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Original Article

# Epigallocatechin-3-Gallate Induces Apoptosis through Up-regulation of Bax and Down-regulation of Bcl-2 in Prostate Cancer Cell Line

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## ABSTRACT

***Article history***

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***Key words***

Apoptosis
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**Background and Aims:** Epigallocatechin-3-gallate (EGCG) is a polyphenolic compound from green tea, which its anticancer effects on many types of cancers have been confirmed, but the molecular mechanism by which EGCG induces apoptosis remains unknown. The aim of the present study was to investigate anti-proliferative properties and apoptotic signaling pathway of EGCG on PC3 human prostate cancer cells.

**Materials and Methods:** Cytotoxic effect of EGCG on prostate cancer cell line (PC3) was evaluated by MTT assay. DAPI staining was carried out to determine the morphological appearance of cells. Finally, the expression of Bax and Bcl-2 (apoptosis-regulating genes) were evaluated by quantitative Real-time polymerase chain reaction (PCR).

**Results:** Cytotoxicity evaluations demonstrated that EGCG prevented prostate cancer cells growth in a time and dose depended manner, but the effect of treatment duration is more significant than effect of concentration. Cell growth inhibition was found to be accompanied by nucleus condensation or chromatin fragmentations which are signs of apoptosis, as assessed by DAPI staining. Quantitative Real-time PCR results demonstrated that EGCG causes up-regulation of Bax as a pro-apoptotic protein, and down-regulation of Bcl-2 as an anti-apoptotic protein, thus shifting the Bax/Bcl-2 ratio in favor of apoptosis.

**Conclusions:** It is tempting to suggest that consumption of EGCG could be an effective strategy to inhibit prostate cancer. Our results demonstrated that increase in the ratio of Bax/Bcl-2, is the probable molecular mechanisms through which EGCG induces apoptosis in PC3 cells.

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## Introduction

Prostate cancer is the second most commonly recognized cancer and the sixth leading cause of cancer death in males, accounting for 14% of the total new cancer cases and 6% of the total cancer deaths [1]. Reportedly prostate cancer is accounted for 7.75% of new cancer cases and it is the seventh most diagnosed cause of cancer death in Iran [2]. Due to unpleasant effects of prostatectomy or radiotherapy, developing novel protective approaches to control this disease is necessity. Consuming of natural dietary substances which could inhibit cancer extension is one of such approaches [3]. Epidemiological studies have shown that consumption of green tea decreases risk of many cancers, including stomach, lung, colon, rectum, liver, breast and pancreas [4]. Green tea, is a significant source of a type of flavonoids called Epigallocatechin gallate (EGCG) [5]. EGCG is an antioxidant compound and it is proposed that this flavonoid suppresses the inflammatory processes that lead to transformation, hyper-proliferation, and beginning of carcinogenesis [6]. In a wide range of in vitro and preclinical studies EGCGs anti-proliferative [7], anti-angiogenic [8] and apoptotic properties on cancer cells have been confirmed [9]. EGCG can increase gap junctional communication between cells and thus protect cells from tumor development. The experimental studies propose an effect of this polyphenol, which may block the promotion of tumor growth by sealing receptors in the affected cells [10]. However, the anticancer effects of EGCG on many types of cancer have been well studied, but the molecular mechanism whereby EGCG caused apoptosis, in general remain unknown.

The aim of the present study was to investigate anti-proliferative and apoptotic properties of EGCG on human androgen independent prostate cancer cells (PC3), which is resistant to androgen-ablative therapies. Furthermore, the probable signaling pathway of EGCG in apoptosis induction and impact on gene expression of Bax and Bcl-2 was investigated.

