RESEARCH ARTICLE

Mitochondrial Effects of *Teucrium Polium* and *Prosopis Farcta* Extracts in Colorectal Cancer Cells

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Abstract

   Background: *Teucrium Polium* and *Prosopis Farcta* have been traditionally employed in cancer treatment. In this study we evaluated the effects of methanolic extracts of these two plants in HT-29 cells. **Methods:** IC50s of extracts were obtained via MTT assay and the levels of ROS production, cell death, collapse of mitochondrial membrane potential and Sirt3 enzyme activity were determined. **Results:** After 48 hours exposure, IC50s for *Teucrium* and *Prosopis* extracts were 3 and 2µg/ml, respectively. Extracts induced higher ROS production after 6 hours than after 12 hours. Mitochondrial membrane potential collapse and cell death rate were also increased; *Teucrium* caused greater cell death than *Prosopis*. Extracts from both plants increased Sirt3 activity in its normal form, but only *Teucrium* extract caused a significant increase in activity of Sirt3 enzyme isolated from cancer cells. **Conclusion:** *Teucrium* and *Prosopis* extracts exert anticancer activity via mitochondrial alterations, as exemplified by increased ROS levels, Sirt3 activity and cell death in HT-29 colorectal cancer cells.

   Keywords: *Teucrium polium*- *prosopis farcta*- Sirt3- ROS- apoptosis

Introduction

   Mitochondrial dysfunction plays an important role in the diabetes (Qi et al., 2016), aging (Pinto et al., 2016), neurological disorders (Raefsky and Mattson, 2016) and cancer (Huang et al., 2010b; Hanselmann and Welter, 2016; Shagieva et al., 2016). Sirtuins are family of protein deacetylases with regulating role on the mitochondrial function (George and Ahmad, 2016). Sirt3 is a member of Sirtuin family localized in the mitochondria and is among the most important regulatory mitochondrial proteins. It has been linked to aging, resistance to stress and reactive metabolites and its appropriate activity is specifically associated with the mitochondrial normal functions (Kincaid and Bossy-Wetzel, 2013; Huang et al., 2014).

   Sirt3 is considerably expressed in tissues with high metabolism rate including kidney, liver, heart, brain and brown adipose tissue (Huang et al., 2010a). It has been suggested that this enzyme is involved in energy homeostasis, regulating of ATP generation and mitochondrial function as well as oxidative stress and cell damage (Jing et al., 2011). Studies indicated that Sirt3 level in skeletal muscle of diabetic rats (type 1 and 2) has been reduced by 50% that might be also involved in the metabolic abnormalities present in diabetic patients.

   Decreased expression of Sirt3 was associated with mitochondrial metabolism alteration, over production of ROS and impaired insulin production or secretion (Jing et al., 2011). In contrast, it was reported that at nutrient distress states such as fasting, caloric restriction and forced exercise, Sirt3 activity is enhanced (Lantier et al., 2015). Sirt3 knockout mice displayed skeletal muscle glucose uptake defects and insulin resistance. Over expression of Sirt3 may protects against cell death triggered by genotoxic and oxidative stress insults (Yu et al., 2016).

   As a regulator of cell growth and a tumor suppressor, Sirt3 suppresses reactive oxygen species (ROS) and HIF-1α, the two factors that have major role in development of tumors (Allison and Milner, 2007; Li et al., 2010; Bell et al., 2011a; Finley et al., 2011). Different studies have shown that the genetic deletion of the mitochondrial Sirt3 deacetylase lead to increased mitochondrial superoxide leakage and tumor development (Tao et al., 2010). This may associated with the mitochondria related diseases like cancer in which studies revealed that mitochondria are silent and working on an aerobic glycolysis state (Fiorentino et al., 2011).

