RESEARCH ARTICLE

Evaluation of Oxidative Stress in Patients of Follicular Thyroid Cancer and Study The Therapeutic Effect of Resveratrol on Oxidative Stress in FTC-133 Thyroid Cancer Cell Line

Basma Talib Al-Sudani, Suhad Faisal Hatem Al-Mugdadi*, Ahmed Ali Mohammed

Department of Clinical Laboratories Sciences/College Of Pharmacy/Mustansiriyah University/ Iraq

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ABSTRACT

The second most common cancer of the Thyroid gland is FollicularThyroid Carcinoma (FTC), second only to papillary carcinoma. Resveratrol, a natural polyphenolic compound that has anti-oxidative, anti-inflammatory and apoptotic properties, appears to have substantial cardioprotection and cancer-prevention properties. Furthermore, resveratrol is thought to be responsible for regulating several biological processes, such as metabolism and aging, through the modulation of the mammalian silent information regulator 1 (SIRT1) of the Sirtuins family. The purpose of this study is evaluating erythrocyte malondialdehyde's (MDA) role in the indication of the oxidative status in follicular thyroid carcinoma patients and investigating the therapeutic effect of resveratrol, a potent antioxidant, upon oxidative stress levels in thyroid cancer *in vitro.* Malondialdehyde was evaluated in erythrocyte of follicular thyroid cancer patients after and before treatment with sodium stibogluconate. Cytotoxicity by MTT assay and intracellular reactive oxygen species (ROS) levels was measured after resveratrol treatment on follicular thyroid carcinoma FTC-133 cell line. The results of this study confirmed that150 μM Resveratrol inhibited proliferation of FTC-133 thyroid cancer cell line *in vitro* by 88% after 72hand treatment with 50-200μM Resveratrol reduced ROS levels. To sum up, considering its mode of action, resveratrol might have an important role in providing a source for natural antitumor agents, a fact that would have great therapeutic potential in integrated oncology.

Keywords: Follicular thyroid carcinoma, Malondialdehyde, Reactive oxygen species (ROS)

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INTRODUCTION

The definition of Oxidative stress is: "an imbalance between oxygen-derived free radicals production and their removal by antioxidants".[1] In cases of mild oxidative stress, the response of the tissues is the increase in antioxidant defenses production, but in cases of severe oxidative stress, cell injury and death often occur. Cell Death induced by free radicals can proceed as necrosis or apoptosis.[2] Furthermore, the causes of increased oxidative stress at a cellular level are numerous, including trauma, infections, toxins, poor diet, exposure to alcohols, medications, cold, radiation, or strenuous physical activity.[3] The oxidants that are typically generated in biological systems such as hypochlorite, hydroxy radical, peroxyl radical, peroxynitrite are highly reactive, and have half-lives of only a few seconds.[4] In contrast, some modified biological macromolecules have long half-lives in comparison, that could be hours to weeks, and therefore, modified lipids, proteins, and nucleic acids in tissues can act as reporter groups indicating the presence of oxidative stress. Among these modified molecules are malondialdehyde, isoprostanes, nitrotyrosine, breath alkanes,[5] and several others that have been established or proposed. Malondialdehyde (MDA) is the most commonly used marker that aims to detect the occurrence of oxidative stress in biological systems. Approximately 20% of end-products derived from oxidative damage of lipids *in vitro* are MDA. Others are short- and long-chain aldehydes, ketones, alkanes, and diens. The cytotoxicity effects of aldehydic carbonyl compounds arise from their ability to react with cellular biomolecules and forming a duct, which in turn cause the cell to lose its biological function.[6] In biological systems, MDA is present in both, free and bound forms. Some MDA results from lipid peroxidation *in vivo*, and goes into the circulation of body fluids either protein-bound or in the free form. Another portion of MDA is formed *in vitro*, as a result of decomposition of lipid hydro peroxides. Malonaldehyde is a significant byproduct of the synthesis of prostaglandins by enzymes