## Materials and methods

### Cell culture and cell viability assay

Human prostate cancer PC3 cells (obtained from national cell bank of Pasteur Institute, Iran) were grown in Roswell Park Memorial Institute-1640 (obtained from the Sigma Aldrich Company) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was applied to evaluate anti proliferative effects of EGCG (purchased from the Sigma Aldrich company) on PC3 cells. In brief, $10^4$ cells/well were seeded in a 96 well plate, followed by 24 hours' incubation to cells attach to the surface of the wells. Then, supernatants from the wells were removed and the cells were treated with serial of different concentrations of EGCG (0.1–100 µM). After incubation for 24, 48, and 72 h, 50 µl of 5 mg/ml MTT dissolved in phosphate buffered saline (PBS) and 150 µl of fresh medium was added to each wells and incubated for 4 hours at 37°C. Then, the blue formazan crystals were dissolved in 200 µl of Dimethyl sulfoxide. The optical density values were measured at 570 nm using a spectrophotometric plate reader, ELx 800 (Biotek, CA, USA) with a background correction at 630 nm, and the cell viability was calculated from the following equation:

$$Cell\,viability(\%) = \frac{OD sample}{OD control} \times 100$$

**4'-6-diamidino-2-phenylindole (DAPI) staining assay**

Nucleus morphology is a simple factor for determination of healthy, apoptotic and necrotic cells. DAPI staining was performed to evaluate morphological alterations of the cells during apoptosis, such as nuclear segmentation and chromatin condensation. In brief, PC3 cells were harvested in six-well plates containing 12 mm cover-slips and consequently treated with EGCG for 48 hours. Then, cells were fixed with 4% paraformaldehyde and washed three times with PBS, permeabilized with 0.1% (w/v) Triton X-100 for 5 min, washed again with PBS and stained by incubation with 400 ng/mL DAPI for 20 min and cells were evaluated under a fluorescence microscope (Olympus microscope Bh2-RFCA, Japan). Triplicate samples were prepared for each treatment and at least 300 cells were counted in random fields for each sample and apoptotic nuclei were identified.

**Analysis of Bax and Bcl-2 Gene Expression by Real-time polymerase chain reaction (PCR)**

The cells were seeded in flasks with concentration of $1\times10^6$ cells/flask. Then, flasks were treated with $IC_{50}$ of 48 and 72 h exposure for 48 and 72 h. For extraction of RNA, RNX-plus solution (Sinaclon, Iran) was used according to the manufacturer's protocol. RNA pellet was dissolved in DEPC-treated water, quantified by optical density measurement (A260/A280 ratio) with NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA). Then, 1µg of total RNA was used as substrate for reverse transcription using Thermo Scientific cDNA synthesis kit (Thermo Scientific, Schwerte, Germany), according to the manufacturer's protocols. Real time PCR method based on the SYBR Green chemistry was performed to analyze the expression levels of Bax and Bcl-2 relative to the housekeeping genes, B-actin. Sequences of primers are shown in table 1. In brief, about 1 µl of each specific primers and 1µl of each cDNA sample was added to PCR tubes containing SYBR-Green Master Mix (7.5 µl), and sterile water (5.5 µl). The sample tubes were placed into the real time rotary analyzer (Rotor-Gene 6000, Corbet Life Science, Australia) with the following settings: 45 cycles of 4-step PCR (95 °C-5min, 95°C-15s, 59°C-35s, 72°C-15s) for both Bax and B-actin and 45 cycles of 4-step PCR (95°C-10 min, 95°C-15s, 63 °C-35s, 72°C-15s) for Bcl-2. The experiments were done in triplicate manner for each sample. The Ethics Committee of Tabriz university of medical sciences, Tabriz, Iran approved the study.

**Statistical analysis**

The alteration of Bax and Bcl-2 gene expression was analyzed by $\Delta\Delta C_t$ method and Microsoft® Excel 2013 (T-test with $p<0.05$) was used for statistical analysis.

**Table 1.** Primers sequences

| Gene | Primers Sequence | Products size |
|---|---|---|
| **Bax** | Forward 5-TTGCTTCAGGGTTTCATCCA-3<br>Reverse 5-GACACTCGCTCAGCTTCTTG-3 | 112 bp |
| **Bcl-2** | Forward 5-GTCATGTGTGTGGAGAGCG-3<br>Reverse 5-ACAGTTCCACAAAGGCATCC-3 | 131 bp |
| **B-actin** | Forward 5-TCCCTGGAGAAGAGCTACG-3<br>Reverse 5-GTAGTTTCGTGGATGCCACA-3 | 131 bp |

