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Research article

Antimelanoma properties of cobra oil

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Abstract

Cobra oil is a mixture of fatty acids proven to be most effective against skin melanoma. The properties of cobra oil were investigated against skin melanoma cancer cells. The anti-melanoma properties of cobra oil were determined using inhibition migration, cytotoxicity, apoptosis cell death and mRNA expression of BRAF V600E on SK-MEL-28 cells following wound healing, lactase dehydrogenase assay using a Cytox 96 Non-Radioactive Cytotoxicity kit, apoptosis cell death using flow cytometry and the expression of BRAF V600E using quantitative real-time polymerase chain reaction, respectively. The results showed that cobra oil at 5%, 10% and 20% (volume per volume; v/v) inhibited the cell migration of SK-MEL-28 cells by 26.80%, 61.85% and 91.43%, respectively. Cobra oil at 2.5% (v/v) had the maximum activity (97.75%) for cytotoxicity. The morphological changes of SK-MEL-28 cells included cellular shrinkage and abnormalities in size and shape. Cobra oil at 5% and 10% (v/v) induced 17% and 25% apoptosis, respectively, while at 2.5%, the cobra oil had no effect to the cells. However, the mRNA expression of the BRAF V600E gene was not significantly decreased by 0.5% cobra oil (0.85-fold) compared with untreated cells (1-fold). In conclusion, cobra oil had pharmaceutical properties that affected skin melanoma cells. An expanded study of cobra oil should be considered for further investigation.

Introduction

Melanoma is the most aggressive form of skin cancer which results in numerous deaths each year (Senft et al., 2012; Bucheit et al., 2014). Skin melanoma is an original tumor which spreads to other parts of the body but with poor symptoms being indicated, it is difficult to detect (Hardy et al., 2010). Treatment of malignant melanoma has traditionally been by surgery or chemotherapy or both. However, efficacy is limited due to chemo resistance and toxicity issues and furthermore, melanoma cell showed rebound progression within 6–8 mth after treatment (Alcalá et al., 2012; Sullivan et al., 2013). Recently, new agents from natural products have been research in the process of finding specificity to cancer cells with no tumor regression and less damage to healthy cells.

Natural oil, extracted from many native crops, has been studied for pharmacological properties such as antimicrobial (Nissen et al., 2010), anti-inflammatory (Cardoso et al., 2011), wound healing (Süntar et al., 2011; Süntar et al., 2012) and also anti-cancer (Nikolakopoulou et al., 2013). The active components of natural oils were identified as polyunsaturated fatty acids (Lim et al., 2009; Mandal et al., 2012; Nikolakopoulou et al., 2013). Animal products including snake oils have been used as traditional remedies for skin problems, local tissue necrosis and wound assessments. The famous snake oil from the Chinese water snake is a rich source of eicosapentaenoic acid (omega-3 PUFA) which has potential for muscle, anti-inflammatory and conferred therapeutic benefits (Kunin, 1989). Fixed oil of *Boa constrictor* has anti-inflammatory and antimicrobial agents (Falodun et al., 2008) and produced a 70% reduction in the proliferation of keloid fibroblasts on human skin (Olaitan et al., 2011). In addition, cobra oil has been used as a traditional remedy for wound healing, muscle pain and local tissue necrosis from animal bites or insect

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stings in remote areas of Thailand. The cobra oil components contain a mixture of polyunsaturated fatty acids which act have antioxidant and cytotoxic activities on cancer cells (Khunsap et al., 2016). Therefore, the purpose of the current work was to investigate the pharmaceutical properties of cobra oil on melanoma cells. Cobra oil may have important properties to counter melanoma cancer.

Materials and methods

Lipids extraction

The fat sack was excised from a dead monocled cobra (*Naja kaouthia*) and stored at -20°C. Lipid from the snake's fat sack was extracted using a simple method. The fat sack was cut into small pieces and put on a Petri dish and incubated at 37°C until the cells had shrunk. The lipid was kept at -20°C until used.