   Given that lowered Sirt3 activity has been correlated to the ROS over production, targeting its activity is potentially be an interesting strategy dealing with

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conditions including diabetes and cancer. To test this, we searched for traditional herbal medicines that were reportedly effective against both diseases. In this way, *Teucrium Polium* and Prosopis Farcta, with both anti-diabetic and anticancer properties were selected for further investigation. In prostate cancer, *Teucrium* extract inhibited the proliferation of cancer cells (Kandouz et al., 2010). Also, it was shown that *Teucrium* has anti-cancer effects in HepG2 cells (Fiorentino et al., 2011) and the cytotoxicity of aqueous and methanolic extract of *Teucrium* in Glioblastoma, shown to be concentration dependent (Eskandary et al., 2007). *Teucrium* and vincristine induced higher apoptosis level than vincristine alone in Skmel-3 and Saos-3 cell lines (Lewandowska et al., 2014). Antidiabetic effect of *Teucrium* was also demonstrated in several studies (Ljubuncic et al., 2005; Ardestani et al., 2008; Kandouz et al., 2010; Rafieian-Kopaei and Nasri, 2013). Antidiabetic and anti-inflammatory effects of Prosopis Farcta have been reported previously (Wong et al., 1998; Hajinezhad et al., 2015; Dashtban et al., 2016). Due to some active ingredients including quercetin (Mollashahi and Tehranipour; Asadollahi et al., 2010), Prosopis reported to improve diabetic complications and has been effective in the lowering of blood glucose (Yaniv et al., 1987). Anticancer effect of Prosopis was also confirmed previously (Kumar et al., 2011; Senthil Kumar et al., 2011; Tehranipour et al., 2012; Direkvand-Moghadam et al., 2015).

In the present study, we examined whether *Teucrium* Polium and Prosopis Farcta with common anticancer and antidiabetic effects can make effective changes in the mitochondrial Sirt3 activity in colorectal carcinoma cell line. Recognition of Sirt3 alteration due to the effect of these plants underscores the significance of Sirt3 activation/inactivation pathway in treating mitochondria associated diseases.

Materials and Methods

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); HT29 cell lines were obtained from Pasture Institute (Tehran, Iran); MitoLight and Apoptosis Detection Kit was purchased from Millipore Co. (USA). Mitochondrial purification kit was purchased from Sigma (USA) and Sirt3 assay kit was purchased from BPS Bioscience (USA).

Extraction

Wild-grown *Teucrium Polium* and Prosopis Farcta were collected from their natural growth place in the south: Ahvaz and west: Kermanshah of Iran respectively during spring 2017 and dried in a shaded place at room temperature. Both plants were identified scientifically in the department of Pharmacognosy, Ahvaz Jundishapur Faculty of Pharmacy. The air-dried plant parts were milled and used for preparation of hydroalcoholic extract. *Teucrium* milled powder (40 g) was mixed with methanol

(90% v/v) and kept incubated for 72 hours. The extract was then shaken and filtered and the solvent was partially removed in a vacuum evaporator for 24 hours so that the final volume reduced to one-third of its first volume. The concentrated extract was freeze-dried and kept in a cool and dry place until testing. Extraction of Prosopis was carried out by maceration method in ethanol 80% v/v. The resulting extract was concentrated by a vacuum evaporator, heated to yield a semi dried extract and stored in the refrigerator until testing.

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. Cells were incubated at 37°C and in a humidified atmosphere of 5% CO_2, seeded at a concentration of 1×10^6 and the viability of cells was determined by trypan blue staining. 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 dimethylsulphoxide (DMSO) upon serial dilution (1:3000) contains extremely low levels of DMSO that are far below the acceptable concentration (0.5% v/v) reported to be nontoxic.

