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Original article

Different Cytotoxic Effects of Caper from Different Geographical Regions May Be Related to Changes in Mitochondrial Sirt3

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Abstract

Background and objectives: Beside its nutritional role, caper (*Capparis spinosa)* has long been used as an analgesic, anti-inflammatory, anti-diabetic and anti-cancer remedy. In the present study, we tested whether this plant can make effective changes in Sirt3 and mitochondrial function in colorectal carcinoma cell line since mitochondrial dysfunction has long been implicated in both cancer and diabetes and benefit confers by caper in these diseases might be due to mitochondrial alterations. **Methods**: Total flavonoids and phenolics were assayed using colorimetric tests. Cytotoxicity of a phenolic-flavonoid rich extract of caper collected from two different geographical regions (south and west) were mechanistically studied in HT-29 cell line. Activity of an essential mitochondrial enzyme, Sirt3 has also been evaluated along with other parameters. IC_{50} of extracts were determined by MTT cytotoxicity assay, cell death and mitochondrial membrane potential were evaluated via flow cytometric analysis. Also, at IC_{50} concentrations, Sirt3 activity was determined fluorimetrically. **Results:** The results showed that caper induced significant cytotoxicity in HT-29 cells followed by mitochondrial membrane potential collapse, ROS overproduction, Sirt3 activity alteration and cell death. **Conclusions:** The above-mentioned cytotoxic parameters were inversely proportional to the phenolic and flavonoid contents of the extract showing that other mechanisms beyond their antioxidant capacities may contribute to their anti-cancer effects. In other term, these results suggest that antioxidant capacity may not directly contribute to the anticancer property.

Keywords: apoptosis; *Capparis spinosa*; mitochondria; ROS; Sirt3

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Introduction

Over the past two decades, numerous researches have been carried out about the therapeutic properties of plants used in traditional medicine [1]. Various plants have been experimentally used as anti-diabetes and anti-cancer in traditional medicine. Currently, cancer and diabetes complications despite improved therapeutic approaches are leading causes of death worldwide [2,3]; therefore, discovering new pathways and new therapeutic agents is

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crucial to developing better approaches for treatment of these diseases.

Based on various studies, disruption of mitochondrial activity is involved in a variety of diseases including diabetes [4-6] and cancer [7-9]. Determining the factors that have the potential to modify the function of mitochondria in cancer cells seems to be a key step in the treatment of the disease. Mitochondria contain large numbers of key molecules that regulate cell survival, death, and metabolic pathways [10,11]. Sirt3 is one of the critical enzymes for maintaining mitochondrial integrity and function [12,13]. Sirt3 is among the most important regulatory mitochondrial proteins that have a role in aging [14], resistance to stress[15,16] and reactive metabolites [16] and its activity is specifically associated with mitochondrial normal functions [17,18]. Sirt3 is significantly expressed in tissues with high metabolism rate including kidney, liver, heart, brain and brown adipose tissue [19]. It has been suggested that this protein is involved in energy homeostasis, regulation of ATP generation and mitochondrial function as well as oxidative stress; furthermore, sirt3 plays an important role in aging and cell damage [17]. Studies have indicated that sirt3 levels in skeletal muscle of diabetic rats (type 1 and 2) have been reduced by 50%, indicating it may play a role in the metabolic disturbances in diabetes. Decreased expression of Sirt3 has been associated with mitochondrial metabolism alteration, over production of ROS and impaired insulin production or secretion.

The interplay between Sirt3 and ROS in different pathological and physiological states might be associated with HIF1-α and SOD, both of which are involved in tumor formation [17]. Activity of Sirt3 decreases HIF-1α and ROS formation, the two major players in the development of tumors [20-23]. Different studies have shown that genetic deletion of the mitochondrial Sirt3 deacetylase leads to increased mitochondrial superoxide, resulting in tumor development [24].

