Original Article

Running Title: Anticancer and Apoptotic Effect of Carob Bean
Received: December 01, 2021; Accepted: December 25, 2022

Anticancer Effect of Carob Bean Extract on Human Prostate Cancer Cell Lines: Apoptosis Induction and Bax/Bcl-2 Ratio Improvement

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Abstract

Background: Carob (*Ceratonia siliqua L.*) has been used to cure various diseases in traditional medicine. This plant has also exerted anti-proliferative effects on certain cancer types. The present study aimed to determine the effects of carob bean extracts on proliferation and apoptotic genes (Bax, Bcl-2, and P53) and Caspase-3, -8, and -9 expressions of human prostate cancer cell lines**.**

Method: In this in vitro experimental study, human prostate cell lines (LNCaP and PC3) were treated with carob bean extract (0, 50, 100, 200, 400, and 800 µg/ml) for 24, 48, 72, and 96 h. Cell viability was investigated via MTT assay. The genes expression: Bax (pro-apoptotic), Bcl-2 (anti-apoptotic), Caspase-3 (required enzyme for the execution of apoptosis), Caspase-8 (mediator of the extrinsic pathway), and Caspase-9 (mediator of the intrinsic pathway) genes as well as P53 (tumor suppressor) were assessed using real-time polymerase chain reaction. Moreover, the nitric oxide in culture media was evaluated.

Results: Carob bean extract suppressed the proliferation of prostate cancer cells in a time and dose-dependent manner by inducing intrinsic apoptotic pathways and decreasing nitric oxide production (P <0.01). The obtained results revealed the overexpression of Caspases-3 and -9, P53, and Bax, but reduction in Bcl-2 expression, giving rise to a higher Bax/Bcl-2 ratio (P <0.05).

Conclusion: The carob bean extracts exerted anti-prostate cancer properties via induction of apoptosis. It could be also suggested as a dietary supplement for patients suffering from prostate cancer.

Keywords: Prostatic neoplasm, Apoptosis, Antineoplastic agents, Locust bean gum

Introduction

Prostate cancer is the second cause of male cancer deaths, with conventional treatment options, including radiation therapy, surgery, and chemotherapy. However, chemotherapy often induces severe side effects, such as diarrhea, fatigue, anorexia, vomiting, hair loss, nausea, constipation, and thrombocytopenia.[1] Prostate cancer treatment is believed to be an essential principle in male diseases, and combination or synergic therapy is considered in cancer management. Our previous work showed that noscapine enhanced the therapeutic effect of paclitaxel in prostate cancer cells (LNCaP and PC3) through the induction of apoptosis.[2]

Ceratonia siliqua (carob), from the *Leguminosae* family, is mainly consumed in the food industry as the source of gum. The plant parts (leaves and fruits) are commonly utilized for alleviating various diseases in traditional medicine. Different parts of carob tree have antioxidant, hypoglycemic, anxiolytic, sedative, anti-microbial, and nephroprotective properties.[3] The major constituents of carob beans are polyphenol and flavonoids, such as caffeic, cinnamic, coumaric, ferulic, gallic, gentisic, and synergic acids, as well as gallocatechin, gallocatechin gallate, apigenin, catechin, genistein, kaempferol, and quercetin. Carob leaf infusion induced apoptosis in a mouse cell line of hepatocellular carcinoma.[4] Furthermore, the aqueous extract of carob fiber suppressed the proliferation of human colorectal cell lines (HT29 and LT97).[5]

Methanolic extracts of carob leaf exerted anti-proliferative, anti-cancer, and cytotoxic effects on HeLa cells.[6] They also demonstrated that carob bean extract (CBE) reduced the viability of breast cancer (MDA-MB-231) and cervical cancer (HeLa) cell lines.[7] In another study, CBE exerted inhibitory effects on some human cancer cell lines (HCT-116, MDA-MB-231, and HeLa cells).[8] A study reported that carob leaf polyphenol triggered the intrinsic apoptotic pathway in colon cancer cells.[9] Since there is no scientific report on the effect of carob bean on prostate cancer, the present study investigated the anti-tumor activity of CBE on two prostate cancer cell lines (LNCaP and PC3) in vitro.

Material and Methods

In this in vitro experimental study, two prostate cancer cell lines, namely PC3 (bone metastasis-derived) and LNCaP (lymph node metastasis-derived), were obtained from the national cell bank. Trypsin, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Co. (Germany). Fetal bovine serum (FBS) and RPMI 1640 (Roswell Park Memorial Institute) medium were obtained from Gibco Company (Denmark).