Cell proliferation assay (MTT)

Cells were plated in 96-well plates at a density of 5×10^3 cells/well and incubated overnight. The cells were treated with 100 µl of each extract dilution contained 10, 5, and 1 µg / ml. After 24, 48, and 72 hours incubation period, MTT test was performed. Supernatant was removed very gently and the cells were incubated with fresh medium containing 1 g MTT/L at 37ºC for 3.5 hrs. After that, 150µl DMSO was added and cells were shaken for 15 min to dissolve formazan crystals. Colored product amount was measured by plate reader (Sunrise Absorbance Reader (USA)) at 590 nm with a reference filter of 620 nm. Viability inhibition was calculated as follows:

Cell viability (%) = Ab (test) /Ab (control) ×100.

Cell death assay

For evaluation of apoptosis and necrosis rates, 2*105 HT-29 cells were plated in 24 well plate exposed to 1 ml of extracts in their IC50 concentrations. Cells were collected after 48 hour centrifuged at 1200×g for 8 min and washed with 5 ml PBS twice. The supernatant removed and cold binding buffer was added to 10^6 Cells/ml and 3µl of Annexin conjugated ApopNexinTM FITC and 2 µl of PI were added and incubated for 15 min in the dark. Rate of apoptosis and necrosis were measured by a Flow cytometer (Galaxy, Serial. No: 0105362). 3 control groups were considered: Negative control including untreated cells and two positive controls: including only PI and Annexin.

ΔΨm measurement

For measuring potential changes in the mitochondrial

inter membrane potential, MitoLight® Mitochondrial Apoptosis Detection Kit (Millipore Company) was used. After treating cells with extracts (48 hour at IC50 concentrations), cells were trypsinized, centrifuged at 500×g and then exposed to MitolightTM reagent. After incubation in 37°C for 15-20 min and centrifugation at 500×g, supernatant was removed and incubation buffer solution was added to cells. All samples were analyzed using FACS Calibure (Becton Dickinson, USA) flow cytometry.

ROS measurement

For measuring of ROS, cells were passaged in 24-well plate. After 24 hour supernatant was removed and cells were treated with the desired extraction concentration (IC50). Rate of ROS production was measured at 6 and 12 hours. For this purpose, cells were treated with trypsin, then washed with PBS and centrifuged at 200×g for 5 min. Supernatant was removed and washed with PBS. Cells were Stained with 500 μl of 10 μM chloromethyl-2, 7 dichlorodihydrofluorescein diacetate (CM-H2DCFDA) for 45 min at 37 ºC. Amount of ROS was measured by Flow cytometry (Becton Dickinson, USA) in FL-1 at 525 nm (32). Positive control cells exposed to 250 μM H_2O_2.

Isolation of mitochondria

Mitochondria purification kit from Sigma was used for isolation of enriched mitochondrial fraction. Using this kit, most of the isolated mitochondrion will contains intact inner and outer membranes. Briefly, $2×10^7$ cells were harvested and washed with cold PBS. Lysis buffer and extraction buffer were added to cells respectively and suspension centrifuged at 600g for 10 minutes at 4°C. Supernatant was removed and centrifuged at 11,000 g for 10 minutes at 4°C. Upon the addition of protease inhibitor, mitochondrial pellet was kept in the storage buffer containing 15% (v/v) percoll. After centrifugation, mitochondrial band at the lowest interface were harvested and diluted with ice-cold storage buffer. Then mitochondria were centrifuged at ~17,000 g for 10 minutes (2–8 °C). The supernatant was removed and the pellet suspended in the storage buffer at a concentration of 1–5 mg-protein/ml.

Sirt3 assay

Fluorogenic Sirt3 Assay Kit was used to determine the Sirt3 activity. Briefly, HDAC fluorometric substrate (HDAC substrate 1) incubated with purified Sirt3 enzyme. The deacetylation sensitizes the substrate so subsequent treatment with the lysine developer produces a fluorophore that can be measured through 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 has also nicotine amide (enzyme inhibitor). Test tubes in addition to the above mentioned materials (HDAC assay buffer, NAD+ and HDAC substrate) contained the extracts. Fluorescence microplate reader (BioTek FLX800) was used to read the fluorescence intensity (EX: 350-380 nm and EM: 440-460 nm).