Cell culture and reagents

Cells of human skin melanoma—SK-MEL-28 (ATCC® HTB-72™)—were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). SK-MEL-28 was cultured in Modification of Minimum Essential Medium (MEM) medium supplemented with 10% fetal bovine serum (FBS), 1 mM glutamine, 100 U/ml streptomycin and 100 U/ml penicillin. LDH enzyme was determined using a Cytox 96 Non-Radioactive Cytotoxicity kit (Promega, Madison, WI, USA). Apoptosis cell death was detected using an Annexin V Apoptosis Detection kit (Santa Cruz, CA, USA). Reagents in the experiments were all analytical grade.

Cytotoxicity analysis

The LDH assay was chosen for determining the cytotoxic activity. Briefly, 1×10^5 cells/mL of SK-MEL-28 were seeded in 96-well plates and incubated at 37°C with 5% CO_2 for 24 hr. The cells were treated with cobra oil (0.625%, 1.25%, 2.5% or 5% [volume per volume; v/v]) for 72 hr. A sample (50 µL) of the supernatant from each experiment was collected and mixed into the CytoTox96 reagent at a ratio of 1:1. The mixture was kept in the dark at room temperature for 30 min. After incubation, a stop reagent was added and then, the absorbance was measured at an optical density of 492 nm (OD_{492}). Untreated cells were lysed using the lysis buffer to obtain maximum cytotoxicity. The cytotoxicity was calculated as $100 \times OD_{492}$ (LDH of sample) / OD_{492} (LDH of positive). A well of the culture medium without cell was used as the negative control whereas untreated cells lysed with the lysis buffer to obtain maximum cytotoxicity was used as the positive control.

Migration inhibition

SK-MEL-28 cells were seeded in 24-well plates at 5×10^5 cells/mL concentration for 24 hr. After incubation, the cells were scratched and washed twice with a medium without FBS. The cells were treated with various concentrations of cobra oil (2.5%, 5%, 10% or 20%, v/v) for 24 hr. Migration inhibition was determined by measuring the distance of the edge scratching. The migration inhibition was calculated as: $100 - [(Z - Tn) / Z) \times 100]$, where Z is the negative distance at time 0 and Tn is the experimental distance at 24 hr.

Apoptosis analysis

SK-MEL-28 cells were seeded in 6-well plates and incubated at 37°C with 5% CO_2 for 24 hr. Various concentrations of cobra oil (0%, 2.5%, 5% or 10% v/v) were added and incubation was continued for 24 hr. Apoptotic cells were detected using the Annexin V Apoptosis Detection kit, with staining according to the manufacturer's instructions. Cells were acquired and analyzed using FACSCalibur flow cytometry and the CellQuest Pro software (Becton Dickinson; Franklin Lanes, NJ, USA).

mRNA expression of BRAF V600E using quantitative real-time polymerase chain reaction

Total RNA was extracted from the SK-MEL-28 cells which had been treated with 0.5% cobra oil for 24 hr. Untreated SK-MEL-28 cells were used as the negative control. A sample (1µg) of total RNA was used to generate cDNA using random hexamer primers. A sample (1µL) of 1:10 cDNA dilution was applied to the polymerase chain reactions (PCRs). The mRNA expression of BRAF V600E and GAPDH was investigated using the specific primers:

BRAF V600E: Forward 5'-AGATTCTCATCCGAAACC GCTCTA-3'
Reverse 5' -GTGGTGGTGAAGCTGTAGCC-3'
GAPDH: Forward 5'-ACCACAGTCCATGCCATC-3'
Reverse 5' -TCCACCACCCTGTTGCTG-3'

Quantitative real-time polymerase chain reaction was performed based on a power SYBR green PCR master mix. The reaction was performed under the following conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s, followed by melting curve analysis using the equation: $\Delta\Delta Ct = \Delta Ct$ (Target gene treated - Reference gene treated) - Δct (Target gene control - Reference gene control). The mRNA expression was calculated based on $2^{-(\Delta\Delta Ct)}$, where Ct is the threshold cycle.

Statistical analysis

Data were expressed as mean ± SE based on independent triplicates. The statistical significance of the results was analyzed using a t test facilitated by a software PRIMER in Biostatistics version 3.02 (The McGraw-Hill Companies, Inc, San Francisco, California, USA). Differences between the treated groups and the negative control were considered significant at $p < 0.05$.

