Research Article

Effect of chebulagic acid on apoptotic regulators in prostate cancer cell line - PC-3

R. Ponnulakshmi[1], B. Shyamaladevi[2], J. Selvaraj[2]*, G. Valli[1], V. Purushothaman[1], Murad Alsawalha[3], Surapaneni Krishna Mohan[4]

ABSTRACT

Background: Prostate cancer (PCa) is the second most typical cancer and also the sixth leading reason behind cancer death among men worldwide with associate degree calculable recorded amount of 1.1 million cases and 307,000 deaths in 2012. Chebulagic acid (CA), a benzopyran tannin, is one of the major bioactive compounds present in the fruits of *Terminalia chebula*. **Aim:** The present study was aimed at assessing the effects of CA on proapoptotic (Bax) and antiapoptotic protein molecules such as B-cell lymphoma-2 (Bcl-2) and Bcl-extra large (Bcl-xl) in PC-3 cell line. **Materials and Methods:** Antiproliferative potential of CA was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. PC-3 cells were treated with different concentration of CA (1, 5, 10, 25, 50, and 100 µM). Pro- and anti-apoptotic proteins were done by western blotting methods. PC-3 cells were treated with the treatment of CA resulted in a dose-dependent inhibition of the growth of PC-3 cells. This was associated with increased levels of proapoptotic protein (Bax) and the reduced levels of the antiapoptotic proteins Bcl-2 and Bcl-xL. **Conclusion:** The present findings clearly suggest that CA induces apoptosis by the regulating intrinsic pathways which could be very useful for the treatment of PCa.

KEY WORDS: Apoptosis, B-cell lymphoma-2, Chebulagic acid, PC-3 cells

INTRODUCTION

Cancer is the second leading cause of death and is becoming the leading one in the old age.[1] Prostate cancer (PCa) is the second most typical cancer and also the sixth leading reason behind cancer death among men worldwide, with associate degree calculable recorded amount of 1.1 million cases and 307,000 deaths in 2012.[2] Incidence rates are comparatively high in certain less developed regions, rock bottom incidence rates are discovered in Central and Eastern European countries and additionally in Asian Populations. Mortality rates are usually high in preponderantly African-American Population.[3]

The most risk factors associated with PCa are as follows: Smoking and drinking, family medical history, race/ethnicity, genetic changes, low intake of fresh fruit and vegetables, geographical area, occupation, use of micronutrients, steroid medicines, inflammation of prostate, obesity, and sexually transmitted diseases.[4,5] Many scientific literatures explained the real burden of diseases, especially in developing countries which reported the importance of effective preventive actions. Hence, the objective of the present study was to examine the molecular mechanisms of the anticancer effect of chebulagic acid (CA) on PCa.

CA, a benzopyran tannin, is one of the major bioactive compounds present in the fruits of *Terminalia chebula*. *T. chebula* fruits have been used in various preparations of Ayurvedic medicines to treat several diseases such as coronary disorders, digestive and allergic problems, and infectious diseases such as cough and skin disorders[6] reported that CA inhibits the cytotoxic T-lymphocyte-mediated cytotoxicity and suppresses arthritis in mice[7] and lipopolysaccharides (LPS)-induced nitric oxide generation in RAW 264.7 mouse macrophage cells.[8] Gao *et al*. also described that CA inhibits the ROS production in phorbol myristate acetate-stimulated leukocytes and alpha-

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[1]Department of Central Research Laboratory, Meenakshi Academy of Higher Education and Research, Chennai, Tamil Nadu, India, [2]Department of Biochemistry, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, Tamil Nadu, India, [3]Department of chemical and Process Engineering, Technology, Jubail Industrial College, Jubail Industrial City, Saudi Arabia, [4]Department of Biochemistry, Chetinad Hospital and Research Institute, Chetinad Academy of Research and Education, Chennai, Tamil Nadu, India.

***Corresponding author:** Dr. J. Selvaraj, Department of Biochemistry, Saveetha Dental College and Hospitals, Saveetha Institute of Medical and Technical Sciences, Savvehtha University, Chennai - 600 077, Tamil Nadu, India. E-mail: jselvaendo@gmail.com

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glycosidase activity.[9] Reddy *et al*.[10] demonstrated that the anti-inflammatory effect of CA was mediated by suppression of nuclear factor kappa B and mitogen-activated protein kinase activation in LPS-induced mouse macrophage cells - RAW 264.7. It has been reported that it acts as cyclooxygenase-2/5-lipoxygenase dual inhibitor and exhibits anti-proliferative effects in varied of human cancer cell line.[11] Hence, in our study, we aimed to investigate the effect and molecular mechanism of CA on apoptotic regulators in PCa cell line.