Extract preparation

Dried carob beans were purchased and authenticated by an academic expert, whose pulp and seeds were then powdered. For extract preparation, 200 g of the powder was added to 800 ml of 70% ethanol, and the mixture was left to macerate at room temperature. Subsequently, the soaked beans were extracted through the percolation method, and the obtained extract was concentrated in a vacuum and dried on a flat surface.[10]

Cell culture

Antibiotic-free RPMI 1640 supplemented with FBS (10%) was used for cancer cell culture. The two prostate cancer cell lines (LNCaP and PC3) were cultured in T25 flasks and incubated in standard conditions (37°C humidified with 5% CO2). 10^5 cells were used in each well of a 24-well culture dish and treated under different concentrations of the extract (0, 50, 100, 200, 400, and 800 µg/ml) dissolved in cell culture media for 24, 48, 72, and 96 h. Three wells

were used for each concentration in each exposure time.

MTT assay

Cell viability was determined using the MTT assay, as described previously.[11] briefly; the cells were cultured in 96-well plates (10^4 cells /well). After exposure to different concentrations of the extract (0, 50, 100, 200, 400, and 800 µg/ml), they were dissolved in cell culture media for 24, 48, 72, and 96 h. Three wells were utilized for each concentration in each exposure time. The media were removed, and MTT solution (0.5 mg/mL) was added to each well and incubated for 4 h. Positive control for cytotoxicity is the cells treated with a cytotoxic drug/chemical (800 µg/ml) +MTT. Negative control for cytotoxicity is the cells left untreated +MTT. In this study, negative control wells were treated with an equivalent amount of a medium alone. The formazan crystals produced by living cells were dissolved using DMSO (100 µL). The resulting solutions' optical density (OD) was measured with an ELISA reader at 570 nm and 630 nm. The percentage of cell viability was calculated according to the following formula:

Cell viability (%) = [OD570, 630 (sample)/OD570, 630 (control)] ×100

Nitric oxide (NO) assay

The Griess colorimetric method was used for NO level assay. After the cell lines (LNCaP and PC3) were treated with CBE (200, 400 µg/ml) for 48 h, the supernatants were collected and deproteinized; 6 mg zinc sulfate powder was added to 400 µL supernatant, following which 100 µL of the supernatant was mixed with 100 µL Vcl3 (vanadium chloride III), 50 µl sulfonamide 2%, and 50 µl naphthyl ethylenediamine dihydrochloride (NEED) (0.1%). It was then incubated at 37° C for 30 min. The absorbance of the samples was measured at 540 and 630 nm with an ELISA reader (Stat fax 100, USA). Each experiment was performed in triplicate. The concentrations of 0, 6.25, 12.5, 25, 50, 100, and 200 µM sodium nitrates were used for standard solution.[12]

Real-time polymerase chain reaction (PCR)

Through the use of Real-time PCR, we analyzed the effects of different extract concentrations on the expression of apoptotic-related genes, as described previously. Briefly, via the RNA isolation kit (DENA Zist, Tehran, Iran), total RNA extraction was carried out, and using the cDNA synthesis kit (BIOFACT. Daejeon, South Korea), complementary DNA (cDNA) synthesis was done. We also utilized SYBR Premix Ex Taq technology (Takara Bio Inc., Shiga, Japan) in real-time PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was served as an internal control. The Applied Biosystems Step One Real-Time PCR System was used (Applied Biosystems7500, Life Technologies, ABI Corporation, Wisconsin, USA). The data were collected and analyzed with the $\mathbf{2^{-\Delta\Delta Ct}}$method. The apoptotic genes include Bax (pro-apoptotic), Bcl-2 (anti-apoptotic), Caspase-3 (required enzyme for the execution of apoptosis), Caspase-8 (mediator of the extrinsic pathway), and Caspase-9 (mediator of the intrinsic pathway) genes as well as P53 (tumor suppressor).

Ethical consideration

Kermanshah University of Medical Sciences financially supported the present study, furthermore, the Ethics Committee of the University approved this work [ethics code: IR.KUMS.REC.1397.897]. This article does not contain any studies with human participant or animals.