Studies have shown that cancer cells obtain their energy requirements from aerobic glycolysis and this will pass over mitochondrial ATP generation [21]. As a result, the main intracellular source for ROS generation will be turned off or attenuated. As mentioned above, decrease in Sirt3 activity was linked to ROS over production and it seems that ROS alteration through Sirt3 manipulation can be one of the important mechanisms for effective medications against diabetes and cancer. So we searched for a special herbal medicine that could be effective against both diseases. Apparently, plants that have been traditionally used in both conditions could be good candidates for our study. Consequently, *Capparis spinosa* L. (caper) from two different geographical regions (south and west) of Iran was selected. It has been shown that the leaves and flowers of caper are useful against HT-29 colon carcinoma cell line [25] and also its fruit extract has reduced blood glucose and lipid levels in diabetes types 1 and 2 [26,27]. In the present study, we examined whether this plant could make effective changes in Sirt3 and mitochondrial activity in colorectal carcinoma cell line.

Material and methods

Chemicals and reagents

Cell culture materials including Dulbecco's Modified Eagle Medium (DMEM) were obtained from Applichem (Germany); penicillin-streptomycin, trypsin, and fetal bovine serum were obtained from Gibco (USA); dimethyl sulfoxide (DMSO) and PBS were obtained from Sigma Chemicals (Darmstadt, Germany); HT-29 cell line was obtained from Pasture Institute (Tehran, Iran); MitoLight and apoptosis detection kit were purchased from Millipore Co. (USA). Mitochondrial purification kit was purchased from Sigma (USA) and Sirt3 assay kit was provided from BPS Bioscience (USA).

Extraction

Wild-grown *Capparis spinosa* was collected from two different geographical regions (south: Ahvaz and west: Kermanshah) of Iran during spring 2016 and dried in a shaded place at room temperature. The air-dried plant aerial parts were milled and used for preparation of hydroalcoholic extract. The plant powder (40 g) was mixed and macerated with methanol (90% v/v) and kept for 72 h. The extract was then filtered and the solvent was partially removed in a vacuum evaporator so that the final volume reduced to one-third. Then, the concentrated extract was freeze-dried and kept in a cool and dry place until testing.

Total flavonoids assay

Total flavonoids were assayed using aluminum chloride colorimetric test [28]. Extract samples were mixed with 1.5 mL of methanol, 0.1 mL of aluminum chloride (10%), 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water

and then kept at room temperature for 30 min. Absorbance of the reaction mixture was recorded at 415 nm with a double beam UV-VIS spectrophotometer (T-90, PG Instrument, England). Calibration curve was attained using quercetin at concentrations of 12.5 to 100 µg/mL in methanol. All Experiments were made in at least triplicates and results were presented as mean ± SEM.

Total phenolics assay

Total phenolics content of the extract was determined using a previously reported method [29]. Appropriate dilutions were oxidized by 2.5 mL of 10% Folin-Ciocalteu reagent (v/v) and neutralized by 2.0 mL of 7.5% sodium carbonate. All mixtures were incubated at 45 °C for 40 min and their absorbances were measured at 765 nm spectrophotometrically. The total phenolics content was then determined using gallic acid as a reference standard.

Cell culture

HT-29 human adenocarcinoma cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal Bovine serum (FBS), 100 U/mL penicillin and 0.1 g/L streptomycin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. They were seeded at a concentration of 1×10^6 in flasks and the viability of cells was determined by using trypan blue staining. The culture medium was replaced at least every two days for all experiments. There were no significant differences between vehicle received and other control groups and the results of MTT assay did not show any toxic effect of vehicle at exposed levels (data not shown). Final concentration of DMSO upon serial dilution (1:3000) contained extremely low levels of DMSO that were far below the acceptable concentration (0.5% v/v) reported to be nontoxic.

Cell proliferation assay (MTT)

The cells were plated in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. After 24 h, once the cells reached 85% confluency, they were treated with different concentrations of the extracts so that 100 µL of the extract containing 1, 5 and 10 µg/mL was added to each well for 24, 48, and 72 h. Subsequently, the supernatant was removed very gently and the cells were incubated with fresh medium containing 0.5 mg/mL MTT-tetrazolium salt 37 ºC for 3.5. Then, the formazan crystals were dissolved by adding 150 µL dimethylsulphoxide (DMSO). Reduced MTT was measured spectrophotometrically by Sunrise Absorbance Reader (USA) at 590 nm with a reference filter of 620 nm. Viability inhibition was calculated as follows:

$$\text{Cell viability (\%)} = \text{Ab (test)} / \text{Ab (control)} \times 100$$

Ab (test) was the test absorbance; Ab (control) was the control absorbance

Apoptosis and necrosis evaluation

HT-29 cells (1.5×10^5) of were seeded and treated with different concentrations of extract for 72 h (obtained from IC_{50}). The cells were then harvested, centrifuged (1200×g, 8 min), washed with ice-cold PBS and stained according to ApopNexin™ FITC apoptosis detection kit instructions for 15 min at room temperature in the dark. The samples were kept on ice before being subjected to flow cytometric analysis. Stained samples were analyzed by Galaxy flow cytometer and 5000 events were measured.

