

Original Article

Running Title: Induction of Apoptosis-by *Cyrtopodion Scabrum* Extract and P53 Signaling Pathway

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Induction of Apoptosis with *Cyrtopodion Scabrum* Extract in Colon Cancer Cells: A Preliminary Study on Targeting P53 Signaling Pathway

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Abstract

Background: *Cyrtopodion scabrum* is a kind of lizard, widely distributed in southwestern, central, and eastern Iran. In our previous studies, we showed the selective anticancer properties of *Cyrtopodion scabrum* extract (CSE) against certain gastrointestinal cancer cell lines with no significant harmful effects on the normal cells.

Method: This in vitro study was conducted to investigate the mechanism of action of CSE in SW742 (colon cancer cell line) using western blotting, real-time PCR, and PI staining/flow cytometry analysis.

Results: Western blot analysis showed that CSE up-regulates P53 protein expression significantly in SW742 cancer cell line while QRT-PCR results revealed that P53 mRNA did not increase accordingly. It is proposed that TP53 protein play its role as a tumor suppressor through the protein stability mechanism and not through the increase in the gene expression. Considering this fact, for TP53 stabilization, two of its target genes, p21 and mdm2, were regulated. We evaluated the mRNA expression of these two genes as well. The obtained data showed a significant increase in both genes by CSE (1000 µg/ml), suggesting that the induction of G2 cell cycle arrest, which we previously reported, may happen through P53 and P21over-expression. No significant decrease was observed in apoptosis when SW742 cells were co-treated with CSE and the inhibitor of P53 transcriptional activity, (PFT)-α, indicating that the induction of apoptosis with CSE did not occur through P53-dependent transcriptional activity.

Conclusion: The obtained results herein revealed that the observed anti-cancer effect of CSE may occur through TP53 up-regulation, yet with P53-independent transcriptional activity.

Keywords: Natural anticancer product, Gecko (Gekko) extract, *Cyrtopodion scabrum*, Western blot analysis, Real-time PCR, flow cytometry, P53 signaling pathway

Introduction

Following heart disease, cancer is one of the main causes of death worldwide¹ and the prevalence of colorectal cancer (CRC) is ranked the third among cancers. CRC is the cause of 9.7% of all deaths in the world.² It has the third and fifth prevalence rank among Iranian women and men, respectively.³ Today, certain therapeutic methods are being utilized to treat cancer patients, including chemotherapy as one of the main powerful treatments. Chemotherapy drugs not only kill the cancer cells, but also intensively affect normal cells; therefore, these types of drugs have several unwanted side effects. To date, many scientists have proposed the use of less toxic and safer drugs, such as natural agents. The use of natural agents, for instance, herbal extracts or animal products and bodies, is one of the traditional medicine (TM) branches. In Traditional Chinese Medicine (TCM), the whole-body extract of a kind of Chinese gecko (*Swinhonia Güenther*) has been applied as a drug to treat various diseases, like cancer, for hundreds of years. In our previous studies, we also showed that a kind of gecko, named *Cyrtopodion scabrum* (so-called rough-tailed home gecko), has anti-proliferative effects on the breast, colorectal,⁷ stomach, and liver cancer cells.⁸ *C. scabrum* is distributed around the Indus Valley to the eastern borders of the Caspian Sea, including Iran, Turkey, Pakistan, Iraq, and so forth.⁹ The current study aimed to investigate the potential effects of *Cyrtopodion scabrum* extract (CSE) on the P53 protein level and gene expression, as well as the role of P53 transcriptional pathway in CSE apoptosis induction. The gene expression of p21 and mdm2, as the two target genes of P53 protein pathway, in response to CSE treatment was also investigated.

Materials and Methods

In the present experimental in-vitro research, we studied the effect of CSE on apoptosis induction and the involvement of some elements of P53 protein pathway.

Preparation of aqueous extract from animals

Cyrtopodion scabrum was provided by Razi Research Institute of Vaccine and Serum, Shiraz, Iran. The extract (CSE) was prepared exactly according to the protocol described previously by Amiri et al.⁷

Cell culture

SW742 colon cancer cell line was purchased from the National Cell Bank of Pasteur Institute of Iran. The cells were cultured in RPMI-1640 (shellmax, China) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of Penicillin, 100mg/ml of Streptomycin, and were incubated at 37°C in a 5% CO₂ incubator.