Statistical analyses

All experiments were performed in at least triplicates. Student's t-test was used to determine statistical significance (P < 0.05). Analysis of variance (ANOVA) was considered when more than two groups were compared and data were expressed as mean ± SD.

Results

Cytotoxicity of Teucrium and Prosopis extracts on HT-29 cells

Cytotoxicity was evaluated after 24, 48 and 72 hours of treatment with extracts. Results for MTT assay at different concentrations of both extracts (10, 5, and 1 μg/ml) shown in Table 1 and Table 2. IC50s were obtained using graph PAD Prism 6 for *Teucrium Polium* at 24, 48 and 72 hours as 4, 3 and 6 μg/ml respectively. These values were 12, 2 and 1 μg/ml for Prosopis Farcta. As shown in the results, Prosopis Farcta exhibited a higher cytotoxicity effect on cells than *Teucrium Polium* at 48 and 72 hrs of treatment. The more potent IC50 of *Teucrium* extracts were selected for further assessments. 2 μg/ml of Prosopis Farcta was selected for more mechanistic evaluations since at 1 μg/ml, a profound decline in cell survival was observed and the percent of cytotoxicity values were comparable to 48 hours for *Teucrium*.
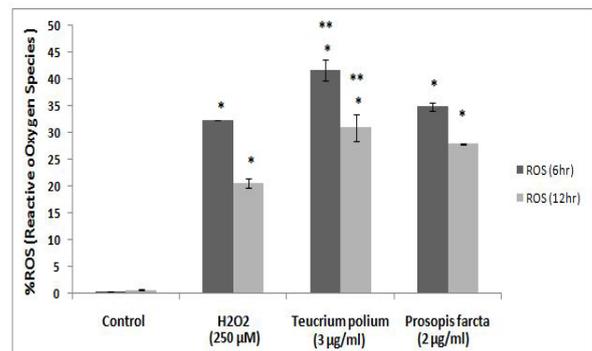

Figure 1. ROS Generated in HT-29 Cells when Exposed to IC_{50} Concentrations of Extract of Teucrium (3μg/ml) and Prosopis (2μg/ml) for 6 and 12 hours. HT29 cells were grown in Dulbecco's Modified Eagle Medium, exposed to Teucrium and Prosopis at IC50s concentrations. Values are mean ± SD in each group measured from three independent experiments. (One way ANOVA followed by Tukey's post – hoc test). * Significant difference in comparison with negative control group (P < 0.05). ** Significant difference in comparison with positive control group (P < 0.05).

Table 1. Cytotoxicity Percent of Hydroalcoholic Extract of Teucrium Polium at Different Concentrations (10, 5, and 1 μg/ml) on HT-29 Cells. Values are mean ± SD in each group and least three independent experiments

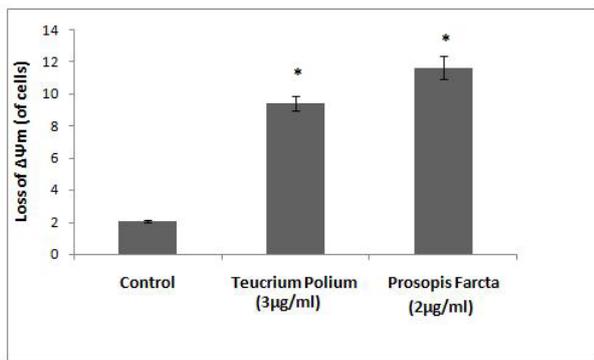| Concentration (μg/ml)	0	10	5	1
Time	(Control)			
24hr.	98.65±0.674	48.44±4.7	49.64±5.9	71.36±12.7
48hr.	98.36±1.4	40.8±5.3	29.84±16.3	46.21±3.09
72hr.	98.36±1.4	45.58±1.4	11.24±26.6	11.11±2.6

Figure 2. Loss of Mitochondrial Membrane Potential in HT-29 Cells Exposed to IC_{50} Concentrations of Extracts of Teucrium (3μg/ml) and Prosopis (2μg/ml) Using MitolightTM Florescent Dye. In healthy cells, the dye accumulates in the mitochondria and yields red fluorescence. In dead cells where mitochondrial membrane potential has been depolarized, the dye aggregates in the cytoplasm and gives off a green fluorescence. Values are mean ± SD in each group measured from three independent experiments. (One way ANOVA followed by Tukey's post – hoc test).* Significant difference in comparison with control untreated cells (P < 0.05).