MATERIALS AND METHODS

Chemical and Reagents

All the chemicals used in this study were of extra pure and analytical grade. CA and dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Pvt., Ltd. (St. Louis, MO., USA), polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Billerica, Massachusetts, USA). Trypsin-EDTA, fetal bovine serum (FBS), antibiotics-antimycotics, Roswell Park Memorial Institute (RPMI) medium, and phosphate-buffered saline (PBS) were purchased from Gibco, United States. Primary antibodies against B-cell lymphoma-2 (Bcl-2), Bcl-extra large (Bcl-xL), and Bax were purchased from cell signaling (Danvers, Massachusetts, United States) and Santa Cruz Biotechnology (Texas, United States). The secondary antibodies, horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG, and goat-anti-rabbit IgG were obtained from Santa Cruz Biotechnology (Texas, United State).

Cell Line Maintenance

PC-3 cell lines were obtained from the National Centre for Cell Science (Pune, India). The cells were grown in T-25 culture flasks in RPMI medium supplemented with 10% FBS with 1% penicillin/streptomycin and 1% amphotericin B. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. On attaining the confluence, the cells were trypsinized and plated.

Cell Viability Assay

The cell viability assay was performed by MTT assay, which determines mitochondrial activity in living cells. Cells were seeded in a 96-well plate at a density of 5×10^4 cells/well and incubated for 24 h at 37°C, 5% CO_2 incubator. After attachment, cells were washed with PBS and then incubated with serum-free medium for 6–12 h. CA was dissolved in DMSO with different concentration CA (1, 5, 10, 25, 50, 100, and 200 μM) and added to the cells. After the treatment period, 20 ml medium was removed and 20 ml of MTT was added and incubated for 30 min. Then, 100 ml of DMSO was added to solubilize the crystals and was kept in dark for 10 min. The intensity of color development was measured at 570 nm in ELISA reader. The cell viability was calculated as follows: Cell viability = absorbance of treated cells/absorbance of control cells ×100%.

Cell Lysate Preparation

PC-3 cells were plated in a Petri dish of 100 mm × 20 mm at a density concentration of 1×10^6 cells/Petri dish and grown in RPMI medium, respectively, with 10% FBS. After 24 h (~70–80% confluent), both the cells were treated with CA (20 and 40 mM/ml) for 24 h. At the end of the treatment, cells were washed once with ice-cold PBS and added 300 ml of ice-cold RIPA buffer with protease inhibitors. The Petri dishes were placed on ice and left on a shaker rocking for 2 min. Cell lysates were collected into a 1.5-ml tube and centrifuged at 14,000× g for 10 min at 4°C. The supernatant was separated and the protein concentration of supernatants was determined.

Western Blotting Analysis

The cell lysates (50 mg) were electrophoresed in 12% sodium dodecyl sulfate-polyacrylamide gel and then transferred onto PVDF membranes. The membranes were incubated with primary antibodies against Bcl-2, Bcl-xl, and Bax, Bad, caspase-9, caspase-3, and β-actin in Tris-buffered saline. After washing, the membranes were incubated with HRP conjugated anti-mouse IgG (1:5000) and goat anti-rabbit IgG (1:5000). The protein bands were detected using chemiluminescence system (ECL kit) and quantified in ChemiDoc XRS Imaging System, Bio-Rad.

Statistical Analysis

Data were expressed as mean ± standard error of the mean. Statistical analyses were performed using one-way ANOVA followed by Duncan's tests for comparison between treatment and control values using the Statistical Package for Student version 17.0 (SPSS Inc., Chicago, IL) software $P < 0.05$ was considered to be statistically significant.

RESULTS

Effect of CA on the Cell Viability

CA reduced the survival of PC-3 cells in a dose-dependent manner (1, 5, 10, 25, 50, and 100 μM) concentration. However, it significantly decreased the viability of PC-3 cells in 24 h with IC_{50} values of 25, 50, and 100 μM/ml, respectively [Figure 1]. Hence, 25 and 50 μM concentrations were used for further studies.

Effect of CA on Bax Protein Expression in PC-3 Cells

To determine whether proapoptotic protein is involved in the CA-induced apoptosis, protein expression analysis was examined. After the CA treatment, the expression of proapoptotic protein, Bax, was significantly increased [Figure 2]. At 50 μM concentration, CA found to have the maximum effect.

