Pharmacogn. Mag.
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ORIGINAL ARTICLE

Evaluation of Apoptotic and Cytotoxic Effect of Robinin in TPC-1 and SW1736 Human Thyroid Cancer Cells

Yawei Shen, Periyannan Velu[1], Xin Huang, Taoping Dang[2]

Department of General Surgery, Xi'an Central Hospital, China, [1]Department of Biochemistry and Biotechnology, Annamalai University, Annamalai Nagar, Chidambaram, Tamil Nadu, India, [2]Department of General Surgery, Tongchuan People's Hospital, Tongchuan, China

Submitted: 17-Jun-2021 Revised: 05-Feb-2022 Accepted: 11-Mar-2022 Published: 07-Jul-2022

ABSTRACT

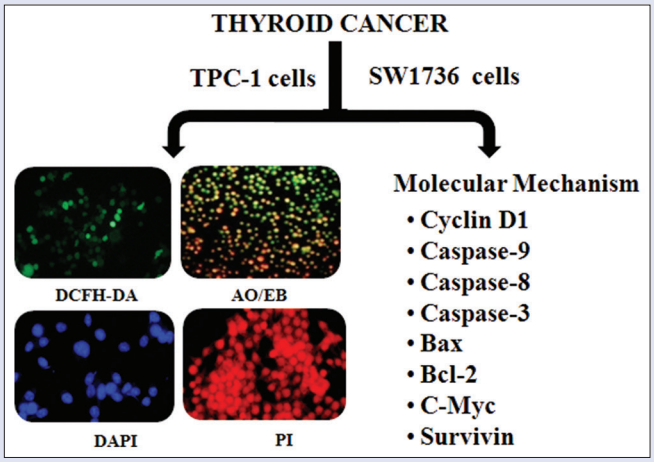
Background: During the past few years, thyroid cancer (TC) has increased in terms of rate of morbidity and mortality. Plant flavonoids have shown positive effect in regulating thyroid tumorigenesis via inhibition of apoptosis. Robinin is a natural flavone glycoside isolated from *Vinca erecta* with potent pharmacological activities. **Materials and Methods:** In this study, we aimed to explore the apoptotic and cytotoxic activity of robinin against TPC-1 and SW1736 cells. **Results:** Robinin (20 μM/mL) significantly suppressed the growth and cell proliferation and induced apoptotic activity in TC cells. According to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test, TPC-1 and SW1736 cancer cells revealed 100% cell viability. Robinin inhibited growth of TC cells in a dose-dependent manner. It was found that reactive oxygen species (ROS) generation in TPC-1 and SW1736 cancer cells was decreased; however, robinin (20 μM/mL) enhanced ROS formation. The study of apoptosis in TPC-1 and SW1736 cells revealed morphological changes and damaged nuclei. Robinin (20 μM/mL) triggered a powerful apoptosis signal and caused loss of membrane integrity in TC cells. It also increased the activity of caspases 8 and 9. Robinin (20 μM/mL) decreased the levels of Bcl-2, c-Myc, and cyclin-D1 and increased the levels of Bax and caspase-3 when compared to control and robinin-treated cells. It exhibited potent antiproliferative and apoptotic activity in TC cells. **Conclusion:** Robinin can be useful in the treatment of thyroid cancer.

Key words: Apoptosis, proliferation, robinin, ROS, thyroid cancer

SUMMARY

- Robinin inhibited cell proliferation of TC cells.
- Robinin increased apoptosis in TPC-1 and SW1736 cells.

Abbreviations used: TC: thyroid cancer; PTC: papillary carcinoma; ATC: anaplastic carcinoma; FC: follicular carcinoma, MTC: medullary carcinoma; PTC: papillary thyroid cancer; ATC: anaplastic thyroid cancer; P13K/AKT: phosphatidylinositol 3-kinase/protein kinase B; DMEM: Dulbecco's Modified Eagle's Medium; FBS: fetal bovine serum; AO: acridine orange; EB: ethidium bromide; MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI: propidium iodide; OD: optical density; DCFH-DA: 2′-7′-dichlorodihydrofluorescein diacetate; DAPI: 4′,6-diamidino-2-phenylindole.