Effect of Teucrium and Prosopis extracts on the ROS formation in HT-29 cells

The levels of ROS production by *Teucrium* and Prosopis extracts were presented in Figure1. Both *Teucrium* and *Prosopis* extracts increased ROS levels higher than negative control at 6 and 12 hours following the treatment. ROS amounts were even higher than positive control for *Teucrium* at mentioned times.

Effect of Teucrium and Prosopis extracts on the mitochondrial membrane potential of HT-29 cells

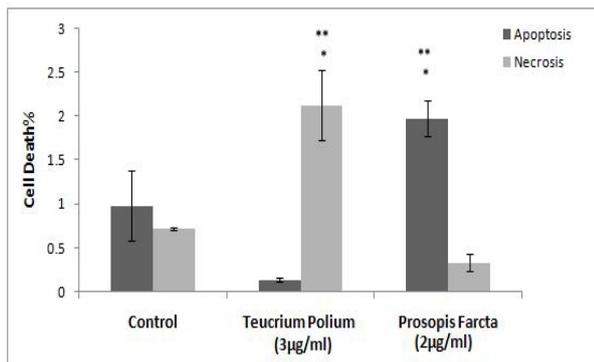As shown in Figure 2, both of *Teucrium* and *Prosopis*

Figure 3. Induction of Apoptosis and Necrosis by Teucrium (3μg/ml) and Prosopis (3μg/ml) in HT29 Cells. HT29 cells were grown in DMEM, exposed to extracts and early apoptosis and necrosis were determined by Annexin and PI staining using flow cytometry. 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. Values are mean ± SD in each group measured from three independent experiments. (One way ANOVA followed by Tukey's post – hoc test). * Significant difference in comparison with control groups (P < 0.05). ** Significant difference in comparison with test groups (P < 0.05).

Table 2. Cytotoxicity of Hydroalcoholic Extract of Prosopis Farcta at Different Concentrations (10, 5, and 1 μg/ml) in HT-29 Cells. Values are mean ± SD in each group measured from three independent experiments.

Concentration (μg/ml)	0	10	5	1
Time	(Control)			
24hr.	98.65±0.67	86.02±14.2	78.07±1	77.2±7.9
48hr.	100±4.2	16.96±12.2	32.2±14.4	59.59±1.7
72hr.	99.9±5.2	6.76±1.2	5.42±0.42	5.37±0.23

extracts induced significant loss of mitochondrial membrane potential. ΔΨm collapsed at higher level by Prosopis compared to *Teucrium*.

The Effect of Teucrium and Prosopis extracts on death rate in HT-29 Cells

Figure 3 shows percentage of cell death measured by propidium iodide (PI)/ Annexin V staining. Results revealed that treatment with *Teucrium* extract induced more necrotic cell death than Prosopis extract in HT29 cells while substantial apoptotic cell death is observable for Prosopis. Percentage of necrotic cell death induced by *Teucrium* extract was significantly higher than Prosopis extract. Inversely, apoptotic cell death induced by Prosopis extract immensely higher than *Teucrium*.