## Results

### Effects of EGCG on PC3 cell proliferation

EGCG can impose cytotoxicity effect on PC3 cells. After treatment of PC3 cell line with different concentrations of EGCG for 24, 48 and 72 h, EGCG $IC_{50}$ was calculated as 67.5 μM for 48 h treatment and 44.6 μM for 72 h treatment (Fig. 1). Microscopic image of PC3 cells after 48 h of EGCG treatment is also shown in Fig 1. In this study, after 24 h treatment, EGCG did not show a considerable cytotoxic effect on PC3 cell line. These results showed that EGCG at the longer treatment duration has a stronger cytotoxic effect on PC3 cell line, so it can be declared that EGCG dose and time dependently decreased the growth of PC3 cells.

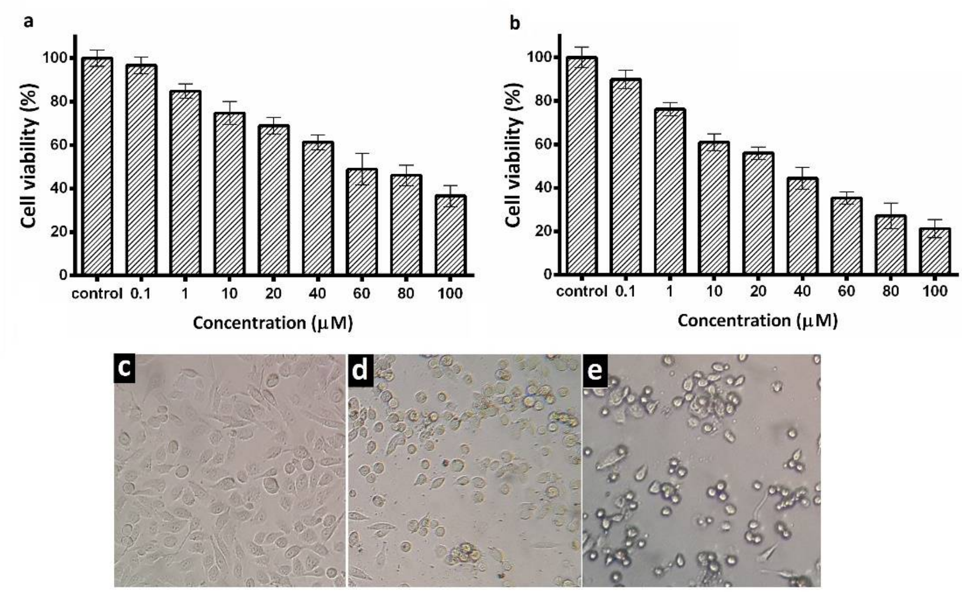


**Fig. 1.** Cytotoxic effects of epigallocatechin-3-gallate in (a) 48 h and (b) 72 h on PC3 cells. 67.5 μM and 44.6 μM EGCG induces cell death in half of cellular population in 48 and 72 h respectively. Data is presented as mean ± standard deviation (n=3). Figure also illustrate the morphological alteration of PC3 cells following a 48 h exposure by (c) untreated, (d) 40 μM and (e) 80 μM epigallocatechin-3-gallate.

### DAPI Staining

Induction of apoptosis was investigated by DAPI staining using fluorescent microscopy in 48 h. Images of DAPI stained cells showed that 44 and 67 μM EGCG after 48 h treatments, induced apoptosis in PC3 cell line about 27.9% and 54.4%, respectively (Fig. 2).

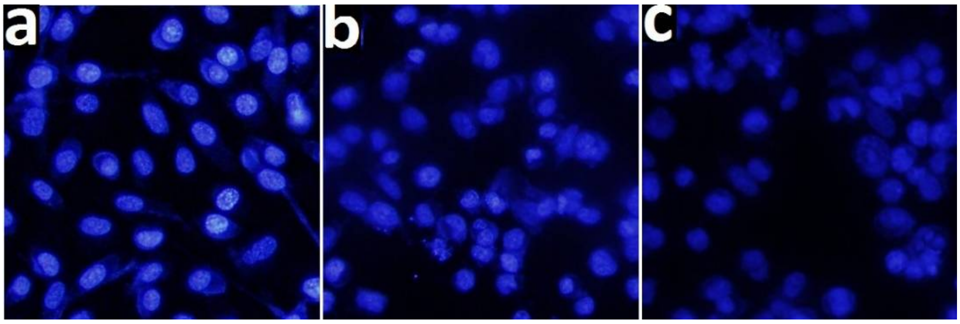


**Fig. 2.** Fluorescent images of DAPI stained PC3 cells following a 48 h exposure by (a) untreated, (b) 44 µM and (c) 67 µM epigallocatechin-3-gallate.