Correspondence:

Prof. Taoping Dang,
Department of General Surgery,
Tongchuan People's Hospital, Tongchuan - 727100,
China.
E-mail: sheffyxu@sina.com

DOI: 10.4103/pm.pm_270_21

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INTRODUCTION

Thyroid cancer (TC) is the most common malignancy which includes papillary carcinoma (PTC), anaplastic carcinoma (ATC), follicular carcinoma (FC), and medullary carcinoma (MTC). Its prevalence is constantly increasing worldwide.[1,2] PTC makes up 85% of all TCs,[3] and ATC accounts for a major part of morbidity and mortality, as it contributes upto 14% - 50% annually among all TCs. ATC is the most dangerous type of TC, which fails to take up iodine.[4,5] At present, most TCs can be managed by a combination of radioiodine and levothyroxine after thyroidectomy. The aggressive growth of ATC and the decreased ability to take up radioiodine make it difficult to treat patients with ATC. Conventional chemotherapeutic treatment is also less effective in ATCs.[6] The rate of occurrence of TCs has increased recently.[7,8] Induction of apoptosis in these carcinomas is one of the novel therapeutic approaches. Therefore, the development of a novel bioactive compound with less toxicity is highly essential to increase the survival rate of patients with TC.

Programmed cell death plays a key role in the growth and homeostasis of human beings. Prevention of apoptosis leads to cancer formation, including TC.[9,10] The genetic changes and their accumulation causes the pathogenesis and progression of TCs.[11] The signal transduction of phosphatidylinositol 3-kinase/protein kinase B (P13K/AKT) is recognized as the key process in human TCs.[12] c-Myc modulates the process of apoptosis.[13] Survivin is

Cite this article as: Shen Y, Velu P, Huang X, Dang T. Evaluation of apoptotic and cytotoxic effect of robinin in TPC-1 and SW1736 human thyroid cancer cells. Phcog Mag 2022;18:360-5.

an inhibitor of apoptosis that modulates cell growth and proliferation.[14] Regulatory cell death protein caspase-3 has profound action in apoptosis and tumorigenesis.[15] *Bax* is a popular proapoptotic gene, whereas Bcl-2 has anti-apoptotic action in cancer cells.[16] It has been shown that Bcl-2 protein possesses antioxidant activity in *in vitro* systems.[17] However, the molecular changes in the pathogenesis of TCs have not been fully evaluated. Therefore, further research to understand the exact mechanisms of tumor progression is highly warranted.

Several studies have suggested that flavonoids have selective antiproliferative and anticarcinogenic role in preventing various cancers.[18–20] Flavonoids regulate thyroid tumorigenesis via inhibition of apoptosis.[21–23] Robinin is a natural flavone glycoside of plant origin with potent biological activities.[24,25] So far, there are no studies conducted to evaluate the beneficial effect of robinin in TC. Therefore, in this study, we aimed to analyze the cytotoxic and apoptotic effects of robinin in TPC-1 and SW1736 cells.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), antibiotics, robinin, and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Maintenance of Cell Culture

TPC-1 and SW1736 cancer cells were obtained from the Peking Union Cell Resource Center (Beijing, China). The cells were routinely grown in DMEM consisting of fetal bovine serum (FBS) (10%), streptomycin (100 μg/mL), and penicillin (100 units/mL). The cells were grown in a humidified incubator at 37°C with 5% CO_2.

Cell Treatment with Robinin

Robinin was administered at a dose of 5, 10, 20, and 30 μM/mL in TPC-1 and SW1736 cells and incubated for 24 h at 37°C.