ΔΨm measurement

Mitochondrial membrane potential changes were assayed by MitoLight® mitochondrial apoptosis detection kit (MILLIPORE Company, Germany). In intact cells, MitoLight® accumulates in the mitochondria and produces a red fluorescence. When mitochondrial membrane potential declines during apoptosis, the dye aggregates outside the mitochondria in cytosol and yields a green fluorescence. Briefly, the cells were exposed to extracts (72 h at IC_{50} concentrations), trypsinized, centrifuged (500×g) and then incubated with pre-diluted florescent dye solution for 15 min at 37 °C according to the manufacturer's instructions. All samples were analyzed using FACS Calibure (Becton Dickinson, USA) flow cytometry.

ROS measurement

Cells (10^5) were seeded in 24 well plates and allowed to adhere overnight. Extracts at IC_{50} concentrations were added and incubated for 6 or 12 h. After that, the cells were washed with PBS afterward trypsinized (250 µL of trypsin /well) and complete culture medium (10% FBS) was added to the cells and centrifugation (5 min at 200 g) was conducted at room temperature. The supernatant was removed (2 runs). The cell pellet was resuspended with H2DCFDA (10 µM). The

cells were incubated in the dark for 45 min. Fluorescence intensity (excitation= 485 nm; emission= 530 nm) was measured by FACS Calibure (Becton Dickinson, USA) flow cytometry. H_2O_2 (250 µM) was used as the positive control.

Isolation of mitochondria

Sigma's mitochondria purification kit was used for isolation of an enriched mitochondrial fraction from the cells. Most of the isolated mitochondria would contain intact inner and outer membranes. Briefly, 2×10^7 cells were harvested and washed with cold PBS. The cells were exposed to the lysis buffer on ice for 5 min. The extraction buffer was added to the contents and centrifuged at 600 g for 10 min at 4 °C. The supernatant was then centrifuged at 11000 g for 10 min at 4°C. Upon addition of protease inhibitor, for further purification of mitochondria Percoll was used. Mitochondrial pellet was kept in the storage buffer containing 15% (v/v) Percoll. After centrifugation, mitochondria that banded at the lowest interface were harvested and diluted with ice-cold storage buffer. Then mitochondria were centrifuged at ~17,000 g for 10 min at 2-8 °C. The supernatant was removed and the pellets were suspended in the storage buffer at a concentration of 1-5 mg-protein/mL.

Sirt3 assay

The fluorogenic Sirt3 assay kit was used to determine the Sirt3 activity. The HDAC fluorometric substrate (HDAC substrate 1) is incubated with purified Sirt3 enzyme. The deacetylation sensitizes the substrate so subsequent treatment with the lysine developer produces a fluorophore that can then be measured using a fluorescence reader. In this assay, all tubes except the blank contained Sirt3 enzyme, HDAC substrate, BSA, NAD^+ and HDAC assay buffer and the negative control also had nicotine amide (enzyme inhibitor). Test tubes in addition to the above mentioned materials (HDAC assay buffer, NAD+ and HDAC substrate) also contained the extracts. Fluorescence Microplate Reader (BioTek FLX800, United States) was used to read the samples fluorescence intensity (EX: 350-380nm and EM: 440-460nm).

Statistical analyses

All experiments were performed in at least 3 replicates. The results have been shown as Mean±SEM and Student's t-test was used to determine statistical significance ($p<0.05$) between two groups. Comparisons between multiple groups were made by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for more than two groups. Differences were considered significant when $p<0.05$.