Statistical analysis

All the experiments herein were performed in triplicate. Data were reported as mean ± SE and analyzed with the PRISM software package (Ver. 8, Chicago, Inc.). Kolmogorov-Smirnov test was used for evaluating the parametric distribution. The

results were analyzed through one-way analysis of variance (ANOVA) and Tukey post-hoc test. $P \leq 0.05$ was considered as the level of significance.

Results

Cell viability

The IC50 values were 577.5, 406.5, 295.1, and 174.4 for LNCaP and 496.2, 216.5, 173, and 115.6 for PC3 cell lines respectively after 24, 48, 72, and 96 h of treatment, using Graph Pad Prism 7 software (Graph Pad Software Inc, San Diego, USA). The MTT assay indicated that various doses of CBE (0, 50, 100, 200, 400, and 800) and its differences in duration of treatment affect the viability of cancer cell lines (LNCaP and PC3), suggesting CBE cytotoxic effect in a dose- and time-dependent manner. The PC3 cancer cells, however, were more sensitive to the extract, as shown by its IC_{50} values (Figure 1).

No production

NO concentration in cell culture media was detected using the Griess assay. A dose-dependent decrease in NO production by prostate cancer cells indicated the effect of different concentrations of CBE after 48 h of treatment (Figure 2). The extract showed a more significant effect on NO production by PC3 cells. Compared to the control group, the difference was significant with the 50, 100, and 200 µg/mL doses for LNCaP and 100 and 200 µg/mL doses for PC3 cells ($P < 0.01$).

Apoptotic-related genes expression

In the treated cancer cells, P53 and Bax expression were up-regulated while *Bcl-2* was down-regulated significantly. Since apoptosis is controlled by the ratio of Bax (pro-apoptotic) to Bcl-2 (anti-apoptotic) protein, the expression levels of Bcl-2 and Bax mRNA in the cells were measured after the treatment. Furthermore, the extract led to increased mRNA expression of Caspase-3 (required enzyme for the execution of apoptosis) and Caspase-9 (mediator of the intrinsic pathway) genes (Figure 3).

Discussion

The present study revealed that CBE had an inhibitory effect on the proliferation of prostate cancer cell lines (PC3 and LNCaP) in a dose- and time-dependent manner. The IC50 values demonstrated that PC3 cells were more sensitive to the extract than LNCaP. The MTT assay confirmed the CBE cytotoxicity against these two distinct prostate cancer cell lines (PC3 and LNCaP). LNCaP cells exhibit the characteristic features of prostate cells whereas PC3 cells show a more aggressive phenotype.[13] Most chemotherapy agents target normal and malignant proliferating cells, thereby causing serious side effects, such as bone marrow suppression and thrombocytopenia.[12] The potential of anti-cancer agents to distinguish between cancer and normal cells is essential in designing and discovering novel chemotherapeutic drugs.[14]

NO has a bi-functional regulatory effect on apoptosis, and its levels are generally associated with angiogenesis, tumor growth and metastasis, and tumor-related immune suppression. Additionally, our findings demonstrated that the extract decreased NO production in prostate cell lines. Inhibitory and NO-inducing effects on apoptotic signaling pathways have been reported in many experimental models (in vitro and in vivo). NO acts as an endogenous apoptosis suppressor by inhibiting caspases, cytochrome c release, and cGMP-dependent inhibition and induction of apoptotic and cytoprotective stress proteins.[15] Decreased NO production by the extract may remove the NO anti-apoptotic effect in cancer cells.

According to the obtained results, CBE had an apoptotic-induction potential in both cell lines in a dose-dependent manner, but PC3 cells were less sensitive to the extract than LNCaP cells. Cancer cells, in most

cases, exhibit resistance to apoptosis to sustain their uncontrolled proliferation, and therefore, any apoptosis-inducing agent is desirable as a chemotherapeutic agent against cancer.[16]

The receptor-mediated (extrinsic pathway) and the mitochondrial-mediated (intrinsic pathway) pathways are the two main apoptotic pathways in cells in which caspases contribute to the intrinsic pathway. Caspase-3 belongs to the aspartate-specific cysteinyl protease family and is an effector member involved in the final step of both apoptotic pathways. In contrast, Caspases-9 and -8 are initiators in the intrinsic and extrinsic pathways.[17]