Proliferation Assay by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to analyze cell toxicity. TPC-1 and SW1736 cells were grown in medium supplemented with 5, 10, 20, and 30 μM/mL of robinin and incubated at 37°C. Then, MTT stain was added at a working concentration (1 mg/mL) to each well and incubated for 4 h at 37°C. The formazan crystals formed were dissolved using 150 μL of dimethyl sulfoxide (DMSO). Then, the blue color developed was read at 490 nm.[26] The inhibitory concentration (IC_{50})value was calculated and the selected dose was employed for further studies.

Apoptosis Analysis

The cell morphology was analyzed by staining the cells with acridine orange (AO)/ethidium bromide (EB).[27] TPC-1 and SW1736 cells were grown in medium supplemented with robinin (15 and 20 μM/mL) followed by application of the dye. Then, the cells were kept for 20 min under dark. The excess dye was washed using phosphate-buffered saline (PBS), and a fluorescence microscope was used for detection.

Analysis of Reactive Oxygen Species Levels by Staining with Dichlorodihydrofluorescein Diacetate Dye

Intracellular reactive oxygen species (ROS) oxidizes nonfluorescent 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) dye into a fluorescent compound by the action of intracellular esterases.[28] Around 2×10^5 TC cells/mL (control and 20 μM/mL robinin) were grown for 24 h and subsequently washed with medium; then, 100 μL of DCFH-DA (10 μM) dye was added. The cells were incubated for 20 min under dark at 37°C. Cells were washed twice using DMEM medium, and 1×10^4 cells/mL were stained with dye and detected through a fluorescence microscope.

Examination of Apoptosis by Staining with 4′,6-Diamidino-2-phenylindole

As per your suggestion, human thyroid cancer cells TPC-1 and SW1736 were seeded in the concentration of 1×105 cells in each well of 6-well plate. Then robinin 20 μM/ml were treated and incubated at overnight. These treated cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to examine the nucleus changes allied with apoptosis by the method described previously.[29] Then, the samples were mounted on a glass slide and observed through a BX51 fluorescence microscope (Olympus).

Analysis of Apoptosis by Propidium Iodide Staining

The propidium iodide (PI) staining assay was used to examine the apoptotic nuclei of human TC cells TPC-1 and SW1736. As per your suggestion, human thyroid cancer cells TPC-1 and SW1736 were seeded in the concentration of 1×105 cells in each well of 6-well plate. Then robinin 20 μM/ml were treated and incubated at 24 hr. Then, they were harvested and stained with PI according to the protocol.[30] The red fluorescence formed from the nuclei was assessed through a fluorescence microscope (Olympus).

Apoptosis Assay for Caspases 8 and 9

Apoptosis was quantified using the enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, Thermo Fisher Scientific Inc.) based on the manufacturer's instructions. Three independent replicates were performed.

Total mRNA Analysis

TRIzol reagent was used to isolate RNA from TC cells. The cDNA Reverse Transcription kitkit (Bio-Rad Laboratories Pvt. Ltd., Hercules, California, USA) was used to reverse transcribe the RNA into cDNA. The FastStart SYBR Green master mix makes use of a stain to analyze the cDNAs. The experimental conditions of PCR thermocycler were as follows: the mix was activated by incubation for 5 min at 95°C, followed by 38 cycles of amplification at 95°C for 46 s and at 60°C and 72°C for 60 s. The mRNA expression was estimated by the previously reported method.[31]

Statistical Analysis

Statistical analysis was performed using GraphPad prism software version 8.0.1 with comparison achieved by one-way analysis of variance (ANOVA) and Duncan's test. P values less than 0.05 were considered as significant.