Results and Discussion

As shown in table 1 total phenolic and flavonoid contents of extracts were much higher for caper from west (Ksh) compared to the south (Ahz) caper. Variations in flavonoid content was previously reported for some other plants due to inferred geographical differentiation [30].

Table 1. Total phenolics and flavonoids contents of hydroalcoholic extract of *Capparis spinosa*

Sample	Total phenolics (mg GAE/100g extract)	Total flavonoids (mg QE/100g extract)
CP Ahz	609.85 ± 0.78	188.75 ± 0.27
CP Ksh	1113.37 ± 0.5	473.05 ± 0.29

Values were expressed as mean±SD; CP Ksh: *Capparis spinosa* from west; CP Ahz: *Capparis spinosa* from south; GAE: Gallic acid equivalent; QE: quercetin equivalent

In this study, MTT assay was used for evaluating the effect of caper methanol extract on the proliferation of HT-29 cells and determination of IC_{50}. The anti-proliferative effect was evaluated after 24, 48 and 72 h of treatment.

The results showed that IC_{50} was 1.3 µg/mL (72 h) and 1.7µg/mL for west and south *Capparis spinosa* extract in HT-29 cells, respectively. Influence of essential oil and aqueous infusion from wild-grown caper on HT-29 cell growth were studied previously [31] and the maximum cytotoxic effect of caper extract was reported to be 0.1 mg/mL. The difference may be due to the type of extract and different geographical region. Also, the MTT results obtained from our research showed that extracts of these plants had different effects. West (Ksh) *Capparis spinosa* exhibited higher cytotoxicity on cells after 24, 48 and 72 h exposure. Basically, our results were consistent with previous studies. The study of 15 types of *Capparis spinosa* based on morphological features showed that there presented different characteristics in different regions [32].

Another important issue for mitochondria during cell death is the ROS over production. In this study the level of ROS was significantly elevated in both *Capparis spinosa* extract treatments. The level of ROS production by caper methanol extract has been given in figure 1. There was a significant difference between level of ROS by *Capparis spinosa* extracts and the negative

control. The ROS formation was increased in 6 and 12 h and was significantly higher than the positive control. South *Capparis spinosa* in 6 h led to higher level of ROS, but in 12 h the level of ROS production was greater in the west *Capparis spinosa* ($p<0.05$). In figure 1, time-dependent increase in levels of ROS has been shown. Again, extracts dissimilarly affect the ROS formation in a way that the south *Capparis spinosa* increased the levels of ROS more considerably.

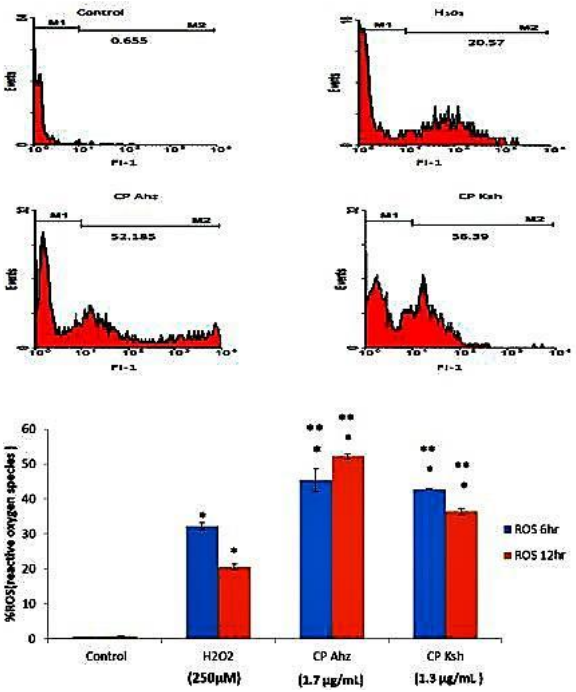

Figure 1. ROS generated in HT-29cells when exposed to IC_{50} concentrations of methanolic extract of CP Ahz (1.7 µg/mL) and CP Ksh (1.3 µg/mL) for 6 and 12 h. CP Ksh: *Capparis spinosa* from Kermanshah; CP Ahz*: Capparis spinosa* from Ahvaz; *significant difference in comparison with negative control group ($p<0.05$); *significant difference in comparison with positive control group ($p<0.05$)

In order to study the influence of *Capparis spinosa* extract on mitochondrial membrane potential of HT-29 cells, MitoLight florescent dye and flow cytometry analysis was used. As shown in figure 2, both *Capparis spinosa* extracts led to loss of mitochondrial membrane potential and there was significant difference between them and the negative control. Also, south caper caused significantly higher loss of mitochondrial membrane potential than the west caper ($p<0.05$). As shown in figure 3, the results revealed that treatment with caper extract induced necrotic cell death in HT-29 cell lines.

