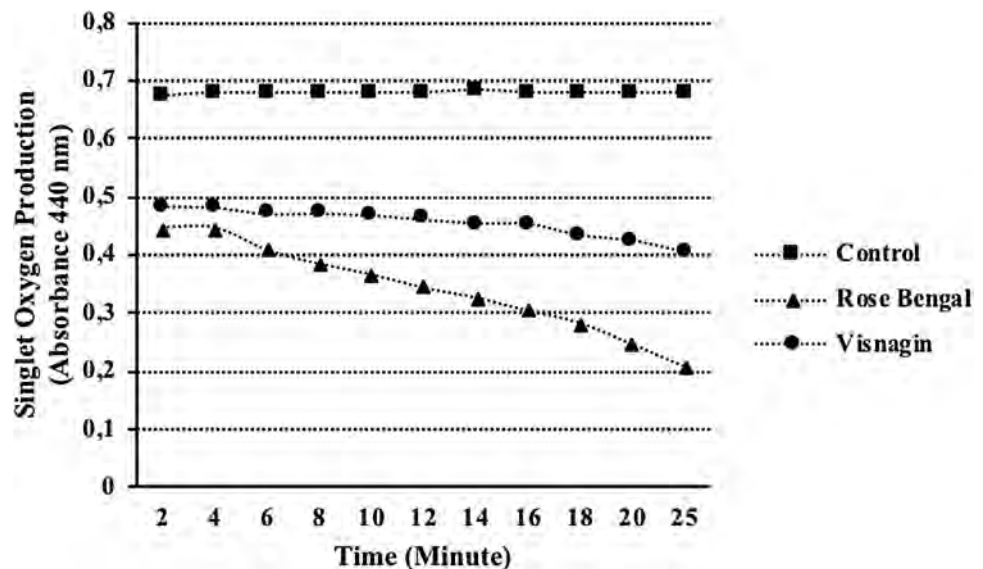
[22, 23]. We compared our results with doxorubicin, used as a positive control in this study. Visnagin showed cytotoxic activity against HT 144 (human malign melanoma) cell lines in dose-dependent manner by the standard MTT assay, and by the illuminated MTT assay. In standard MTT assay, it showed 68.09 ± 1.06, 76.46 ± 2.39, 82.07 ± 1.94, and $80.93 \pm 2.01\%$ ($p < 0.05$) inhibitory activity against HT-144 cancer cell line at 12.5, 25, 50, and 100 μg/mL concentrations, respectively. Visnagin was more potent to inhibit the growth of melanoma cells, depending on the concentration by the standard MTT assay. When visible light was applied, the inhibition values reduced to 36.52 ± 19.3, 45.96 ± 4.10, 52.86 ± 5.08 and, $63.19 \pm 2.94\%$ ($p < 0.05$) at 12.5, 25, 50, and 100 μg/mL concentrations, respectively (Fig. 2). It has been observed that the light exposure had no desirable effect on the cytotoxicity by increasing concentrations. The inhibition value of visnagin was 8.06% at 100 μg/ mL against 3T3 cell lines; therefore, found nontoxic.

A few studies have been conducted on the cytotoxic and antitumor activity of visnagin, and its derivatives. Sayed et al. [24] have found that visnagin isolated from the aerial parts of *Cyperus rotundus* L., exhibited potent cytotoxic activity against the L5178y (mouse lymphoma) cell line. It induces 94% inhibition at 3 μg/mL concentration. The cytotoxic activity of visnagin, isolated from *Cimicifugae rhizoma* (Ranunculaceae), against HL-60, MCF7, and A549 cell lines were also evaluated. Visnagin was found to exhibit inhibitory activity against HL 60 cell line with the IC_{50} value of 18.1 μM. It has been observed that the cytotoxic activity against other cell lines was weak [13]. The antitumor activity of some heteroaromatic benzofurans derived from visnagin against the HepG2 cancer cell line was compared with 5-fluorouracil and doxorubicin, known anticancer drugs. Some of the benzofuran derivatives exhibited a strong growth inhibition in a dose-dependent manner when compared to 5-fluorouracil, and doxorubicin [14]. Pakfetrat et al. [15] suggested that ethanolic extract
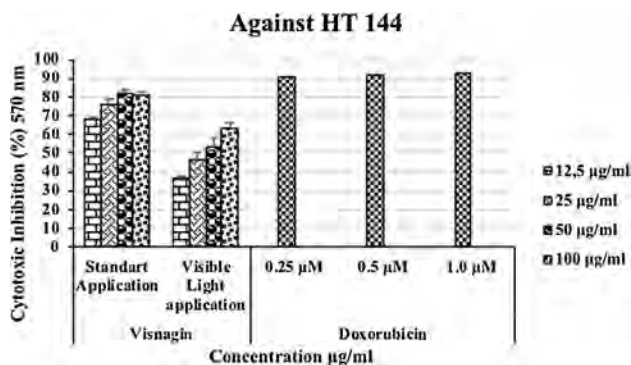
of *A. visnaga* has inhibitory effects on the cell growth of MCF-7 (human breast adenocarcinoma), and HeLa (human cervical cancer) cell lines. Visnagin isolated from *Ammi visnaga* was evaluated against HeLa, HepG2, HCT 116, and MCF-7 cell lines. The highest cytotoxic activity of visnagin exhibited activity against the HepG2 cell lines with the IC_{50} value of $10.9 + 0.68$ μg/mL. Moreover, it showed activity against HCT 116, MCF-7, and HeLa cancer cell lines with IC_{50} values of 12.3 ± 0.94, 13.7 ± 0.942, and 35.5 ± 1.2 μg/mL, respectively [25].