Results and Discussion

Cytotoxicity analysis

Cobra oil at concentrations of 0.625%, 1.25%, 2.5% and 5% (v/v) induced cytotoxicity on the SK-MEL-28 cells by 14.96%±1.4%, 92.88%±5.44%, 97.75%±10.51% and 82.31%±9.03%, respectively. The cytotoxicity levels of the untreated cells (the cells without oil) and positive controls were 6.27%±0.96% and 100.04%±0.05%, respectively (Fig. 1). The morphological changes of the cells caused by cobra oil were clearly visible under microscopic examination (Fig. 2) whereby cobra oil caused cell shrinkage in a dose-dependent manner.

Apoptosis analysis

Cobra oil at 5% and 10% (v/v) could induce 17% and 25% apoptosis, respectively, while 2.5% did not affect the cells (Fig. 3).

Migration inhibition analysis

The results showed that cobra oil at 5%, 10% and 20% (v/v) inhibited migration of SK-MEL-28 cell by 26.80%±6.7%, 61.85%± 0.61%% and 91.43% ±6.64%, respectively. Meanwhile, 0% and 2.5% cobra oils could not inhibit migration of the cells for 24 hr (Fig. 4).

Analysis of mRNA expression of BRAF V600E

Cobra oil at 0.5% was able to slightly decrease mRNA expression of BRAF V600E on SK-MEL-28 cells after 24 hr. However, there was no significant difference to the untreated cells under the same conditions (Fig. 5).

Cobra oil has plenty of saturated and unsaturated fatty acids, especially vaccenic acid, palmitic acid and linoleic acid, which act as cytotoxic acid (GLA) and α-linolenic acid (ALA) that can kill tumors both *in vitro* and *in vivo* (Scheim, 2009; Murray et al., 2015). Cobra oil mechanisms had not been studied before the current work. Cobra oil might possibly have the same action as other essential oils. Fatty acids could pass through the cell wall and cytoplasmic membrane to disrupt the polysaccharides, fatty acids and phospholipid of structural layers. Such disruptions have led to the leakage of macromolecules and lysis, causing cell injury and death (Bakkali et al., 2009). A morphological change in the SK-MEL-28 cells was observed following cobra oil induction as shown in Fig. 2. This change involved cellular shrinkage, reduced cell viability and size, and a shape change which were similar to the bladder carcinoma J82 apoptosis cell death following frankincense oil stimulation (Frank et al., 2009). Shrinkages in apoptotic cell size and shape are typically involved in cell death in an apoptotic series. At 24 hr, the highest amounts of apoptotic cells (25%) of SK-MEL-28 cells was slightly induced by 0.5% cobra oil.

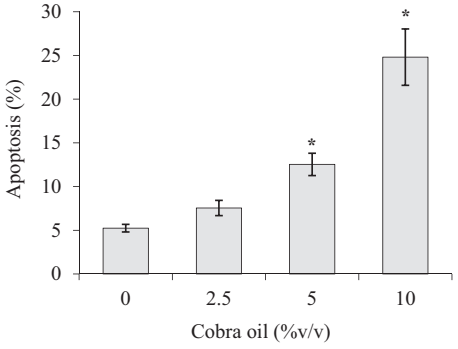

Fig. 3 Apoptosis of SK-MEL-28 cells induced by cobra oil (% volume per volume; v/v) after 24 hr, where * indicates significant difference between each treated group and the control (0% v/v) at $p < 0.05$ and error bars indicate SE.

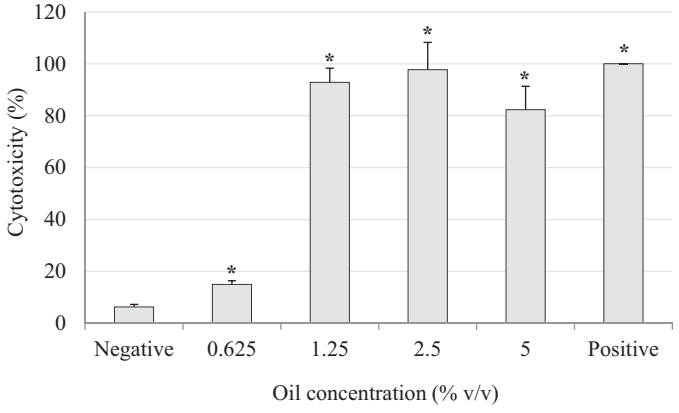

Fig. 1 Cytotoxicity activity of skin melanoma cells induced by various cobra oil concentrations (% volume per volume; v/v) after 72 hr, where * indicates significance at $p < 0.05$ and error bars indicate SE