RESULTS

Analysis of Cell Proliferation by Robinin on TC Cells

MTT assay showed 100% cell viability in TPC-1 and SW1736 cancer cells. TPC-1 and SW1736 cells supplemented with robinin showed significant inhibition of proliferation in a dose-dependent manner. Robinin (20 μM/mL) significantly ($P < 0.05$) reduced the viability of TPC-1 cells and SW1736 cancer cells. Exposure of TPC-1 and SW1736 cells to 30 μM/mL robinin caused significant ($P < 0.05$) reduction in TC cells' proliferation. Robinin was tested further at a concentration of 20 μM/mL (IC_{50}) in TPC-1 [Figure 1a] and SW1736 cancer cells [Figure 1b].

Effect of Robinin-Induced Apoptosis via Generation of ROS

Control TPC-1 and SW1736 cancer cells showed low levels of ROS, whereas the levels were increased in robinin (20 μM/mL)-treated cells. Treatment with 20 μM/mL robinin significantly ($P < 0.05$) increased ROS generation in TPC-1 and SW1736 cancer cell lines [Figure 2a and b].

Influence of Robinin on Apoptosis

The AO/EB staining was performed to visualize the apoptotic cells. TPC-1 and SW1736 cancer cells showed evenly green-colored cells, which were viable. Robinin caused apoptotic changes in TPC-1 [Figure 3a] and SW1736 [Figure 3b] cells, compared with the control cells. Robinin (20 μM/mL)-treated cells revealed late apoptotic stage in TPC-1 and SW1736 cells. Highest apoptotic activity was obtained for 20 μM/mL robinin.

Detection of Apoptosis by DAPI Staining

Untreated TC cells stained with DAPI revealed the presence of normal viable cells. Supplementation of robinin influenced apoptosis in TPC-1 [Figure 4a] and SW1736 [Figure 4b] cancer cells. Robinin (20 μM/mL) caused chromatin condensation, loss of nuclear envelop, and cellular fragmentation in TPC-1 and SW1736 cells. These results indicated that robinin brings about apoptosis.

Detection of Apoptosis by PI Staining

Robinin induced programmed cell death inTPC-1 and SW1736 cancer cells, as measured by PI staining technique. PI staining procedure corresponds with the membrane alteration-associated apoptosis. PI penetrates into the cells with a damaged cell membrane. This shows that robinin (20 μM/mL) increased the apoptotic effects on TPC-1 [Figure 5a] and SW1736 [Figure 5b] cancer cells.

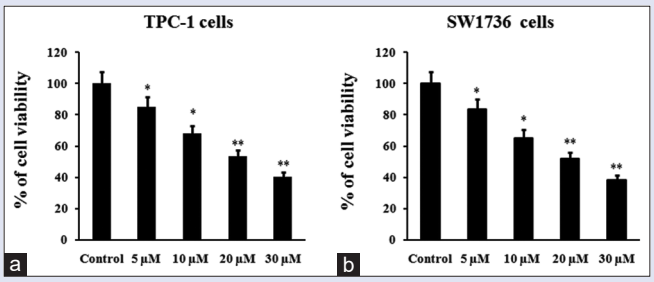

Figure 1: Effect of robinin on TC cell proliferation. a & b) Cell viability of TPC-1 cells and SW1736 with different 5, 10, 20 and 30 μM/ml concentration of robinin showed the growth reduction in a dose dependent manner at 37°C for 24 h incubation respectively. MTT assay was used to measure the viable cells. Results are presented as mean ± SEM. *$P < 0.05$ as compared to the control cells. MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, SEM = standard error of the mean, TC = thyroid cancer

Measurement of Activities of Caspases 8 and 9 by ELISA

Robinin (20 μM) significantly ($P < 0.05$) increased the activities of caspases 8 and 9 than those seen in control cells [Figure 6a and b].

Analysis of Cyclin-D1, Bcl-2, Bax, Caspase-3, c-Myc, and Survivin mRNAs in Robinin-Treated TPC-1 and SW1736 Cells

Figure 7a and b depicts the mRNA analysis of robinin-treated TPC-1 and SW1736 cancer cells. The expression of cyclin-D1, Bcl-2, c-Myc, and survivin mRNAs was upregulated, whereas Bax and caspase-3 mRNAs were downregulated. TPC-1 and SW1736 cells supplemented with 20 μM/mL of robinin showed significant downregulation ($P < 0.05$) in the expression of cyclin-D, Bcl-2, c-Myc, and survivin mRNAs, whereas the expression of Bax and caspase-3 was upregulated when compared to that in control TPC-1 and SW1736 cells.