In the present study, the ROS formed in HT 144 cells was detected by using DCFH-DA (2′,7′-dichloro-dihydro-fluorescein diacetate). The fluorescence of DCFH-DA, which was oxidized by ROS to DCF (dichlorofluorescein), was measured at the excitation, and emission of 495/520 nm, respectively. Compared to H_2O_2, which was used as a standard, visnagin induced intracellular ROS production. Martelli et al. [11] reported that visnagin is a potent inducer of the intracellular reactive oxygen species (ROS) production, particularly for superoxide radicals and singlet oxygen.

The dual staining with FITC Annexin V/PI identified the percentage of apoptosis by employing flow cytometry technique. The percentage of apoptosis in the HT 144 cell lines treated with 100 μg/mL of visnagin was 25.88%. The visnagin induced 11.47% early apoptosis (An+/PI-) and induced 14.41% late apoptosis (An+/PI+), and 8.71% of cells were necrotic (An-/PI+) (Fig. 3). Effect of visnagin on TNF-α production was determined by Human TNF-α ELISA kit. We found that visnagin has no effect on TNF-α secretion (Fig. 4). Our data are in line with the previous studies that apoptosis is induced due to the increased production of ROS in the apoptotic pathway, where death receptors play an essential role [26–29].

Our data demonstrated that visnagin has an antiproliferative effect on HT 144 cells in a dose-dependent manner. Visible light treatment inhibited more than 50% of cells at concentrations of 50, and 100 μg/mL. Visnagin induced apoptosis due to the intracellular generation of ROS. However, it has no effect on TNF-α secretion. Thus, it can be said that other death receptors may induce the apoptosis.

Fig. 2 Cytotoxic activity of visnagin against HT 144 human malignant melanoma cell line by the standard and illuminated MTT assays. Values are mean $\pm$ SEM, $n = 3$, $p < 0.05$

Conclusions

Visnagin may be a potential lead for the treatment of melanoma. Our data suggest that visnagin can inhibit the proliferation of human malignant melanoma cells by the induction of the intracellular ROS production, and the activation of the proapoptotic pathway. However, further molecular studies are required to investigate its effectiveness as an anti-cancer agent of visnagin.
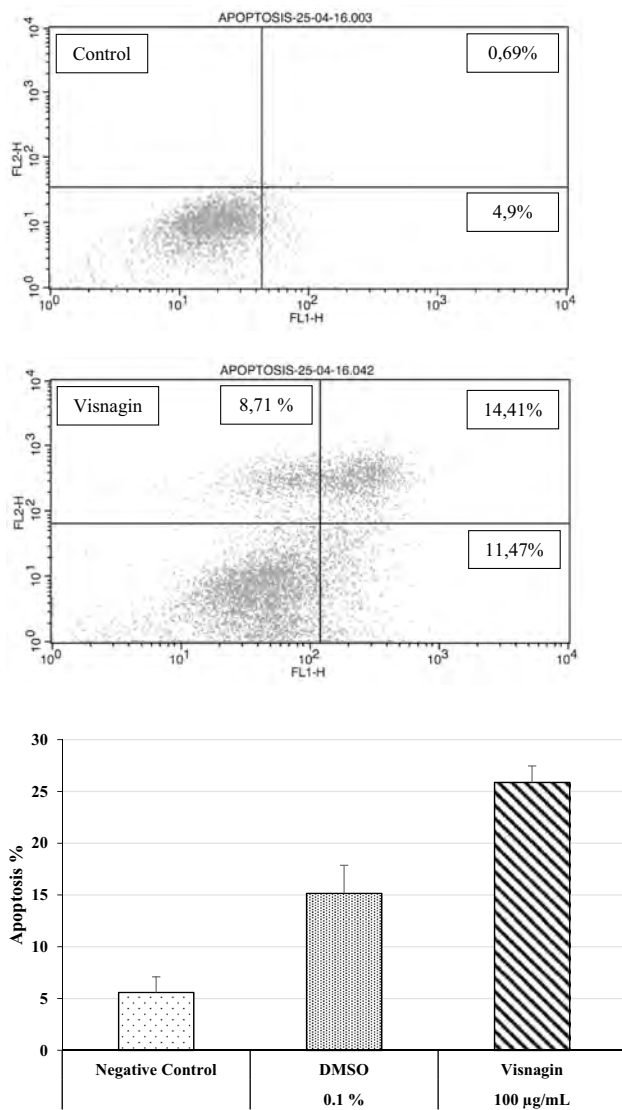
Fig. 3 Flow cytometry detection of apoptosis in HT 144 cell lines treated with visnagin
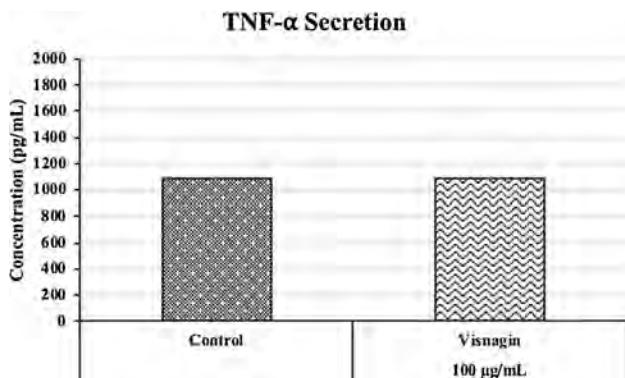
Fig. 4 TNF-α secretion (pg/ml) of visnagin. Values are mean ± SEM, $n = 3$, $p < 0.05$

Compliance with ethical standards

Conflict of interest The authors declare that is no conflicts of interest associated with this publication.

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