**Author for Correspondence: suhadhatim@yahoo.com*

and is removed by renal clearance. During Throxin synthesis by utilization of H_2O_2, it is common for oxidative stress to develop in the thyroid tissue, or when ROS is produced by the inflammation, and when the tumor has active proliferation.[7,8] Follicular thyroid cancer (FTC) is a relatively rare form of thyroid cancer, as it accounts for about 4% to 39% of combined malignancies of the Thyroid,[9] and they don't constitute a large proportion of the study cohorts for thyroid cancer.[10-16] Despite classified collectively as well-differentiated papillary (PTC) and follicular thyroid cancer (FTC), the two types each have distinct clinical and pathologic features, biologic behavior, and different clinical outcomes.[12-15] FTC is more aggressive than PTC and has a worse prognosis. Patients were suffering from FTC present with diseases in a more advanced stage, and with a higher rate of distant metastases due to the tumor's tendency to invading the vascularity. This study was carried out to explain the effect of the thyroid carcinoma on the lipid peroxidation process by measuring the MDA level in the erythrocyte patients of follicular thyroid cancer to look if it can mark the degree and type of the disorder. Additionally, in the present studies, treatment of human thyroid cancer cells with the naturally occurring polyphenol, Resveratrol, that has been tested as a prophylactic anti-cancer agent andreduced ROS levels by activated SIRT1 enzyme via the mechanisms as shown in Figure 1.

It is predicted that the elevation in SIRT1 enzyme's level in the cancer cells treated with resveratrol lead to inhibited the NF-κB by decreasing nuclear p65 protein, the subunit of NF-κB and decreasing DNA binding through direct deacetylation, (Merksamer *et al.,* 2013) which reported that the increased activity of SIRT1 enzyme leads to inhibiting NF-κB by SIRT1, which can be blocked cytokine-induced NF-κB and led to downstream gene iNOS.[18] This, in turn, led to decrease ROS level as was found in the results obtained in this study. According to the results of mechanisms, the treated resveratrol on FTC-133 thyroid cancer cell line and its ability to inhibited these cancer cell line, resveratrol can be used in the future to the FTC treatment.

MATERIAL AND METHODS

Samples

A total of one hundred cases were enrolled in the study. A total of 60 cases were collected from the medical city hospital in Baghdad, being newly diagnosed with follicular thyroid carcinoma (FTC), and followed up after 4 weeks of complete chemotherapy using Sodium Stibogluconate. A control group of 50 healthy people was selected, having similar ages and sex as the therapy group. Group of the patients before treatment were also compared with their follow-up.

EDTA vials were used to take 5mL of blood as samples. In this study, we used chemicals and reagents of analar grade unless otherwise specified and were obtained from Sigma chemicals USA, Fluka, A.G., Germany, (BDH) chemicals Ltd., England and Hopkins and Williams, England.

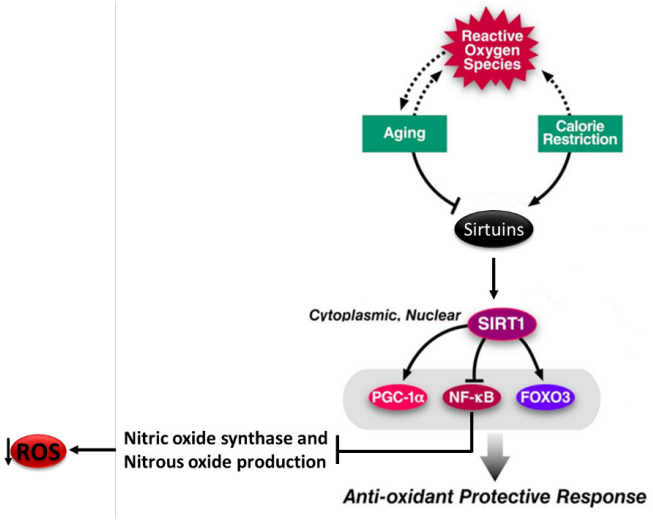
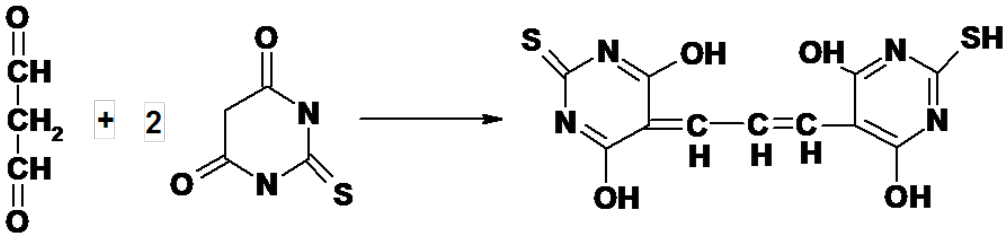


Figure1: Relationship between ROS and SIRT1 (17).