**Results for quantitative real-time PCR**

The expression levels of Bax and Bcl-2 after 48 and 72h EGCG treatment were analyzed using real time quantitative realtime-PCR. The 24h time exposure was not included in the experiments, because EGCG treatments in 24h exposure did not display significant cytotoxic effects on PC3 cell line. The level of Bax and Bcl-2 mRNA were normalized to mRNA level of the regularly expressed housekeeping gene, B-actin, within each sample. The relative mRNA level of Bax and Bcl-2 after treatment with different concentrations of EGCG has shown in table 2. The real time quantitative PCR data demonstrated that treatment of PC3 cells with EGCG causes up-regulation of Bax mRNA level and down-regulation of Bcl-2 mRNA levels in time and dose dependent manner, but treatment in longer duration is more effective (Fig. 3). In other words, it seems that the effect of treatment duration was more significant than the effect of concentration in up-regulation of Bax and down-regulation of Bcl-2.

**Table 2.** The effect of EGCG on Bax and Bcl-2 gene expression in PC3 cells.

| Samples | EGCG Cocentration (µM) | Bax gene expression (fold change) | Bcl-2 gene expression (fold change) |
|---|---|---|---|
| **Control** | 0 | 1 | 1 |
| **48 h** | 44 | 2.23 | 0.87 |
| | 67 | 3.24 | 0.61 |
| **72 h** | 44 | 3.57 | 0.64 |
| | 67 | 5.52 | 0.39 |

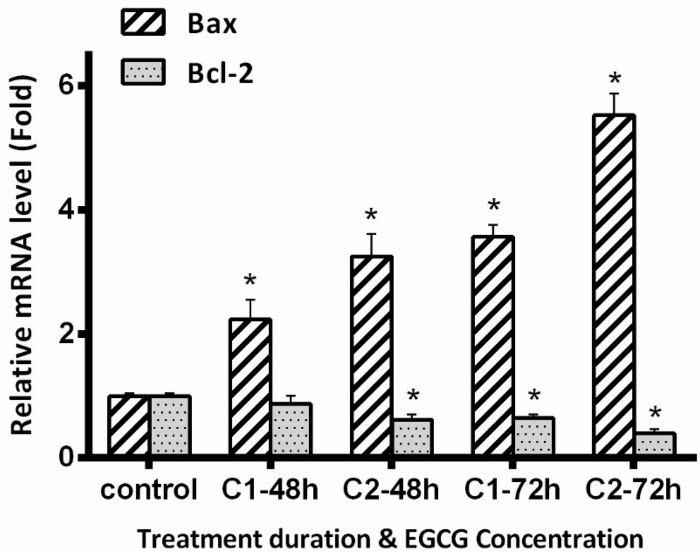


**Fig. 3.** Effects of epigallocatechin-3-gallate (EGCG) on Bax and Bcl-2 mRNA expression levels in PC3 cells after 48 and 72h. As this figure shows, in higher EGCG concentrations and treatment durations, the expression of BAX increases and expression of Bcl-2 decreases. Bax/Bcl-2 ratio is more dependent on treatment duration than EGCG concentration (C1=44 µM and C2=67 µM). *Alteration in gene expression is significant in comparison to the control group. ($p<0.05$)