Western blotting

The cells were treated for 72 hours with complete medium for the control and complete medium containing three different concentrations of CSE (IC₅₀: 250, 2×IC₅₀:500 and 4×IC₅₀: 1000µg/ml) for the tests. According to our previous study, IC₅₀ for SW742 cells was calculated as 251±13µg/ml.⁸ The cell lysates were then prepared and the total protein concentrations were determined using Bradford assay. The samples were separated on a 12% SDS-PAGE gel and then transferred to the nitrocellulose membranes for western blotting. The following antibodies were utilized for probing: TP53 Antibody (Biogen BTP53-12, UK) for tests and β-actin (Abcam ab6276, USA) for controls. Image J software analysis was employed to determine the band intensity.¹⁰

Quantitative real-time polymerase chain reaction

2.6×10⁵ SW742 cells were seeded into 25 cm² (T-25) cell culture flasks and after 12 hours of incubation, the cells were treated with different concentrations of CSE for

72h, as mentioned in section 2.3. Following the total RNA extraction using the corresponding mini kit (Yekta Tajhiz Azma, Iran), cDNAs were synthesized with cDNA synthesis kit (Thermo Scientific, USA). Quantitative real-time PCR was performed using real-time thermocycler (Applied Biosystems-7500, USA). RPLP0 was utilized as a housekeeping gene. The primer sequences were 5'-ACTGACATTCTCCACTTCT-3' and 5'-CTTCTGACGCACACCTAT-3' for P53, 5'-GGGACAGCAGAGGAAGAC-3' and 5'-GCGTTTGGAGTGGTAGAAA-3' for p21, 5'-GCTCCGTGTTTGGTCAGTGG -3' and 5'-GATGTGGTCAGGGTAGATGGGT-3' for mdm2 and 5'-CCCTGAAGTGCTTGATA -3' and 5'-CCGTTGATGATAGAATGG -3' for RPLP0 genes. The thermal program was a denaturation step for 10 min at 95°C, followed by 40 cycles of 95°C for 10 sec, annealing (59.4 °C for P53, 63 °C for p21, 53.3 °C for mdm2 and 58.4 °C for RPLP0) for 15 sec, and 72°C for 30 sec.¹¹

P53 transcriptional activity inhibition

To investigate the possible relationship between the observed apoptosis in SW742 cells and the P53 transcriptional pathway, pifithrin (PFT)- α , the inhibitor of P53 transcriptional activity, was used, followed by flowcytometry analysis. 2.6×10^5 SW742 cells were seeded into each one of the four 25 cm² (T-25) cell culture flasks and incubated for 12h in the CO₂ incubator. Once reaching 70% confluency, the old media were removed and the flasks were treated as follows: one with vehicle (DMSO, 0.1% in media) as the control, one with CSE (1000 μ g/ml), and one with Pifithrin- α (30 μ M). The fourth flask was treated with Pifithrin- α (30 μ M) for 1 h, followed by CSE treatment (1000 μ g/ml), and incubated in a 5% CO₂ incubator at 37°C for 72h.¹² To evaluate the P53 transcriptional activity inhibition, PI staining/Flow Cytometry was

employed. The cells were stained with PI staining solution at 20 μ g/ml concentration followed by Flow-Cytometry according to Riccardi et al.'s method.¹³ Following the incubation time, the fluorescence intensities were read with Becton Dickinson (FACS Calibur, USA) and the results were analyzed via FLOWJO software (version 7.6, USA).

Statistical analysis

All the experiments were carried out in three independent experiments and the data were presented as mean \pm SEM. Statistical analyses were performed using SPSS 17 software (Chicago, USA). We compared the groups with one way ANOVA and LSD, as a post hoc test. *P* values ≥ 0.05 were considered to be significant.

Results

The effects of CSE on the expression of protein and mRNA of P53 in SW742 colon cancer cell line

As shown in figure 1, CSE induced the expression of P53 protein in SW742 cells. Western blot analysis of P53 protein expression after 72 h of incubation with CSE showed that this induction was significant in the 2 \times IC₅₀ and 4 \times IC₅₀ of the extract, yet insignificant in IC₅₀ (251 \pm 13 μ g/ml) compared to the control untreated cells (Figure 1A).

According to the results summarized in figure 1B, P53 mRNA expression level showed no significant changes in different CSE concentrations.

The effects of CSE on the gene expression of two P53 target genes, p21 and mdm2, in SW742 colon cancer cell line

Both p21 and mdm2 mRNA expression level significantly increased at 4 \times IC₅₀ (1000 μ g/ml) of CSE compared to the control (~2.8 and 2.7-times, respectively; *P*<0.05) (Figures 2A and 2B). The increase in p21 and mdm2 mRNA expression levels was not significant in the IC₅₀ and 2 \times IC₅₀ of CSE.