Our results showed that Caspase-3 gene expression significantly increased upon treatment with CBE in a dose- and time-dependent manner, leading to DNA fragmentation and cell death. Caspases-3, -9, and -8, P53, Bax, and Bcl2 genes were evaluated in order to determine which apoptotic pathway the extract induced. The apoptosis process is controlled by various tumor suppressor genes, including P53 and proto-oncogenes. Bax plays a crucial role in apoptotic initiation while Bcl2 acts conversely to retain cellular survival. In our case, overexpression in Caspases-3, and -9, P53, and Bax, without changes in Caspase-8, indicated that CBE activated the intrinsic pathway. Furthermore, CBE treatment down-regulated Bcl2 accompanied by enhanced Bax expression, which gave rise to higher Bax/Bcl2 ratios regarded as a driving force to prompt apoptosis.

Different parts of the carbo bean, such as fruits, leaves, and seeds, contain large amounts of polyphenols, tannins, quercetin, myricetin, gallotannins, methyl gallate, and gallic acid, some of which, such as gallic acid, have been shown to possess potent antioxidant and chemopreventive effects.[18-22] Regarding the limitations of this study, we could mention the lack of assessment of the genes associated with extrinsic apoptotic pathway in addition to not using any standard drugs for prostate cancer treatment.

Conclusion

The findings of present study could suggest carob as a potent therapeutic agent against prostate cancer. Carob beans could be beneficial for developing novel dietary supplements for cancer treatment.

Acknowledgments

This study was funded by the Kermanshah University of Medical Sciences [grant number: 97812].

Conflict of Interest

None declared.

References

1. Yedjou CG, Mbemi AT, Noubissi F, Tchounwou SS, Tsabang N, Payton M, et al. Prostate cancer disparity, chemoprevention, and treatment by specific medicinal plants. *Nutrients.* 2019;11(2):336. doi: 10.3390/nu11020336.
2. Rabzia A, Khazaei M, Rashidi Z, Khazaei MR. Synergistic anticancer effect of paclitaxel and Noscapine on human prostate cancer cell lines. *Iran J Pharm Res.* 2017;16(4):1432-42.
3. Faramarzi A, Aghaz F, Golestan Jahromi M, Bakhtiari M, Khazaei M. Does supplementation of sperm freezing/thawing media with Ceratonia siliqua improve detrimental effect of cryopreservation on sperm parameters and chromatin quality in normozoospermic specimens? Cell Tissue Bank. 2019;20(3):403-9. doi: 10.1007/s10561-019-09779-2.
4. Khazaei AH, Faramarzi A, Khazaei M. Therapeutic and functional application of ceratonia siliqua: tradition remedies and new research finding. Journal of Knowledge & Health in Basic Medical Sciences

2022;17(3):18-25. doi.org/10.22100/jkh.v17i3.2862
5. Klenow S, Glei M, Haber B, Owen R, Pool-Zobel BL. Carob fibre compounds modulate parameters of cell growth differently in human HT29 colon adenocarcinoma cells than in LT97 colon adenoma cells. *Food Chem Toxicol.* 2008;46(4):1389-97. doi: 10.1016/j.fct.2007.09.003.
6. Custódio L, Fernandes E, Escapa AL, López-Avilés S, Fajardo A, Aligué R, et al. Antioxidant activity and in vitro inhibition of tumor cell growth by leaf extracts from the carob tree (Ceratonia siliqua). *Pharm Biol.* 2009;47(8):721-8.
7. Custódio L, Escapa AL, Fernandes E, Fajardo A, Aligué R, Alberício F, et al. In vitro cytotoxic effects and apoptosis induction by a methanol leaf extract of carob tree (Ceratonia siliqua L.). *J Med Plant Res.* 2011;5(10):1987-96.
8. Custodio L, Fernandes E, Escapa A, López-Avilés S, Fajardo A, Aligue R, et al. Antiproliferative and apoptotic activities of extracts from carob tree (Ceratonia siliqua L.) in MDA-MB-231 human breast cancer cells. *Planta Medica.* 2008;74(09):PA48. doi: 10.1055/s-0028-1084046.
9. Ghanemi FZ, Belarbi M, Fluckiger A, Nani A, Dumont A, De Rosny C, et al. Carob leaf polyphenols trigger intrinsic apoptotic pathway and induce cell cycle arrest in colon cancer cells. *J Funct Foods.* 2017;33:112-21. doi: 10.1016/j.jff.2017.03.032.