Reaction between MDA and TBA

Biochemical test includes erythrocyte (MDA) was determined in controls and FTC patients (before and after treatment) as an indicator for the oxidative status in follicular thyroid carcinoma patients.

MDA assay:

Ohkawa *et al.*'s 1979[19] method with minor modification from Hirayama *et al.* 2002 (20) was used in the assay of (MDA). Condensation of 2 molecules of TBA with 1-molecule of MDA was done, and the reaction resulted in the formation of thiobarbituric acid-reactive substances (TBA-RS). This generated a reddish chromogen that could absorb light that had the wavelength of (532) nm.

Hemoglobin concentration:

A Hemoglobin kit (Randox) procedure no. 540-UV was used to follow Hb. Hemoglobin is oxidized to Methemoglobin through the action of alkaline potassium ferricyanate, which reacts with potassium cyanide afterward forming Cyanomethaemoglobin, which is capable of absorbing light of wavelength (540) nm. The total Hemoglobin concentration directly relates to the intensity of absorbance.

Cell lines and Cultures:

Human follicular thyroid carcinoma (FTC-133) cells were cultured in DMEM: Ham's F12 (1:1) + 2mM Glutamine + 10% Fetal Bovine Serum (FBS) + 1% Penicillin-Streptomycin-Amphotericin B. Cells were cultured in 75 cm^2 flasks and incubated in 5% CO_2/95% humidified air at 37°Celcius.

***In Vitro* Cytotoxicity Assay:**

In 96-well plates, 5,000 /4,000 /3,000 FTC-133, cells were seeded in 100µL of complete medium per well for test periods of 24, 48 and 72h respectively. The next day, cells were treated with 150 µM Resveratrol. The plates were then incubated at 5% CO2/95% humidified air at 37°Celcius until the day of assay. MTT (3-[4,5-dimethylthiazol-2-yL]-2,5 diphenyltetrazolium bromide) was prepared at 3mg/mL in PBS, 30µL of this solution was added to each well and incubated for 4 hours at 37°Celcius. Following incubation, the liquid was removed, and 100µL of DMSO were placed in each well with lightly shaking for 15 minutes. The optical density of the solution was read at 540nm and corrected for background absorbance at 650nm on a Multiscan reader.

Measurement of intracellular reactive oxygen species (ROS) production:

Intracellular reactive oxygen species (ROS) production was evaluated in 50-200 µM Res. treated cells and in control, cells using 2', 7'-dichlorofluorescein diacetate (DCFDA) (Abcam, ab113851, UK). A 100µl from (FTC-133) cells suspensions were dispensed into 96-well flat clear-bottom dark sided tissue culture plates (Falcon) at concentrations of 25,000 cells per well and incubated 24h under standard conditions. After 24-hour, the cells were treated with 50-200 µM resveratrol, then exposed to 50 µM tert-butyl hydrogen peroxide (TBHP) before and after 4-hour from adding the resveratrol. After a recovery period 6h, the cell culture medium was removed and washed the cells in 100 µl/well 1x buffer, then 1x buffer was removed and cells stained by adding 100 µl/well of the DCFDA solution (10 µL of 20 Mm DCFDA solution with 10 mL 1x buffer). The Culture was incubated with the DCFDA solution for about 45min at 37°C. After that DCFDA solution was removed and 100 µl/well 1x buffer were added. In each well, Fluorescent units were measured using luminometer microplate readers (BMG LABTECH, Germany) fluorescence measurement system with an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

STATISTICAL ANALYSIS

All isolated values, expressed as mean ± SD, were analyzed with the Student's t-test. P-values less than 0.05 and 0.01 were measured to be significant and highly significant respectively. Results were obtained for three independent experiments, and analysis of data was performed using the software SPSS package.

RESULTS

Table (1) showed the mean ± SD of erythrocyte (MDA) level expressed (n mol/gHb) in healthy (control) and follicular

Table 1: Biostatistical calculations and student (t-test) of erythrocyte (MDA) level for healthy control and FTC patients (group1 and group2).