PI^+ /Annexin V^- HT29 cells had a higher percentage in caper extract than negative control, but there was no significant difference between PI^- / Annexin V^+ and the control group. Percentage of cell death induced by south extract was significantly higher ($p<0.05$). Treatment with *Capparis spinosa* extract induced necrotic cell death in HT-29 cell line. Caper extract did not show any effect on apoptosis in HT-29 cell line. In agreement with our results, no apoptotic effect was detected for caper essential oil and aqueous infusion in HT-29 cancer cells [31].

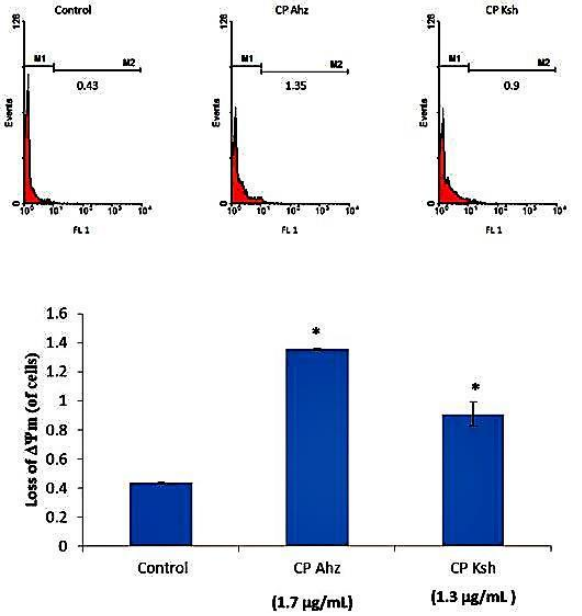

Figure 2. Loss of mitochondrial membrane potential inHT-29 cellsexposed to IC_{50} concentrations of the methanolic extract of CP Ahz (1.7 µg/mL) and CP Ksh (1.3 µg/mL) using MitoLight_florescent dye. CP Ksh: *Capparis spinosa* from Kermanshah; CP Ahz: *Capparis spinosa* from Ahvaz; *significant difference in comparison with control group ($p<0.05$)

In contrast, findings of Lei Yu showed apoptotic effects of *Capparis spinosa* on SGC7901 cell line [33]. We then compared the necrotic cell death produced by south extract with that induced by west extract; all experiments were performed in triplicate. The results were significantly higher for the south (16%) rather than the west extract (4%).

Cell death in HT-29 cells exposed to the caper extract may be due to mitochondrial dysfunction and changes in mitochondrial membrane potential. In this study, caper extract caused loss of mitochondrial membrane potential in HT-29 cells. Loss of $\Delta\psi m$ is considered a key event in the control of apoptosis and necrosis. Reportedly, mitochondrial membrane potential had been severely reduced during cell death [34]. Also, these results suggest that the mechanism of cell death in HT-29 cells may be related to

intracellular ROS generation.