10. Khazaei F, Farzaei MH, Khazayel S, Khazaei M . Trifolium pratense extract induces apoptosis and decreases nitric oxide secretion in human umbilical vein endothelial cells, WCRJ. 2019; doi: 10.32113/wcrj_20203_1508
11. Khazaei S, Khademi A, Nasr Esfahani MH, Khazaei M, Nekoofar MH, Dummer PMH. Isolation and differentiation of Adipose-derived stem cells into Odontoblast-like cells: A preliminary in vitro study. *Cell J.* 2021;23(3):288-93. doi: 10.22074/cellj.2021.7325.
12. Khazaei F, Ghanbari E, Khazaei M. Protective effect of royal jelly against cyclophosphamide-induced thrombocytopenia and spleen and bone marrow damages in rats. *Cell J.* 2020;22(3):302-9. doi: 10.22074/cellj.2020.6703.
13. Dozmorov MG, Hurst RE, Culkin DJ, Kropp BP, Frank MB, Osban J, et al. Unique patterns of molecular profiling between human prostate cancer LNCaP and PC-3 cells. *Prostate.* 2009 1;69(10):1077-90. doi: 10.1002/pros.20960.
14. Crawford S. Is it time for a new paradigm for systemic cancer treatment? Lessons from a century of cancer chemotherapy. *Front Pharmacol.* 2013;4:68. doi: 10.3389/fphar.2013.00068.
15. Kim YM, Bombeck CA, Billiar TR. Nitric oxide as a bifunctional regulator of apoptosis. *Circ Res.* 1999;84(3):253-6. doi: 10.1161/01.res.84.3.253.
16. Tor YS, Yazan LS, Foo JB, Armania N, Cheah YK, Abdullah R, et al. Induction of apoptosis through oxidative stress-related pathways in MCF-7, human breast cancer cells, by ethyl acetate extract of Dillenia suffruticosa. *BMC Complement Altern Med.* 2014;14:55. doi: 10.1186/1472-6882-14-55.
17. Elmore S. Apoptosis: a review of programmed cell death. *Toxicol Pathol.* 2007;35(4):495-516. doi: 10.1080/01926230701320337.
18. Owen RW, Haubner R, Hull WE, Erben G, Spiegelhalder B, Bartsch H, et al. Isolation and structure elucidation of the major individual polyphenols in carob fibre. *Food Chem Toxicol.* 2003;41(12):1727-38. doi: 10.1016/s0278-6915(03)00200-x.
19. Papagiannopoulos M, Wollseifen HR, Mellenthin A, Haber B, Galensa R. Identification and quantification of polyphenols in carob fruits (Ceratonia siliqua

L.) and derived products by HPLC-UV-ESI/MSn. *J Agric Food Chem.* 2004;52(12):3784-91. doi: 10.1021/jf030660y.
20. Lepley DM, Li B, Birt DF, Pelling JC. The chemopreventive flavonoid apigenin induces G2/M arrest in keratinocytes. *Carcinogenesis.* 1996;17(11):2367-75. doi: 10.1093/carcin/17.11.2367.
21. Kumazawa S, Taniguchi M, Suzuki Y, Shimura M, Kwon MS, Nakayama T. Antioxidant activity of polyphenols in carob pods. *J Agric Food Chem.* 2002;50(2):373-7. doi: 10.1021/jf010938r.
22. Kasai H, Fukada S, Yamaizumi Z, Sugie S, Mori H. Action of chlorogenic acid in vegetables and fruits as an inhibitor of 8-hydroxydeoxyguanosine formation in vitro and in a rat carcinogenesis model. *Food Chem Toxicol.* 2000;38(5):467-71. doi: 10.1016/s0278-6915(00)00014-4.

Table 1. The primer sequences of genes

Gene	Primer sequences	Product length
Tp53	F: 5′-TAACAGTTCTGCATGGGCGGC-3 R: 5′-AGGACAGGCACAAACACGCACC-3′	121 bp
Bax	F: 5′-CCTGTGCACCAAGGTGCCGGAACT-3′ R: 5′-CCACCCTGGTCTTGGATCCAGCCC-3′	99 bp
Bcl2	F: 5′-TTGTGGCCTTCTTTGAGTTCGGTG-3′ R: 5′-GGTGCCGGTTCAGGTACTCAGTCA-3′	114 bp
Caspase -3	F: 5´-CAAACTTTTTCAGAGGGGATCG-3' R: 5´-GCATACTGTTTCAGCATGGCAC-3´	119 bp
Caspase -8	F: 5´-CCTGATGACATGAACCTGCTG-3´ R: 5´-GCTCTTGTTGATTTGGGCACAGAC-3´	128 bp
Caspase -9	F: 5´-CATCCTTGTGTCCTACTCCACC-3' R: 5´-CAGCTTTTCCGGAGGAGTAAGT	115 bp
GAPDH	F: 5´-GTCTCCTCTGACTTCAACAGCG-3´ R: 5´- ACCACCCTGTTGCTGTAGCCAA-3´	120 bp