		Follicular thyroid carcinomapatients	
Erythrocyte (MDA) level mol/gHb	*Control*	*Group (1)*	*Group (2)*
Sample size (n.)	50	60	40
Mean ±SD	4.34 ± 1.32	8.18 ± 2.3	5.11 ± 2.07
Probability		p< 0.001**	p< 0.005**

p- Value ≤ 0.05.* significant, p- value<0.01** highly significant

Table 2: Hemoglobin concentration for healthy control and FTC patients (group1 and group2) using student (t-test)

		Follicular thyroid carcinoma patients	
Hb concentrationg/dL	*control*	*Group (1)*	*Group (2)*
Sample size (n.)	50	60	40
Mean ± SD	13.0 ± 1.39	6.96 ± 2.5	10.9 ±1.63
Probability		p< 0.005**	p< 0.001**

p- Value ≤ 0.05.* significant, P- value< 0.01** highly significant

thyroid carcinoma (FTC) patients. The patients divided into group1 (before treatment)and group 2 (after 4-week treatment). Erythrocyte (MDA) level was significantly higher in group 1 (8.18 ± 2.3) compared with control (4.34 ± 1.32) with *p-value* < 0.001**. After treatment group 2, erythrocyte (MDA) level was decreased, but it remained high significantly above the control (p <0.005**). Hemoglobin (Hb) concentration was significantly lower in group 1 (6.96 ± 2.5) versus to control (13.0± 1.39) with *p -value*< 0.005**. Also, after treatment group 2, Hb concentration was increased, but it remained significantly below of the control (p <0.001**) as shown in Table 2.

Effect of Resveratrol on FTC-133 cells viability

In the current study, the effect of resveratrol on FTC-133 thyroid cancer cell line viability was evaluated. FTC-133 cells were treated with 150 µMResveratrol and 50µM of Cizplatin as control at 24, 48, and 72h. The results show that the Resveratrol significantly decreased the viability of the cells of FTC-133 in this concentration (38.5, 33 and 11.7%) at 24, 48 and 72 hours respectively versus to 50µM of Cizlatain (37, 20and 11%) at the same incubation time, as illustrated in Figure 2.

ROS production of FTC-133 cells line

To investigate antioxidative effect of resveratrol, the ability to avoid the production of ROS in FTC-133, was evaluated. As shown in Figure 3, FTC-133 cell lines were incubation with different concentration of Resveratrol (50-200µM) at 6h were slightly decreased the levels of endogenous ROS by using the fluorescent probe called DCF. When cells were exposed to 50 µM TBHP (tert-Butyl hydroperoxide (tBuOOH) is an organic peroxide widely used in a variety of oxidation processes), a marked increase was observed in the intracellular ROS level (p < 0.005). In fact, when cells were pre-incubated with different concentration of resveratrol (50-200µM) for 4h ourbefore being exposed to 50 µM TBHP, the levels of ROS noticed after 45 minute were increased than the same cells which were pre-treated with Resveratrol only (p < 0.05). When the same cells were exposed to 50 µM TBHP before adding 50-200µM resveratrol, the ROS level observed after 45 min were increased significantly than cells of control and cells were pre-treated with Resveratrol firstly (p< 0.005). The 200µM Resveratrol was significantly decreased the ROS level on FTC-133 cell lines compared with 50µM resveratrol, p< 0.005.

DISCUSSION

Current work has study the erythrocyte malondialdehyde (MDA) as a sign for the oxidative status in patients suffering from follicular thyroid carcinoma. In addition, data about the inhibitory effect of resveratrol on cells viability and on ROS

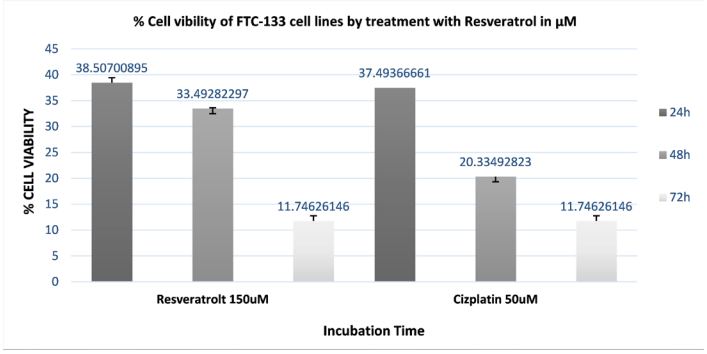


Figure 2: *In vitro* study, cell viability percentage of the FTC-133 thyroid cancer cell line was evaluated by MTT assay in 96-well plates following 24, 48 and 72h treated with 150 µM Resveratrol and 50µM of Cizplatin. Results are represented as % mean ± SEM of cells viability for triplicate experiments.