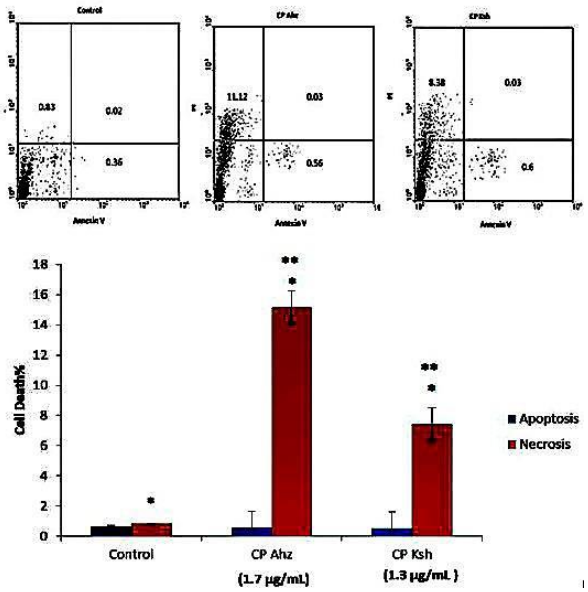

Figure 3. Induction of apoptosis and necrosis by IC_{50} concentrations of the methanol extract of CP Ahz (1.7 µg/mL) and CP Ksh (1.3 µg/mL) in HT-29 cells. In the Dot plot view, early apoptotic cells labeled with annexin V through phosphatidyl serine externalization over cell membrane (lower right quadrant) and necrosis was labeled with PI in the upper left quadrant. CP K: *Capparis spinosa* from Kermanshah; CP Ahz: *Capparis spinosa* from Ahvaz; *significant difference in comparison with control groups ($p<0.05$); **significant difference in comparison with test groups ($p<0.05$)

To evaluate the changes in Sirt3 activity, HT-29 cell mitochondria was isolated and the proteins were purified. The fluorogenic Sirt3 assay kit and HDAC fluorometric substrate and nicotine amide (enzyme inhibitor) negative control were used to determine the Sirt3 activity. Fluorescence measurement showed that south caper extract in contrast to west caper had an increasing effect on purified Sirt3 activity compared to the negative control (figure 4). Decreased expression of Sirt3 was associated with mitochondrial metabolism alteration, over production of ROS and impaired insulin production or secretion. The interplay between Sirt3 and ROS in different pathological and physiological states might be associated with HIF1-α and SOD, both of which are involved in tumor formation [17]. As a controller of cell growth and tumor suppressor, Sirt3 suppresses reactive oxygen species (ROS) and HIF-1α, the two factors that play major roles in the development of tumors [19-22]. Different studies have shown that genetic deletion of the mitochondrial Sirt3 deacetylase leads to increased mitochondrial superoxide, resulting in tumor development [23].

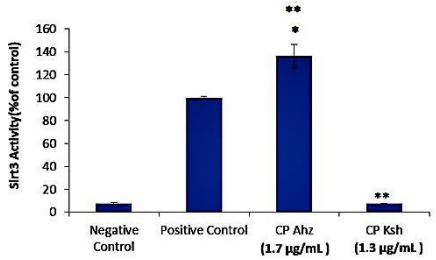

Figure 4. Activityof Sirt3 in HT-29andnormal cells during exposure to the methanol extract of CP Ahz and CP Ksh. Negative control: beside other reagents contains an inhibitor (Nicotinamide). CP Ksh: *Capparis spinosa* from Kermanshah; CP Ahz: *Capparis spinosa* from Ahvaz; *significant difference in comparison with Negative control group ($p<0.05$); *significant difference in comparison with positive control group ($p<0.05$)

Increased levels of ROS can be associated with Sirt3 activity. Many metabolic processes, such as oxidative phosphorylation, fatty-acid oxidation, and the TCA cycle are dependent to deacetylation by Sirt3 [17,35]. Sirt3 deacetylates some important molecules in electron transport chain and also deacetylates and modulates several enzymes of the TCA cycle that contribute to ROS production [36-38]. Sirt3 influences the mitochondrial metabolic processes in various ways so that the changes in Sirt3 activity can be followed as a therapeutic target for cancer [39]. Sirt3 activity is increased or decreased in various cancers depending on the cell type and may participate as tumor suppressor or promoter [40,41]. For example, high levels of Sirt3 have been detected in several human oral cancer cells, fibrosarcoma, cervical cancer, and bladder cancer. Sirt3 acts as a tumor promoter in these cell lines. In contrast, there are decreased levels of Sirt3 in breast cancer, glioblastoma, prostate, head, and neck cancer. Based on previous studies, Sirt3 is also reduced in human colon carcinoma [40,42,43]. Bell et al. have demonstrated a tumor suppressor role for Sirt3 in human colon carcinoma cells. Thus increasing the activity of Sirt3 in these cell lines can be a suppressing factor for tumorigenesis [20,40].