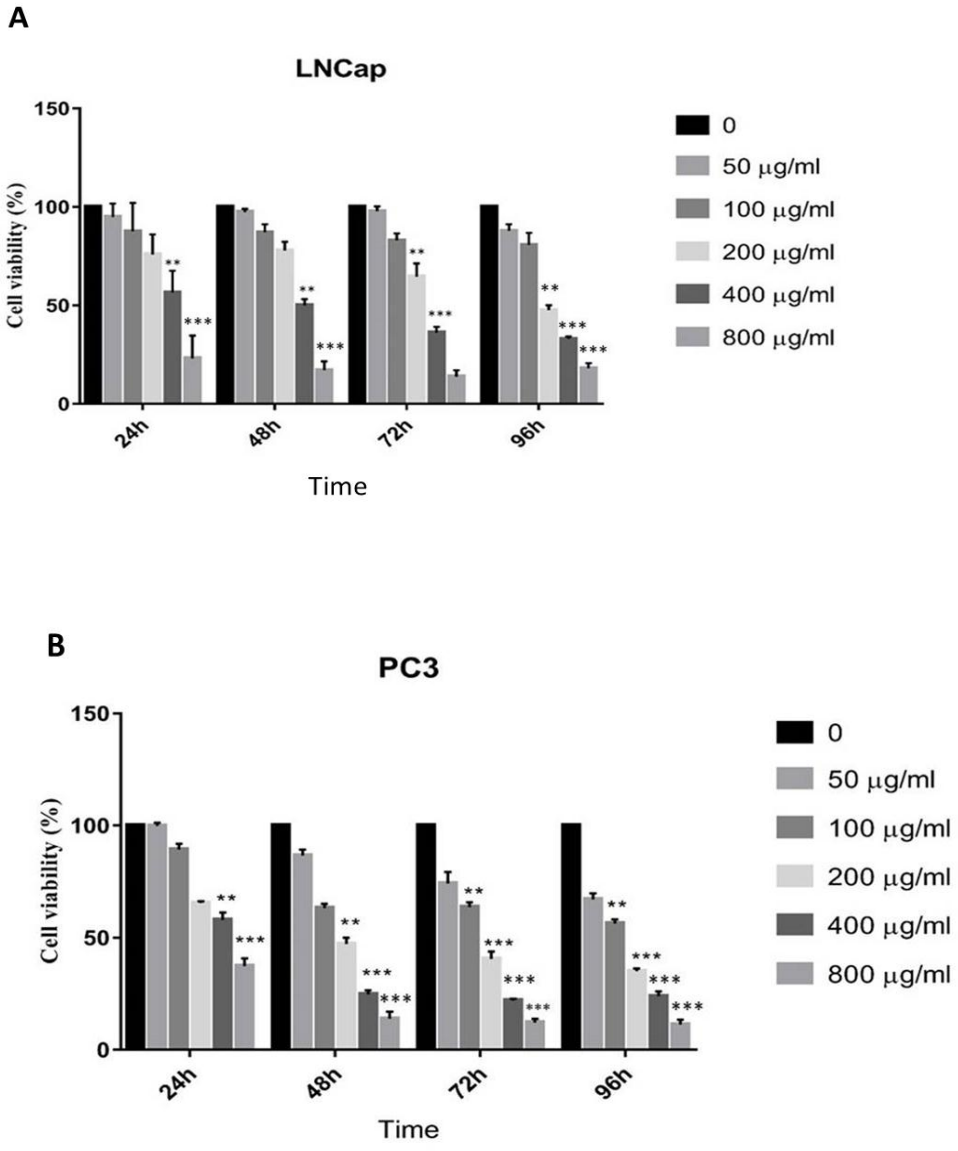

Figure 1. The effect of different doses of CBE on the cell lines LNCaP (A) and PC3 (B) for 24, 48, 72, and 96 h. Cells viability was assessed using the MTT assay. One-way ANOVA was utilized with GraphPad Prism 7 software (GraphPad Software Inc, San Diego, USA). *$P < 0.05$; **$P < 0.01$, ***$P \leq 0.001$ compared with the control