The relationship between apoptosis and P53 transcription pathway

Considering the fact that the Sub-G1 fraction percentage represents apoptotic cells, in this part of the experiment, the percentage of apoptosis was checked with and without PFT- α in SW742 cells treated with CSE. Figure 3 summarizes the results of flowcytometry analysis. As could be seen, the percentage of Sub-G1 fraction in the control and PFT- α treated groups was the same, showing that this concentration of PFT- α was not toxic to the cells. The comparison of the profile of SW742 cells treated with CSE alone or pretreated with PFT- α revealed that treatment with the combination of PFT- α and the extract had no significant effects on the Sub-G1 fraction percentage. However, treatment with both the extract and/or combination treatment (PFT- α and the extract) demonstrated a significant increase in Sub-G1 fraction percentage compared to the controls. The data suggested that the induction of apoptosis via CSE does not occur through the P53-dependent transcriptional pathway.

Discussion

In the present study, aiming to clarify the mechanism of action of CSE on apoptosis induction and the involvement of the P53 transcriptional pathway in this process, we applied SW742 colorectal cancer cells and performed a series of in-vitro experiments. Our findings indicated that the treatment of SW742 cells with different concentrations of CSE leads to increased expression of P53 protein in a dose-dependent manner. While P53 mRNA expression did not show any significant up-regulation, p21 and mdm2 mRNA expression, as two important P53 target genes, were up-regulated significantly, particularly at the highest concentration (1000 $\mu\text{g/ml}$) of CSE. The up-regulation of p21 mRNA was compatible with the results of our previous studies

which implied cell cycle arrest as the result of treatment of SW742 cells with CSE. Furthermore, flowcytometry analysis with PI staining did not show any significant effects on the subG1 population as the representative of apoptotic cells, following the inhibition of P53 transcription activity with Pifithrin (PFT)- α and CSE.

In the present study, we utilized SW742 cells since it was the most inhibited cell line among digestive cancer cells treated with CSE according to our previous work. The IC₅₀ value for CSE was also calculated as 251 ± 13 $\mu\text{g/ml}$. According to the data obtained in this part, the expression of TP53 increased in SW742 cells treated with different concentrations (IC₅₀, 2 \times IC₅₀, and 4 \times IC₅₀) of CSE in a dose-dependent manner. This up-regulation was significant in the cells treated with 2 \times IC₅₀ (1.5 fold) and 4 \times IC₅₀ (1.8 fold) of CSE compared to the control untreated cells. These results are in agreement with those obtained from apoptosis assessment in our previous study. The results of P53 mRNA expression revealed that it increased neither accordingly nor significantly in comparison to its protein, TP53. Such inconsistency between P53 protein and mRNA expression has also been reported previously¹⁵; it has been mentioned that “the induction of the P53 response upon stress occurs largely through alterations in the P53 protein and the changes in the rate of P53 transcription play a minor role, or no roles, in such induction”.¹⁶ P53 is known as one of the key tumor suppressor proteins that plays essential roles in appropriate progression of the cell cycle, thereby having the main role in cancer prevention.¹⁴ Herein, we demonstrated that CSE treatment affects the P53 protein pathway in SW742 cells through up-regulating the P53 tumor suppressor protein (TP53) expression.

P53 plays its role through the downstream genes, including p21 and mdm2, which

results in cell cycle arrest and apoptosis. The results of the expression pattern profiling of p21 gene showed a significant increase in 4×IC50 (1000 µg /ml) and not at IC50 or 2×IC50 of CSE. As mentioned above, at IC50, the P53 protein level was not significantly up-regulated compared to the controls; therefore, no increase in p21 mRNA was expected. However, at 2×IC50, despite the significant increase in the P53 protein level, the p21 mRNA level did not increase significantly. This result is in agreement with that of a study which showed the same inconsistency.¹⁵ However, it seems that a higher level of CSE and thereupon, is needed for induction of p21 mRNA expression, as seen at 4×IC50, with about 2.8 fold up-regulation compared to the untreated control cells. This significant induction of p21 gene is in line with previous reports about cell cycle analysis and G2 arrest of SW742 cells at the concentration of 4×IC50.⁸ p21(Waf1/Cip1) is an important target gene of P53,^{17, 18} which helps regulation of the cell cycle. P53 can transcriptionally up-regulate p21,¹⁹ which inhibits G1/S and G2/M phases; thus, P53 is capable of controlling both G1 and G2/M checkpoints through p21 function. Interestingly, the same profile was observed for mdm2 gene expression; accordingly, at the concentration of 4×IC50, about 2.7 fold up-regulation was observed compared to the control cells. In unstressed conditions, mdm2, as a P53 target gene, provided a negative feedback loop; therefore, its gene product, MDM2 protein, binds to and efficiently inhibits P53 protein.²¹ Mdm2 plays this role in two major ways: binding to the transactivation domain of P53²² and acting as an E3 ubiquitin ligase,²³ leading to changes in the localization and proteasomal degradation of TP53. In stressed conditions, the interaction between P53 and MDM2 will be disrupted through certain mechanisms, including P53 and MDM2 phosphorylation