Sirt3 activity is up regulated by Teucrium Polium and Prosopis Farcta extract treatment

To evaluate the changes in Sirt3 activity, mitochondria from HT-29 cells were isolated and their enzymes were purified. In Figure 4, the activities of the Sirt3 enzyme purified from HT-29 and normal cells are presented under treatment of IC50s (MTT results) of the extracts of Prosopis Farcta and *Teucrium Polium*. The results showed that Sirt3 activity increased in cells exposed to Prosopis Farcta and *Teucrium Polium* extract. In HT-29 cells
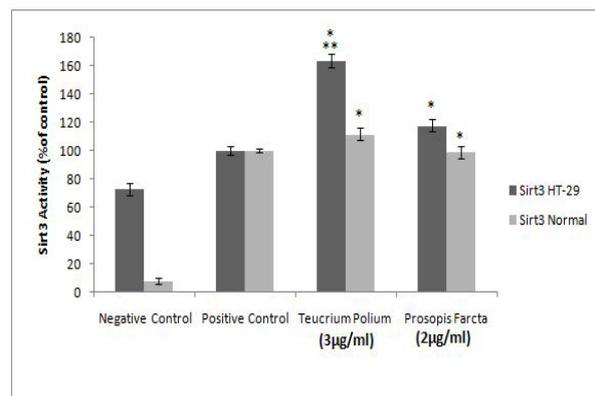

Figure 4. Activity of Normal Sirt3 and Sirt3 from HT-29 Treated with Teucrium (3μg/ml) and Prosopis (3μg/ml) Extract. Values are mean ± SD in each group measured from three independent experiments. (One way ANOVA followed by Tukey's post – hoc test). Negative Control: beside other reagents contained an inhibitor (Nicotinamide). * Significant difference in comparison with Negative control group (P < 0.05). **Significant difference in comparison with Positive control group (P < 0.05).

exposed to Prosopis Farcta extract Sirt3 were significantly different compared with negative control (without NDA, an inhibitor of the enzyme) but there was no significant difference compared with the positive control group (with NDA, an inhibitor of enzyme). Results showed that Sirt3 activities for positive and negative controls of *Teucrium Polium* have also been significantly increased. There is a significant difference between the activity of Sirt3 purified from normal cells and Sirt3 purified from HT-29 cancer cells exposed to *Prosopis Farcta* and *Teucrium* Polium. Fluorescence measurement showed that *Teucrium* extract elevated purified Sirt3 activity compared with negative control. In contrast, there were no significant differences between *Teucrium*, Prosopis and positive control (Figure 4).

Discussion

Various plants have been used traditionally in both cancer and diabetes therapy. Recently some anti-diabetic drugs were reported to having anticancer as well (Eskandary et al., 2007; Lewandowska et al., 2014; Shlomai et al., 2016). Based on their uses in both cancer and diabetes, we used *Teucrium* (Eskandary et al., 2007; Ardestani et al., 2008; Kandouz et al., 2010; Rafieian-Kopaei and Nasri, 2013) and Prosopis (Mollashahi and Tehranipour; Asadollahi et al., 2010; Kumar et al., 2011; Tehranipour et al., 2012; Direkvand-Moghadam et al., 2015; Dashtban et al., 2016) for evaluating their mutual mechanism in cancer cells.

Sirt3 has a major role in mitochondrial protein deacetylation and the main purpose of this deacetylation is for metabolic stability (Anderson, 2012; Ansari et al., 2017a). In response to caloric restriction, Sirt3 activates isocitrate dehydrogenase 2 and glutamate dehydrogenase (GDH) enzyme in the Krebs cycle (Lombard et al., 2011). Also, Sirt3 plays a role in mitochondrial aerobic respiration (Choi et al., 2016). Among the seven types of Sirtuins, Sirt3 is more involved in the mitochondrial function regulation. One of the main common points in cancer and diabetes pathology might be the changes in the mitochondrial Sirt3 activity and levels. This suggestion supported by findings that revealed mitochondrial defects in the both diseases.