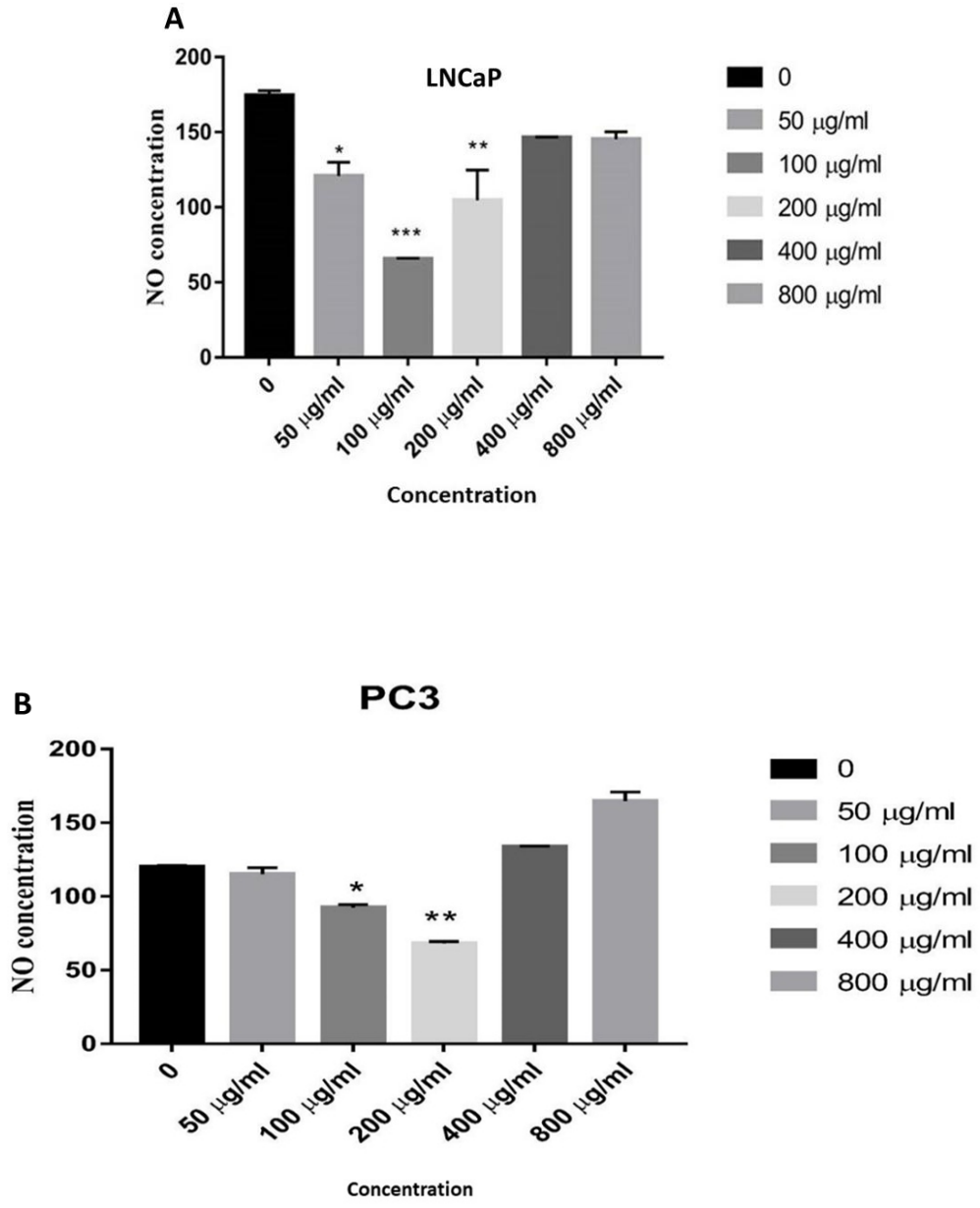

Figure 2. The effect of carob extracts on NO production LNCaP (A) and PC3 (B) was measured based on Griess reaction after 48 h of treatment. The data (means ± SD) are presented as the percentage of the control cells. One-way ANOVA was utilized with GraphPad Prism 7 software. *P < 0.05; **P < 0.01, ***P ≤ 0.001 compared with the control