and sequestration of MDM2 by p14 ARF.²⁴ The results of this section revealed that the CSE at the concentration of 1000 µg/ml (4×IC50) plays a positive role in the inhibition of cell proliferation by up-regulating these two genes as the consequence of P53 up-regulation.

The last part of this study was designed to investigate whether apoptosis occurs through P53 transcription pathway. The findings implied that blocking P53 transcriptional activity with PFT-α in SW742 cells treated with CSE did not affect the percentage of apoptotic cells. Pifithrin (PFT)-α is a small molecule that binds to the DNA binding domain of P53. It also blocks the interaction of P53 with the transcriptional cofactor p300, thereby inhibiting P53 transcriptional activity.¹² The name of Pifithrin is rooted in p-fifty three inhibitor.²⁵ P53 induces apoptosis through two mechanisms, including transcriptional-dependent and independent mechanisms, such as a direct localization of P53 to the mitochondria.²⁶ Our results are in accordance with those of another study that reported apoptosis induction through the P53 transcriptional independent pathway despite P53 protein elevation.¹² Therefore, CSE-induced apoptosis in SW742 cells might occurs independently of P53 transcriptional activity rather than via a P53 transcription-dependent mechanism.

In conclusion, the present in vitro study partially unfolded the role of CSE in P53 signaling pathway and showed that it induces P53 protein as well as p21 and MDM2 gene expression, which all play pivotal roles in cell cycle arrest. Furthermore, it clarified that induced apoptosis does not occur through the P53 transcriptional pathway. These findings are valuable since we tried to partially explore the mechanism of action of a putative natural anticancer drug which harbors no undesirable effects on normal cells.

However, numerous proteins and genes are involved in cancer progression in the cells, which may be affected by CSE treatment, yet were not included in our study. Therefore, further investigation concerning the mechanism of growth suppression is needed. In addition, we could suggest further evaluation of the efficacy of the extract in the animal model, which is fortunately under way in the parallel projects.

The current study is part of a mega project with the ultimate objective of applying CSE in clinical trial and the evaluation of its efficacy and safety as complementary and/or alternative medicine for patients suffering from cancer.

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Conflict of Interest

None declared.

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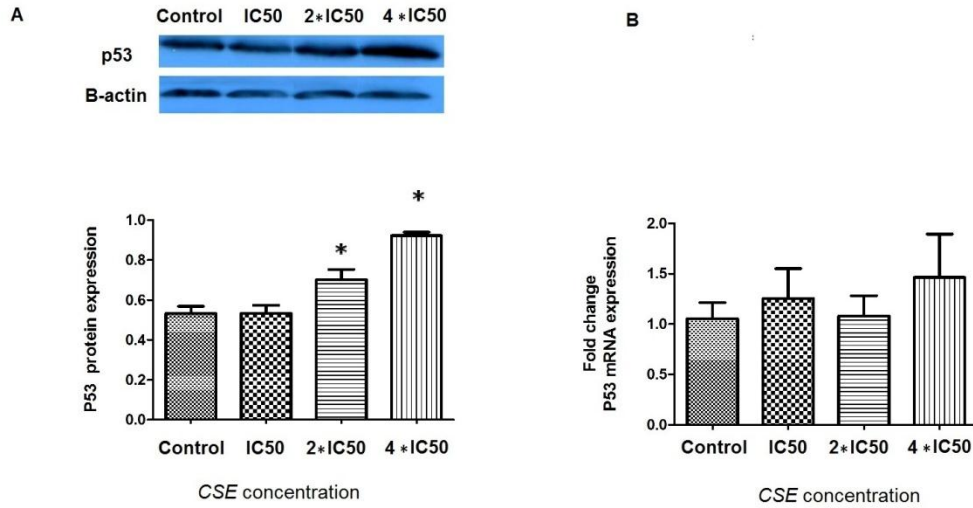


Figure 1. The expression of P53 protein and gene in SW742 treated with CSE. (A) Western blot analysis of P53 protein and its quantification in the control and CSE-treated groups after 72h of treatment. The graph represents the mean values \pm SEM of three independent experiments. (B) Quantitative RT-PCR of P53 gene in the control and CSE-treated groups after 72h of treatment. The graph represents the mean values \pm SEM of three independent experiments. * Significant vs. control ($P < 0.05$). CSE: *Cyrtopodium scabrum* Extract