DISCUSSION

TC is the most common endocrine malignancy, and globally, its incidence rate is gradually increasing.[32,33] PTC is the most frequent thyroid tumor.[34] In ATC, there is presence of invasive tumors and metastasis, which do not respond to chemotherapy.[4,35] Aggressive thyroid tumors do not respond well to conventional chemotherapeutic agents. Previous findings have revealed that molecular and genetic changes ultimately increase the prognosis of ATC.[36] The potential mechanism involved in PTC and ATC metastasis remains unknown. Therefore, a novel bioactive compound is essential for the treatment of TCs. Hence, we evaluated the apoptotic and antiproliferative efficacy of robinin through regulation of P13K/AKT pathway in human TC cells, namely, TPC-1 and SW1736.

Robinin is a chemical compound isolated from *Vinca erecta*[37] or from the common locust *Robinia pseudoacacia*.[38] It is a flavone glycoside based on kaempferol. Many pharmacological activities of robinin have been documented. For example, Janeesh and Abraham[24] have reported the cardioprotective effect of robinin, wherein they reported that robinin modulated doxorubicin-induced apoptosis in heart. Furthermore, Janeesh *et al.*[25] have extensively studied the modulation of robinin in Toll-like receptors (TLRs)/ nuclear factor-kappaB (NF-κB) signaling pathway. Therefore, in this study, we selected robinin to analyze its antiproliferative and apoptotic activity in TPC-1 and SW1736 TC cells.

According to the results, robinin inhibited the proliferation of TC cells in a dose-dependent manner. The IC_{50} value of robinin was 20 μM/mL, which was used to conduct further analysis.[39]

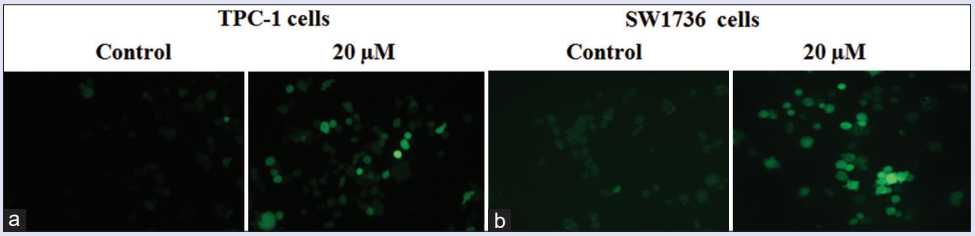

Figure 2: (a and b) Effect of robinin on ROS generation in TC cells. TPC-1 and SW1736 cells were grown in medium treated with robinin 20 μM/ml of robinin. The ROS generation was assessed by DCFH-DA. DCFH-DA = dichlorodihydrofluorescein diacetate, ROS = reactive oxygen species, TC = thyroid cancer

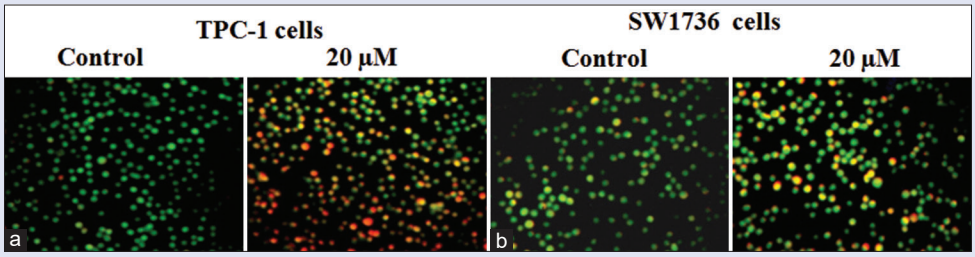

Figure 3: (a and b) Effect of robinin on TC cells' apoptosis assessed by AO/EB staining. TPC-1 and SW1736 cells were grown in medium treated with robinin 20 µM/ml of robinin. AO/EB = acridine orange/ethidium bromide, TC = thyroid cancer