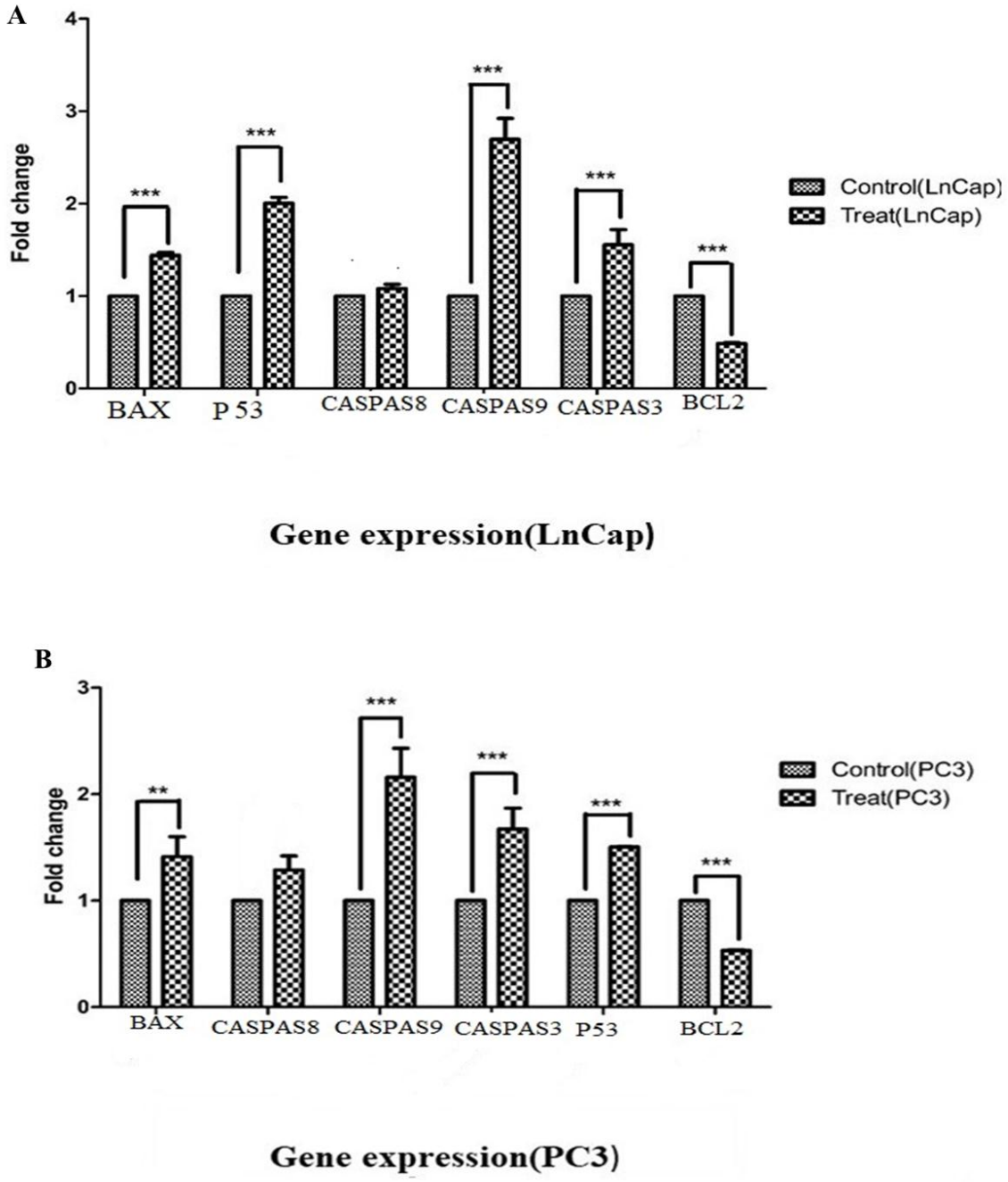

Figure 3. The effect of different doses of CBE on the expression level of *P53*, *Bax*, *Bcl-2*, *Caspase-3*, *-8*, and *-9* genes in LNCaP (A) and PC3 (B) cells at 48 h. The data (means ± SD) are reported as the percentage of the control cells. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ compared with the control.