Effect of CA on Bcl-2 and Bcl-xL Protein Expression in PC-3 Cells

To examine the status of intracellular signaling molecules in the CA-treated cells, protein expression analysis was performed. CA treatment significantly decreased the antiapoptotic proteins such as Bcl-2 and Bcl-xL [Figures 3 and 4]. However, the 50 µM dose of CA showed the maximum effect compared to 25 µM.

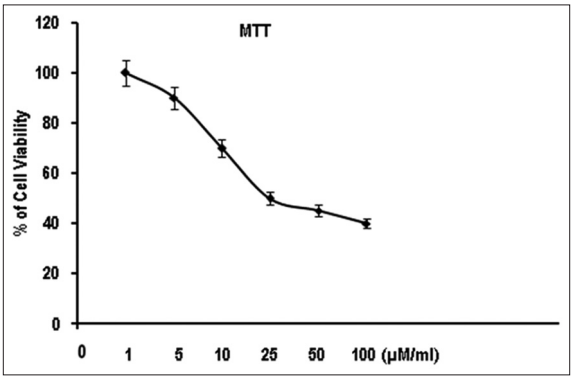

Figure 1: Effect of chebulagic acid (CA) on the viability of prostate cancer (PCa) cells. PC-3 cells were cultured in Roswell Park Memorial Institute medium supplemented with 10% fetal bovine serum and incubated with indicated concentrations of CA for 24 h. For cell viability assay, cells were exposed to different doses (0–100µM) of CA for 24 h. CA inhibits growth (as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay) of human PCa cells. Each bar represents the mean ± standard error of the mean of five independent observations and the statistical significance between control and the treated groups at $P < 0.05$ level

DISCUSSION

Apoptosis or programmed cell death, an extremely preserved cellular mechanism, is concerned in homeostasis of tissue. Dysregulation of programmed cell death troubling the balance between cell proliferation and death contributes to the development and progression of internal organ malignancy.[12] Programmed cell death is a mechanism in which the cells are actively uses a genetically controlled program to cause its own end throughout the tissue reworking of embryogenesis. It could be a key mechanism inflicting cell death and organ diseases, failure of apoptosis is currently understood to contribute to the event of human malignancies. Induction of tumor cell death by apoptosis has been accepted as one of the fundamental objectives of cancer therapy and identified as a novel mechanism of potential chemopreventive drug.[13]

Bcl-2 family proteins are central regulators of caspase-mediated cell death and act totally on the

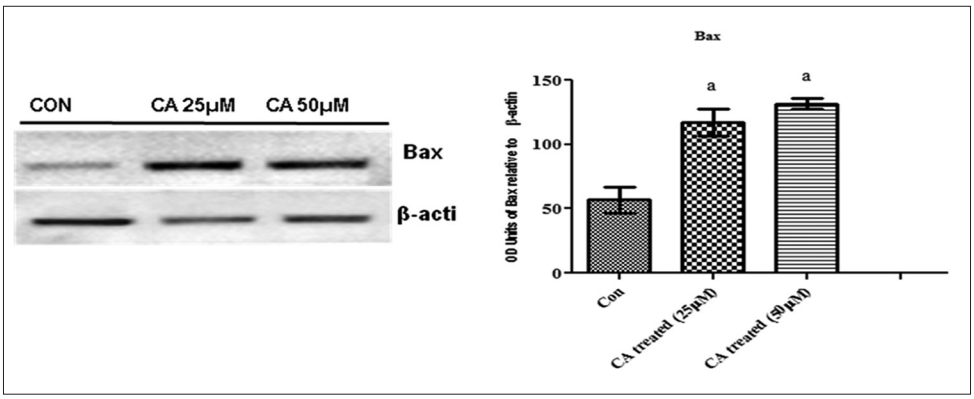

Figure 2: Effect of chebulagic acid (CA) on proapoptotic (Bax) protein expression in PC-3 cells. Cells were treated with 25 and 50 µM of CA for 24 h. Protein levels were quantified using densitometry analysis and are expressed in relative intensity arbitrary unit. Each bar represents mean ± standard error of the mean of six independent observations. a: Compared to control