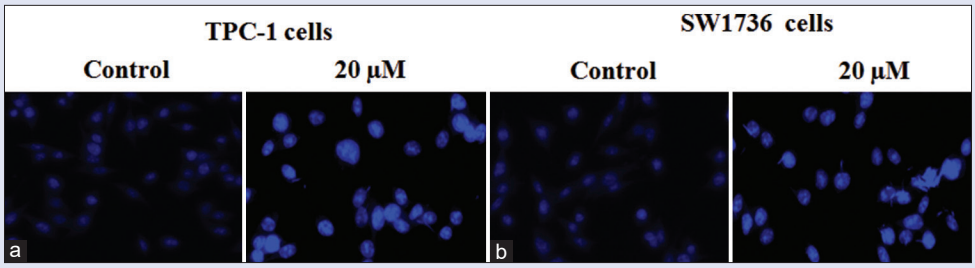

Figure 4: (a and b) Effect of robinin on TC cells' apoptosis measured by DAPI staining. TPC-1 and SW1736 cells were grown in medium treated with robinin 20 µM/ml of robinin. DAPI = 4′,6-diamidino-2-phenylindole, TC = thyroid cancer

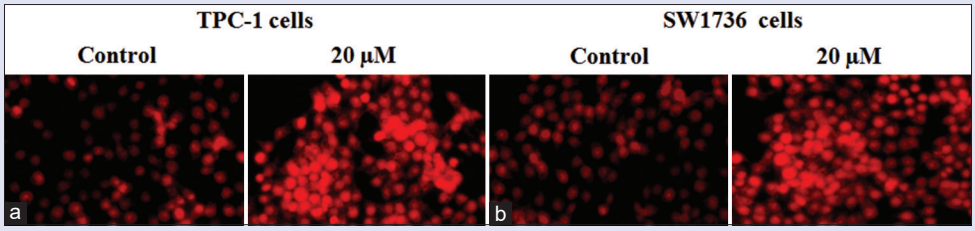

Figure 5: (a and b) Effect of robinin on TC cells' apoptosis measured by PI staining. TPC-1 and SW1736 cells were grown in medium treated with robinin 20 µM/ml of robinin. PI = propidium iodide, TC = thyroid cancer

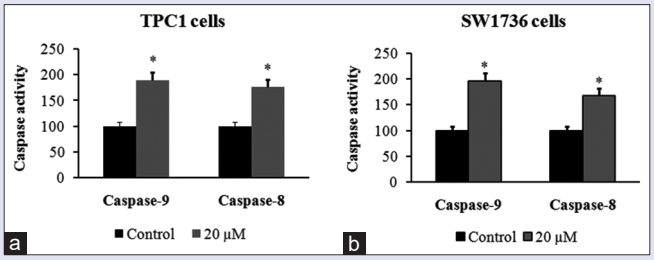

Figure 6: (a and b) Effect of robinin on the activities of caspase-9 and caspase-8 in TPC-1 and SW1736 cells. TPC -1 and SW1736 cells were grown in medium treated with robinin 20 µM/ml. The cell caspase was estimated by ELISA. Results are presented as mean ± SEM. *P < 0.05 versus 20 µg/mL robinin-treated cells. ELISA = enzyme-linked immunosorbent assay, SEM = standard error of the mean

Literature suggests that high levels of ROS can initiate programmed cell death.[40] According to our results, ROS generation was high in robinin-treated TPC-1 and SW1736 cells based on DCFH-DA staining method. Increased ROS generation caused by the anticancer therapeutic drug leads to the oxidative damage of cancer cells.[41] ROS generation is the primary mechanism responsible for the cytotoxicity.[42] Elevated levels of ROS may stimulate mitochondrial apoptosis cascade.