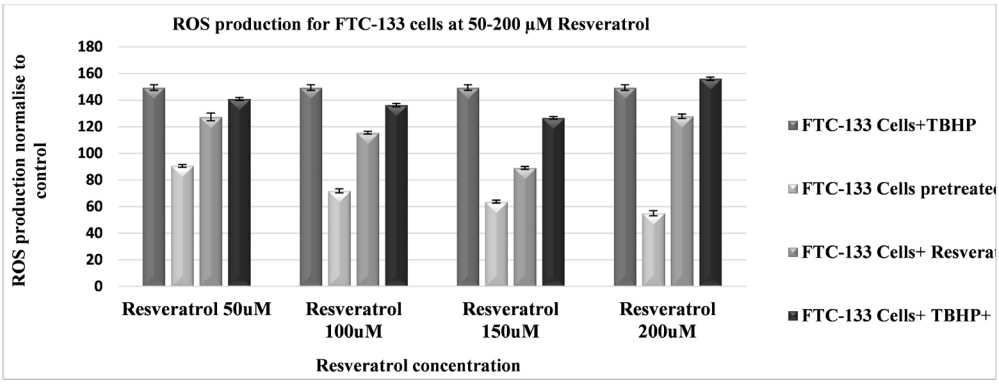


Figure 3: Effects of Resveratrol on intracellular ROS production in FTC-133 cells, using DCF as a fluorescent probe. Cells were pre-incubated with the 50-200 µM of Resveratrol, then cells were exposed to 50 µM TBHP (Tert-Butyl Hydrogen Peroxide) before and after 4h from adding the Resveratrol. After a recovery period 6h, the culture was incubated with the DCFDA solution for about 45min at 37° C. DCF fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The results represent the mean ± SEM of three different experiments performed in triplicate. p< 0.005.

production as an antioxidant in thyroid cancer cells (FTC-133) were obtained.

The fact of the antioxidant scavengers cannot be able to scavenge the excess produced oxygen free radicals; the oxidative stress will take place. Then these free radicals can damage important biomolecules like lipids, proteins, and DNA. When peroxidation of these vital molecules occurs, a variety of carcinogen and mutagen factors will produce.[21,22]

Yeh*et al.* and Sheeba*et al.*reported an increase in the concentration of MDA in breast cancer patients compared to normal ones.[22,23] In addition to many studies, cited that MDA level was increased in lung cancer, gastric cancer, cervical cancer compared to normal.[24-26]This data agreement with our result that showed a high concentration of MDA in cancer patients after and before treatment compared to the healthy group (Table 1).

Another study demonstrated the mean of MDA level in the healthy group (5.107±2.32 nmol/ml) while the mean of MDA level in oral pre-cancer and oral cancer was 9.33 ± 4.89 nmol/mL and 14.34 ± 1.43 ηmol/mL respectively. That means there was a significant increase in serum MDA of the oral pre-cancer and oral cancer patients.[27] This lead to conclude that high level of MDA in cancer leads to damage DNA of these cells via lipid peroxidation induction[28] or may via the role of ROS in aetiopathogenesis of cancers.

According to the present data, the hemoglobin concentration was reduced in cancer patients before treatment (6.96 ± 2.5) and again elevated after treatment (10.9 ± 1.63) but still less than normal (13.0 ± 1.39) as illustrated in Table 2.