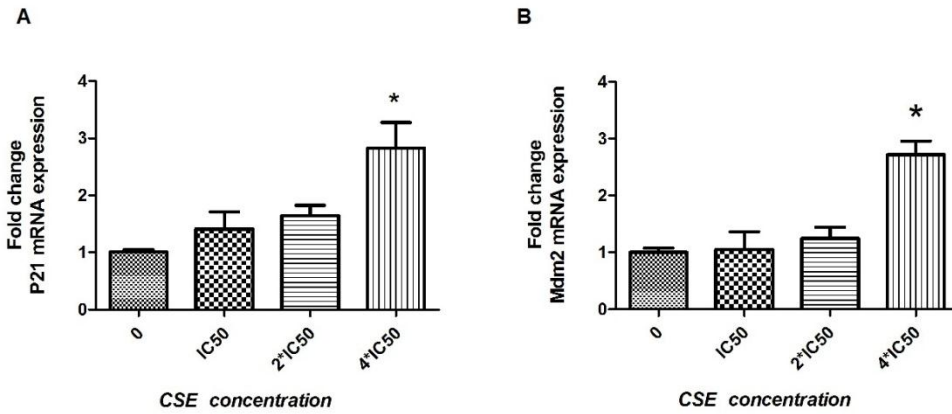


Figure 2. The expression of p21 and mdm2 genes in SW742 treated with CSE. Quantitative RT-PCR analysis of (A) p21 and (B) mdm2 genes in the control and CSE-treated groups after 72h of treatment. The graph represents the mean values \pm SEM of three independent experiments. * Significant vs. control ($P < 0.05$)

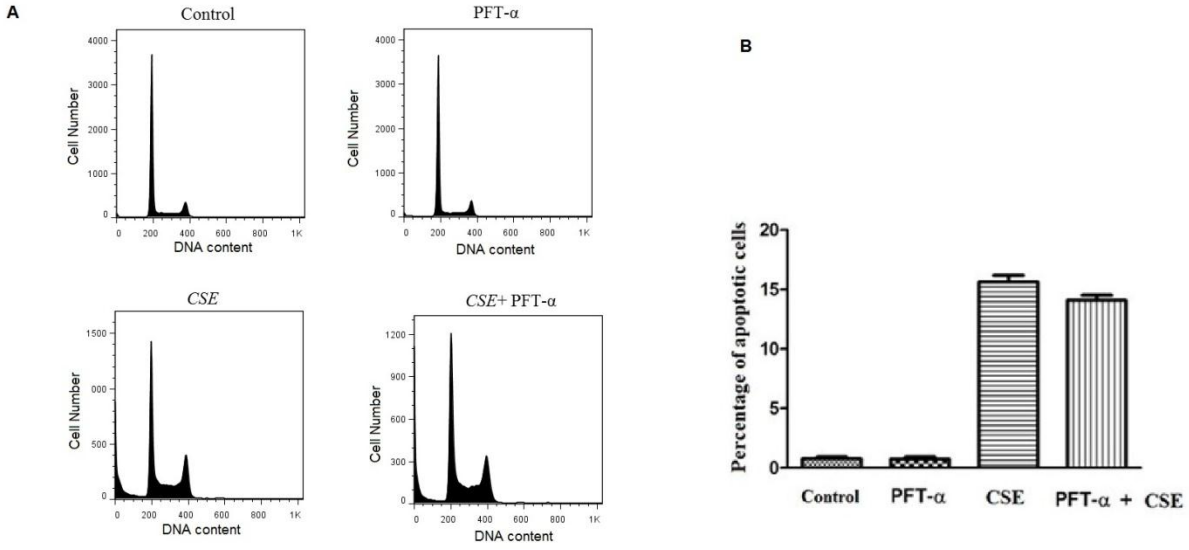


Figure 3. Flowcytometry analysis of the apoptotic SW742 cells treated with CSE and PFT- α . Controls, PFT- α , and CSE groups were treated with vehicle (DMSO, 0.1% in media), PFT- α (30 μ M), and CSE (1000 μ g/ml), respectively. For PFT- α +CSE, the cells were pre-treated with 30 μ M of PFT- α for 1 h, then incubated with CSE for 72 h. The obtained data are presented as mean values \pm SEM of three independent experiments.*Significant vs. control ($P < 0.05$).CSE: *Cyrtopodion scabrum* Extract, PFT- α : pifithrin- α .