Our results implied that robinin reduced proliferation and enhanced apoptosis in TPC-1 and SW1736 cells. AO is a vital dye that stains both live and dead cells, whereas EB stains only those cells that have lost their membrane integrity.[39] Green-stained cells represent live cells, early apoptotic cells reveal yellow color, and late apoptotic cells show reddish orange color. In this study, robinin (20 µM) caused morphological changes, such as chromatin condensation, swelling of membrane, burst nuclei, and late apoptosis, in TPC-1 and SW1736 cells. These results show that robinin was effective in inducing apoptosis in TPC-1 and SW1736 cells.

Induction of apoptosis by robinin was also determined through DAPI and PI staining of TPC-1 and SW1736 cells. Robinin (20 µM/mL) induced chromatin condensation, loss of nuclear envelop, and cellular fragmentation in TPC-1 and SW1736 cells. These results show that robinin induced programmed cell death.[43]

Measurement of the activities of caspases 8 and 9 was performed by ELISA. Robinin increased the activities of caspase-8 and -9 in TPC-1 and SW1736 cells, when compared to control cells. Caspases

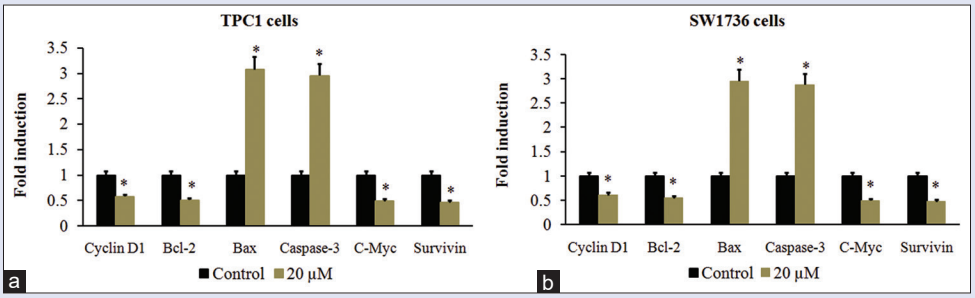

Figure 7: (a and b) Effect of robinin on TPC-1 and SW1736 cells' mRNA gene expression by RT-PCR. TPC-1 cells were grown in medium treated with robinin 20 µM/ml of robinin. The levels of cyclin D1, Bcl-2, Bax, caspase-3, c-Myc, and survivin in TPC-1 cells were analyzed by RT-PCR. Results are presented as mean ± SEM. *$P < 0.05$ as compared to the control cells. RT-PCR = reverse transcription-polymerase chain reaction, SEM = standard error of the mean

are essential mediators of apoptosis. Caspase-8 mediates the extrinsic pathway and caspase-9 initiates the intrinsic pathway of apoptosis.[44]

Next, we analyzed the mRNA expression of cyclin-D1, Bcl-2, Bax, caspase-3, c-Myc, and survivin in TPC-1 and SW1736 cells. The control cells showed increased mRNA levels of cyclin-D1, Bcl-2, c-Myc, and survivin, whereas Bax and caspase-3 were decreased. Robinin (20 µM) significantly downregulated the expression of cyclin-D1, Bcl-2, c-Myc, and survivin and upregulated the expression of Bax and caspase-3, as compared to the control cells. Cyclin-D1 is upregulated in tumor cells, which increases the rate of cell proliferation and carcinogenesis.[45] Our results are in line with these findings.

CONCLUSION

In conclusion, the results of this study demonstrate that under *in vitro* conditions, robinin acts as a potent anti proliferative and apoptotic agent, as tested against TPC-1 and SW1736 cells. These results might be useful to develop an advanced therapeutic treatment for TC.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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