In diabetes type I and II, by decreasing in the Sirt3 activity, oxidative stress will be upregulated. Consequently, insulin secretion and effect would be changed. Studies suggested an interrelationship between Sirt3 and ROS production in pathological and physiological conditions that has a role in tumor growth and suppression (Jing et al., 2011). Sirt3 is a tumor suppressor that works via suppressing the ROS and HIF1-α, factors that have main role in the tumor development (Bell et al., 2011b; Finley et al., 2011). In contrast to high fat diet, exercise, fasting and caloric restriction increased the expression of Sirt3 (Huang et al., 2010b; Signorile et al., 2016). Marcin Buler (in 2012) reported that metformin reduced the Sirt3 expression in rat liver mitochondria and possibly contributed to the reduction of ATP production in the treatment of diabetes. Also Song Yuping in 2014 showed that increased expression of Sirt3 in the L6-IR of muscle

of the diabetic mouse treated with metformin induced increased glucose uptake and reduced ROS and insulin resistance.

It has been shown that the Sirt3 enzyme deacetylates acetyl-CoA synthetase 2 (AceCS2) which is involved in the formation of the required acetyl-CoA for the Krebs cycle and provides the necessary electrons to activate the mitochondrial respiratory chain. Sirt3 also plays a role in the deacetylation of Complex I (activation of Complex I). Hence, the inhibition or reduction of activity of Sirt3 (which occurs in cancer) results in the blockade of the Krebs cycle and the mitochondrial respiratory chain and leads to the over production of ROS (Ansari et al., 2017b). In many studies, the primary dysregulation of Sirt3 in cancer has been confirmed (Torrens-Mas et al., 2017b).

Cancer cells, evade the mitochondria from generating energy (ATP) and aerobic glycolysis takes place (Finley et al., 2011). Consequently, normal leakage of ROS from mitochondria would be decreased. This situation could be related to Sirt3 modifications. While *Teucrium* was reported to have antioxidant properties in some sort of situations, our results indicated that in mitochondria ROS produced from *Teucrium* was overwhelmingly increased. These amounts of ROS induced necrosis in cells possibly through the mitochondrial pathway and Sirt3 modifications. At the same time, Prosopis, induced lower levels of ROS in cancer cells which might be contributes to programmed cell death rather than necrosis. In non-cancerous state, ROS-triggered Sirt3 upregulation results in the diminution of ROS and oxidative stress (Koyama et al., 2011; Xie et al., 2017). The role of Sirt3 in cancer cells upon the exposure to a cytotoxic is still controversial. Though, Sirt3 protects several cell types from toxic insults by maintaining their mitochondrial functions, its overexpression in cancer cells promotes survival signals enhancing tumorigenesis. Other reports suggested a proapoptotic and growth arrest role for Sirt3in several colorectal carcinoma (Alhazzazi et al., 2011). It seems here that extracts induced mitochondrial dysfunction, ROS overproduction and oxidative damage in cancer cells. These cells in turn escaped death by over expression of Sirt3. Many implications can be attainable through the published reports Marfe et al., (2009). Accordingly, it would be reasonable inhibiting of Sirt3 for achieving better consequences in cancer treatment (Torrens-Mas et al., 2017a).

Our Results showed that *Teucrium* in contrast to Prosopis, increased Sirt3 activity in HT-29 derived Sirt3. Considering our short evaluating time, it seems that these biochemical changes were not related to gene expression and modifications (Figure 4). Results from the cancer cells isolated Sirt3, showed a decrease in Sirt3 enzyme activity in comparison with normal enzyme (Figure 4). Sirt3 activity in normal state was almost 7 times higher than HT-29 derived enzyme. In cancer cells it seems that, Sirt3 has changed in a way in which has lower sensitivity to nicotine amide and changes in cancerous Sirt3 made it more sensitive to the plants extract.

Results from this study showed that *Teucrium* and Prosopis conducted their anticancer activities via the mitochondrial alterations. These mitochondrial alteration

increased ROS levels, Sirt3 activity and cell death in HT-29 colorectal cancer cells.

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)

Conflict of interest

The authors disclose no potential conflicts of interest.

Acknowledgments

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