## Discussion

EGCG is the most abundant and active catechin which has been extensively studied for its cancer chemopreventive activity [11]. It also verified that EGCG induced apoptosis in oral squamous carcinoma cells, while has no effect on normal human epidermal keratinocytes [12]. Several investigations have formerly established that EGCG inhibits PC-3 cell proliferation via cell cycle arrest [13]. Our results indeed verified that treatment of PC3 cells with EGCG resulted in a dose- and time-dependent inhibition of cell proliferation and a simultaneous decrease in cell viability. These results suggest that EGCG induced cell death of PC3 cells. Consequently, morphological alterations recognized to be associated with apoptosis were investigated. The advent of an apoptotic process is confirmed by chromatin condensation and fragmentation, trailed by the formation of apoptotic bodies. Although EGCG seems to have a wide range of possible targets, the specific cellular mechanisms responsible for apoptosis induction are not well understood [11]. Inhibition of receptor tyrosine kinase activity [14, 15], down-regulation of cyclo-oxygenase 2 [16], motivation of p53 tumor suppressor [17], and repression of telomerase activity [18] have been offered as molecular mechanisms for EGCG effects. In the present study, an apparent increase in Bax and decrease in Bcl-2 mRNA expression levels were observed when the PC3 cells were treated with EGCG. Bax and Bcl-2 are pro-apoptotic and anti-apoptotic members of Bcl-2 family, respectively. As the BCL-2 family members exist in upstream of irreversible cellular damage and focus much of their efforts at the level of mitochondria, they play a fundamental role in deciding whether a cell will live or die [19]. In reaction to apoptotic stimuli, Bax translocates to the mitochondria and places in mitochondrial outer membrane, resulting in the disruption of mitochondrial membrane potential and release of cytochrome C from the mitochondria, which finally leads to apoptosis. In contrast, Bcl-2 supports cell survival by inhibiting factors which activate caspases [20, 21]. Bcl-2 has been shown to form a heterodimer complex with the Bax, thereby counteracting its pro-apoptotic effects. Therefore, the ratio of Bax /Bcl-2 is important in susceptibility to apoptosis [22]. Considering the results of the present study, the ratio of pro-apoptotic proteins to the anti-apoptotic proteins were altered in favor of apoptosis when PC3 cells were treated by EGCG. Therefore, EGCG could play the apoptotic effect by induction of mitochondria dependent pathway on PC3 cells line.

## Conclusions

In conclusion, this study demonstrated that EGCG treatment inhibits cell proliferation in prostate carcinoma cells. Increasing the ratio of Bax/Bcl-2 level is the probable mechanism by which EGCG stimulates apoptosis in PC3 cancer cell line. Based on the present findings, it is tempting to propose that EGCG could be developed as a potential anticancer agent against human prostate cancer cells, but more investigations are required to study anti-cancer efficacy of EGCG on cancer animal models, before using it in the clinical trial studies.

## Conflict of Interest

The authors have declared that they had no conflict of interest.

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## References

[1]. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA: a cancer journal for clinicians. 2011; 61(2): 69-90.

[2]. Mousavi SM. Toward prostate cancer early detection in Iran. Asian Pac J Cancer Prev. 2009; 10(3): 413-18.

[3]. Syed DN, Khan N, Afaq F, Mukhtar H. Chemoprevention of prostate cancer through dietary agents: progress and promise. Cancer Epidemiol Biomarkers Prev. 2007; 16(11): 2193-203.

[4]. Punathil T, Tollefsbol TO, Katiyar SK. EGCG inhibits mammary cancer cell migration through inhibition of nitric oxide synthase and guanylate cyclase. Biochem Biophys Res Commun. 2008; 375(1): 162-7.

[5]. Kelly T, Owusu-Apenten R. Effect of Methotrexate and Tea Polyphenols on the Viability and Oxidative Stress in MDA-MB-231 Breast Cancer Cells. J Appl Life Sci Int. 2015; 2(4): 152-59.

[6]. Thawonsuwan J, Kiron V, Satoh S, Panigrahi A, Verlhac V. Epigallocatechin-3-gallate (EGCG) affects the antioxidant and immune defense of the rainbow trout, Oncorhynchus mykiss. Fish Physiol Biochem. 2010; 36(3): 687-97.

[7]. Wang P, Heber D, Henning SM. Quercetin increased the antiproliferative activity of green tea polyphenol (-)-epigallocatechin gallate in prostate cancer cells. Nutr Cancer 2012; 64(4): 580-87.