In Two previous studies, the authors recorded that MDA levels increased and rat fragility of red blood cells decreased after radiotherapy, that mean the radiotherapy effect on biophysical characteristics of the erythrocyte(RBCs) membrane.[29,30] During treatment, the effect of free radicals on theRBCs membrane and cytoskeleton can cause defect and hemolysis in the red blood cells and hemoglobin leakage.[31] While another study concluded different results,Plasma level of MDA and erythrocyte osmotic fragility (OF) increased during after and before treatment with chemotherapy and radiotherapy, but without significant statistical difference due to resistance of the erythrocyte membrane to hemolysis.[32]

Present data revealed that 150 μM of Resveratrol significantly decrease the viability of the cells of FTC-133 (38.5, 33 and 11.7%) at 24, 48 and 72h respectively versus to 50μM of Cizplatin (37, 20and 11%) at the same of incubation time (Figure 2).

Recently, in order to evaluate the effects of Resveratrol (RES) and Triacetylresveratrol (TRES) on cell viability of pancreatic cancer include PANC-1 and BxPC-3 cells lines, these two drug used at a concentration range of 0–200 μMin 24 h, 48 h, and 72 h. BxPC-3 cells were more susceptible to TRES and RES, especially when the concentrations of them reached 50 μM after 48 hours. Compared to TRES, RES had significantly effects to inhibit the cell viability in both cells line at different time, Also in this study, they noted that after seeded with 100 μM TRES and RES for 48 h, the cell viability of PANC-1 cells were 90.66 ± 1.89% and 60.81 ± 5.39% ($p < 0.01$), and the cell viability of BxPC-3 cells were 56.94 ± 2.10% and 34.11 ± 1.38%, respectively with $P < 0.01$. These outcome suggested that the up-regulation of Bim and Puma proteins and down-regulation of Mcl-1 protein might be leading to apoptosis induced by RES and TRES in pancreatic cancer cells.[33] These results go in line with our study, which means the follicular thyroid carcinomas sensitive to resveratrol and this treatment had anticancer activities especially after72 hour (Figure 2).

Another study investigated that treatment with resveratrol (0–50 mmol/L for 24 hours) in androgen-responsive human prostate carcinoma (LNCaP) cells. The results in this study cited that a significant drop off in cell viability: decrease of clonogenic cell survival: inhibition of androgen (R1881)-stimulated growth and induction of apoptosis in LNCaP cells. While the same concentrations of resveratrol not affected on the viability or apoptosis in normal prostate cells.This may attribute to resveratrol causes inhibition of phosphatidylinositol 3-kinase/Akt activation that in sequence, modulation in Bcl-2 family proteins in such a mode that the apoptosis of LNCaP cells is promoted.[34]

Generally, low resveratrol doses (0.1–1.0 μg/mL) has been considered to increase cell proliferation, but high doses (10.0–100.0 μg/mL) induces apoptosis and decrease mitotic activity on endothelial cells and human tumors.[35] Two resveratrol actions on colon cancer cells (HT-29) death and growth were observed, at low concentrations (1 and10 μmol/L), resveratrol increased cells number, while at higher doses (50 or 100 μmol/L) this drug reduced cells number and increased apoptotic or necrotic cells percent.[36]

As we know, resveratrol significantly decreased cell viability and induce cell death in many human cancer cells.[37-39]

Notably within present data, ROS production significantly reduced in (FTC-133)cells seeded with many concentration (50,100,150 and 200 μM) of Resveratrol using 50 μM TBHP before and after 4h incubation time (Figure 3). This agrees with new research demonstrated that Resveratrol inhibits pancreatic cancer cells (PCCs) invasion, migration and glycolysis throughout suppression of ROS in addition to miR-21-mediated activation, which the expression in PSCs was down-regulated after RSV treatment in these cells.39 In addition, after treatment with Resveratrol(50 μM), the expression and activity of sirtuin-1(SIRT1) were activated leading to induces chondrosarcoma cell apoptosis via deacetylating the p65-NF-κB complex, that is mean the p65-NF-κB will reduce.[38]

In the other side, our results differ from another study reported that the use of resveratrol drug may increase intracellular ROS in ovarian cells CSCs and other cancer kinds. They recommend that increase intracellular ROS in ovarian cells might be useful, with or without resveratrol, as cancer therapy.[37] Resveratrol caused apoptosis and autophagy via the

production of ROS, which indicate that ROS controls apoptosis and autophagy in Res-treated HT-29 colon cancer cells.[40]

CONCLUSIONS

Considering the mode of action, resveratrol might be considered as a promising source of natural antitumor agents which might have therapeutic potential in integrated oncology.

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