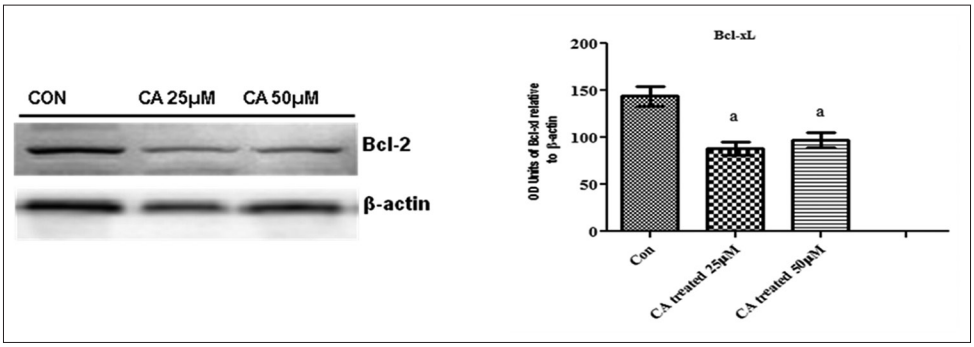

Figure 3: Effect of chebulagic acid (CA) on Bcl-2 protein expression in PC-3 cells. Cells were treated with 25 and 50 µM of CA for 24 h. Protein levels were quantified using densitometry analysis and are expressed in relative intensity arbitrary unit. Each bar represents mean ± standard error of the mean of six independent observations. a: Compared to control

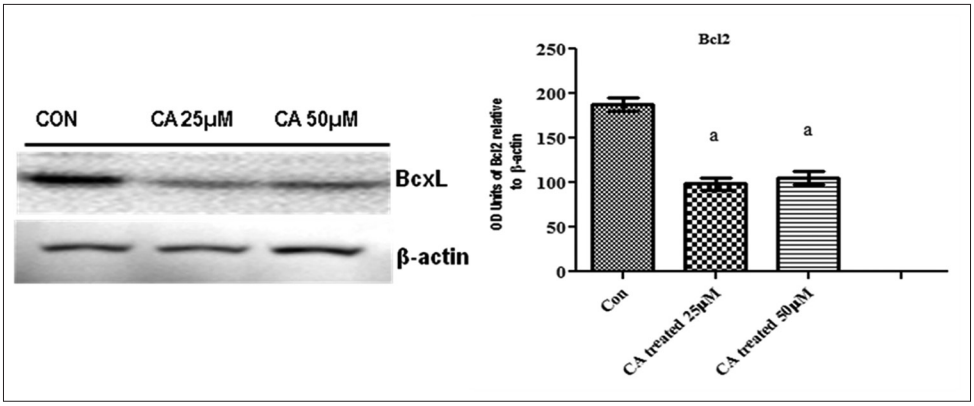

Figure 4: Effect of chebulagic acid (CA) on B-cell lymphoma-extra large-2 protein expression in PC-3 cells. Cells were treated with 25 and 50 μM of CA for 24 h. Protein levels were quantified using densitometry analysis and are expressed in relative intensity arbitrary unit. Each bar represents mean ± standard error of the mean of six independent observations. a : Compared to control

mitochondria. They are divided into antiapoptotic (Bcl-2, Bcl-xL, Mcl-1, and Bcl-w) and proapoptotic proteins (Bax, Bak, Box, Bid, Bim, and Bad) based on the structural and functional characteristics.[14] Impairment in the balance between the proapoptotic and antiapoptotic leads to disruption of apoptosis pathways in the affected cells which promotes cancer. It has been widely proposed that downregulation of Bax mRNA combined with overexpression of Bcl-2 mRNA in malignant condition is one of the underlying mechanisms in the pathogenesis of all.[15]

In our results, we observed a significant increase in the protein level of antiapoptotic protein Bcl-2 and Bcl-xL, which inhibit mitochondrial apoptotic pathway by blocking the release and oligomerization of proapoptotic proteins and promote angiogenesis through increasing vascular endothelial growth factor (VEGF) expression substantially[16] in PCa cell line, whereas CA treatment reduced the protein expression of Bcl-2 and Bcl-xL to the normal level. This may be due to the antiangiogenic effect of CA mediated by blocking both VEGF/VEGF receptor 2 and cell-cell contact-dependent downstream signaling pathway.[17] On the other hand, overexpression of proapoptotic members of the Bcl-2 family, such as Bax, was observed in CA-treated cell line which initiates mitochondrial apoptosis by facilitating pore formation and cytochrome C release from the inner mitochondrial membrane with subsequent activation of caspases resulting in tumor cell death.[12]

CONCLUSION

Based on the preliminary findings, it is very clear that CA due to its antioxidant potentials it exerts its anticancer activity in PCa cells through the regulation of pro- and anti-apoptotic signaling molecules. Hence, it is concluded that CA may have a therapeutic role in PCa. Further studies are needed to elucidate the whole mechanisms of the action of CA.

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