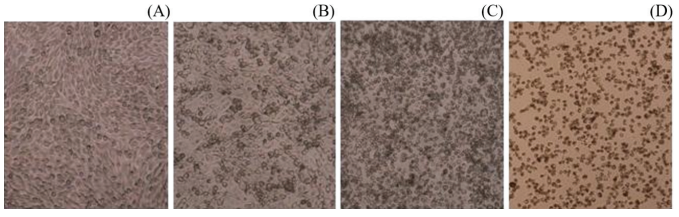

Fig. 2 Morphological changes in human skin melanoma cells following cobra oil stimulation after 72 hr under 10× inverted microscope: (A) untreated cells; (B) cell shrinkage caused by the lowest cobra oil concentration (0.625% v/v); (C and D) the shrinkage of the treated cells increased with concentrations of cobra oil.

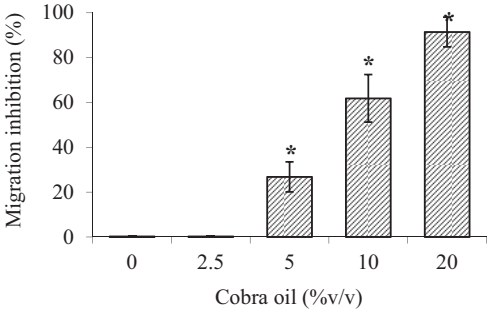

Fig. 4 Migration inhibition of SK-MEL-28 cells by cobra oil (% volume per volume; v/v) after 24 hr, where * indicates difference between each treated group and the control (0% v/v) at $p < 0.05$ and error bars indicate SE.

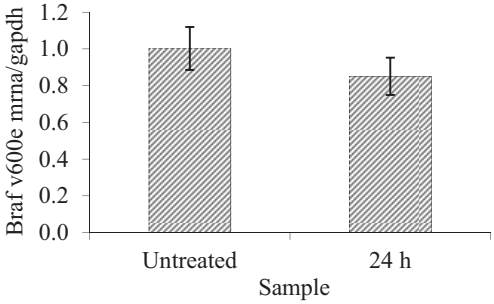

Fig. 5 mRNA expression of BRAF V600E gene on SK-MEL-28 cells treated with 0.5 cobra oil (volume per volume) for 24 hr compared to untreated cells, where error bars indicate SE.

However, the Annexin V-FITC kit is a tool for detecting the early phase of apoptosis. Cobra oil might also induce the other phases of apoptosis and other programs of cell death. Further study should be considered on other cell death programs caused by cobra oil. In addition, this work was extended to study the migration inhibition of cobra oil on SK-MEL-28 cells. Cobra oil at 20% exhibited produced than 90% inhibition migration on SK-MEL-28 cells, similar to the effect of snake venom (Khunsap et al., 2011; Khunsap et al 2016). Lastly, mRNA expression of the BRAF V600E gene on SK-MEL-28 cells was slightly decreased by 0.5% cobra oil for 24 hr compared with untreated cells. However, there was no significant mRNA expression of the genes at 24 hr. Melanoma cancer has been characterized by the BRAF V600E mutation gene (Maddodi et al., 2010). This gene was reported to promote cancer cell survival and proliferation in most malignant melanomas (Senft et al., 2012; Wang et al., 2013). However, BRAF V600E has a complicated function and is related to multi-genes. Thus, the related genes should be considered for further study. Cobra oil induced apoptosis and mRNA expression of the BRAF V600E gene on SK-MEL-28 cells in a mild manner. Nonetheless, it promoted extreme inhibited migration and induced cytotoxicity in SK-MEL-28 cells which are important anticancer properties. Cobra oil could be a good candidate as a potential alternative anti-cancer agent against melanoma.

Conflict of Interest

The authors declare that there are no conflicts of interests.

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