Studies have have indicated that Sirt3 levels in skeletal muscle of diabetic rats (type 1 and 2) have been reduced by 50% and so, may be involved in the metabolic disturbances in diabetes. It has been shown that caper fruit extract reduced blood glucose and lipid levels in diabetes types 1 and 2 [27,44]; besides, the leaves and flowers were effective against HT-29 colon carcinoma cell line [31].

In conclusion, our findings suggest that the traditionally and experimentally established effectiveness of caper extract on cancer and diabetes might be due to the Sirt3 activity modifications (figure 5).

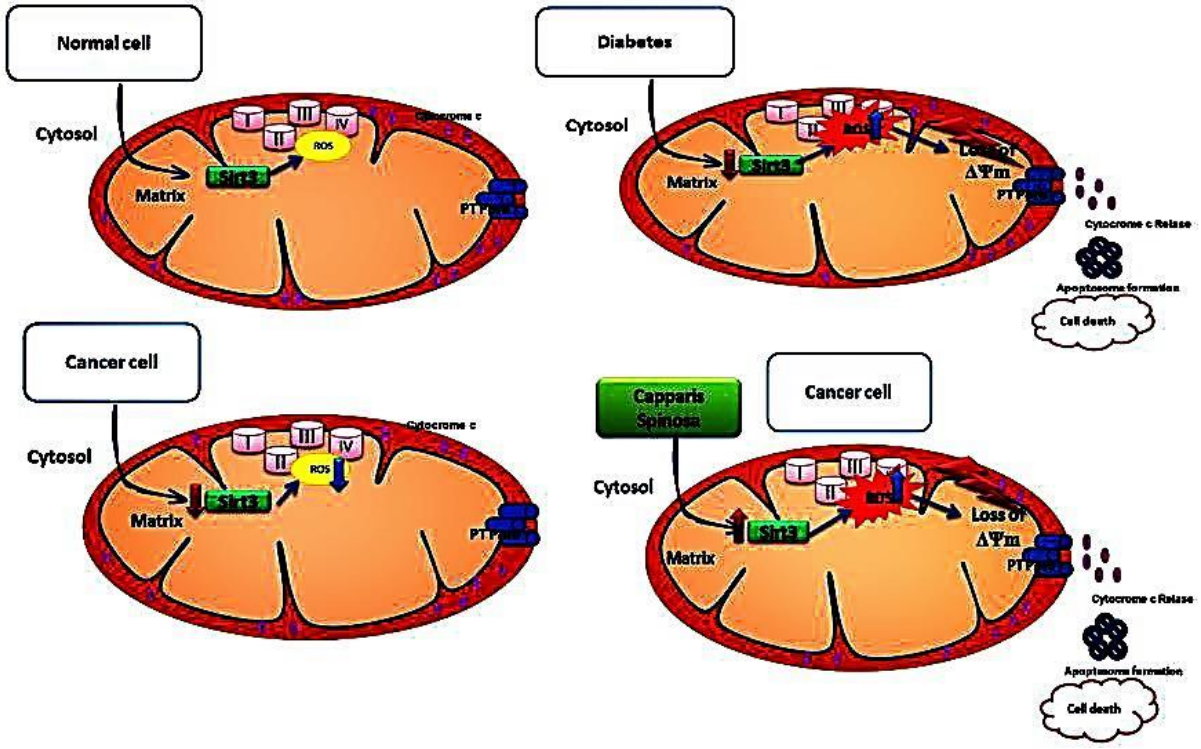

Figure 5. By regulating Sirt3 activity in cancer cells, caper induces mitochondrial ROS over production and opening permeability transition pore followed by cell death.

Acknowledgments

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Author contributions

Mohsen Rezaei designed the study and critically reviewed the paper; Forouzan Khodaei conducted the experiments and prepared the early manuscript; Mahmood Reza Moein determined phenolic compound; Amir Siahpoosh supervised the identification and extraction of the plants; Marzieh Jafari prepared the discussion questions Mahmoud Hashemitabar critically reviewed the experiments.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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Abbreviations

ROS: reactive oxygen species; Sirt3: silent mating type information regulation 2 homolog 3; SOD: superoxide dismutase; HIF-1α: hypoxia-inducible factor 1-alpha; MTT: dimethyl thiazolyl diphenyl tetrazolium bromide