[8]. Wang CC, Xu H, Man GCW, Zhang T, Chu KO, Chu CY, et al. Prodrug of green tea epigallocatechin-3-gallate (Pro-EGCG) as a potent anti-angiogenesis agent for endometriosis in mice. Angiogenesis 2013; 16(1): 59-69.

[9]. Shuntaro T, Keisuke H, Motofumi K, Yoko G, Kaori S, Takafumi S, et al. Green tea polyphenol

EGCG induces lipid-raft clustering and apoptotic cell death by activating protein kinase Cdelta and acid sphingomyelinase through a 67 kDa laminin receptor in multiple myeloma cells. Biochem J. 2012; 443(2): 525-34.

[10]. Singh BN, Shankar S, Srivastava RK. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. Biochem Pharmacol. 2011; 82(12): 1807-821.

[11]. Choi Y-J, Lim S-Y, Woo J-H, Kim Y-H, Kwon YK, Suh S-I, et al. Sodium orthovanadate potentiates EGCG-induced apoptosis that is dependent on the ERK pathway. Biochem Biophys Res Commun. 2003; 305(1): 176-85.

[12]. Nihal M, Ahmad N, Mukhtar H, Wood GS. Anti-proliferative and proapoptotic effects of (−)-epigallocatechin-3-gallate on human melanoma: Possible implications for the chemoprevention of melanoma. Int J Cancer 2005; 114(4): 513-21.

[13]. Shenouda NS, Zhou C, Browning JD, Ansell PJ, Sakla MS, Lubahn DB, et al. Phytoestrogens in common herbs regulate prostate cancer cell growth in vitro. Nutr Cancer 2004; 49(2): 200-208.

[14]. Shimizu M, Deguchi A, Hara Y, Moriwaki H, Weinstein IB. EGCG inhibits activation of the insulin-like growth factor-1 receptor in human colon cancer cells. Biochem Biophys Res Commun. 2005; 334(3): 947-53.

[15]. Shimizu M, Deguchi A, Lim JT, Moriwaki H, Kopelovich L, Weinstein IB. (-)-Epigallocatechin gallate and polyphenon E inhibit growth and activation of the epidermal growth factor receptor and human epidermal growth factor receptor-2 signaling pathways in human colon cancer cells. Clin Cancer Res. 2005; 11(7): 2735-746.

[16]. Shimizu M, Deguchi A, Joe AK, McKOY JF, Moriwaki H, Weinstein IB. EGCG inhibits activation of HER3 and expression of cyclooxygenase-2 in human colon cancer cells. J Exp Ther Oncol. 2005; 5(1):69-78.

[17]. Muthusami S, Prabakaran D, An Z, Yu J-R, Park W-Y. EGCG suppresses Fused Toes Homolog protein through p53 in cervical cancer cells. Mol Biol Rep. 2013;40(10): 5587-96.

[18]. Berletch JB, Liu C, Love WK, Andrews LG, Katiyar SK, Tollefsbol TO. Epigenetic and genetic mechanisms contribute to telomerase inhibition by EGCG. J Cell Biochem. 2008; 103(2): 509-19.

[19]. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 1999; 13(15): 1899-911.

[20]. Mbazima VG, Mokgotho MP, February F, Rees DJG, Mampuru L. Alteration of Bax-to-Bcl-2 ratio modulates the anticancer activity of methanolic extract of Commelina benghalensis (Commelinaceae) in Jurkat T cells. Afr J Biotechnol. 2008; 7(20): 3569-576.

[21]. Li Z, Huang Y, Dong F, Li W, Ding L, Yu G, et al. Swainsonine promotes apoptosis in human oesophageal squamous cell carcinoma cells in vitro and in vivo through activation of mitochondrial pathway. J biosci. 2012; 37(1): 1005-16.

[22]. Malik A, Afaq S, Shahid M, Akhtar K, Assiri A. Influence of ellagic acid on prostate cancer cell proliferation: A caspase–dependent pathway. Asian Pac J Tropic Med. 2011; 4(7): 550-55.