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Original Research Article

Juglans regia Hexane Extract Exerts Antitumor Effect, Apoptosis Induction and Cell Circle Arrest in Prostate Cancer Cells *In vitro*

Wei Li[1], De-Yuan Li[2]*, Hai-Dong Wang[2], Zhe-Jun Zheng[2], Jie Hu[2] and Zong-Zhe Li[2]

[1]Department of Food Science and Technology, Huazhong Agricultural University, [2]Department of Nutrition and Food Research Institute, Wuhan Economic College, Wuhan 430035, China

For correspondence: **Email: deyuanli3265@gmail.com; **Tel/Fax:** 0086-027-83810935*

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Abstract

Purpose: *To elucidate the anticancer activity of Juglans regia leaf extract and its effect on cell cycle analysis, apoptosis and cancer cell morphology.*
Methods: *Hexane extract of the leaves of Juglans regia was prepared by hot extraction. The anticancer activity of Juglans regia extract against human prostate cancer (PC3) cells was evaluated by MTT assay. Flow cytometry, using propidium iodide as a staining agent, was used to study the effect of the extract on cell cycle phase distribution. Apoptosis induced by the extract was evaluated by Annexin V binding assay using flow cytometer. Alterations in cell morphology following apoptosis were studied by inverted phase contrast microscope.*
Results: *The extract of Juglans regia exhibited a potent and dose-dependent anti-proliferative activity against human prostate cancer cells in vitro. The extract also induced significant apoptosis in these PC3 cancer cells as revealed by annexin V binding assay as well as inverted phase contrast microscopy. It triggered a significant formation of apoptotic bodies after treatment with varying concentrations of the extract. Within 48 h of incubation, approximately 9.5, 15.5 and 26.3 % of the cells underwent early apoptosis after treatment with 5, 50 and 100 µg/mL of the extract, respectively. Similarly, 5.2, 11.2 and 18.9 % of the cells underwent late apoptosis after treatment with 5, 50 and 100 µg/mL of the extract, respectively. Treatment with different concentrations of the extract for 48 h induced an increase in the population of cells in the sub-G1 phase and a slight decrease in the G2/M phase.*
Conclusion: *The hexane extract of Juglans regia inhibits growth of human prostate cancer cells by inducing apoptosis with concomitant alterations in cell cycle phase distribution.*

Keywords: *Prostate cancer, Juglans regia, Apoptosis, Flow cytometry, Cell cycle phase, Sub-G1 phase*

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INTRODUCTION

Prostate cancer (PC) is the second most widespread malignancy and the second leading cause of cancer-related deaths among men in many western countries including USA. Clinically, PC is generally diagnosed in men over 50 years of age; with increasing life expectancy the prevalence of PC is likely to increase worldwide [1,2]. For PC chemoprevention at the present time there is considerable emphasis in identifying novel botanicals that selectively induce apoptosis and growth arrest of PC cells without producing cytotoxic effects on normal cells. Because of inadequate treatment options available for prostate cancer, there is an urgent

requirement for developing innovative preventive approaches for this malignancy. For a variety of reasons naturally occurring plant compounds and dietary substances are attaining increasing response as cancer chemopreventive agents [3-7]. The majority of anticancer drugs are natural products or their derivatives. More than 200 drugs derived from natural products are in preclinical or clinical development and evaluation [8,9].

J. regia belongs to the family Juglandaceae which includes 3 species: *J. nigra, J. cinerea*, and *J. regia*. *Juglans regia* L. commonly known as walnut tree is a well-known member of Juglans genus, constituting an important species of deciduous trees found primarily in temperate areas. *J. regia* extract contains ellagitannins which contains anti-cancer agent and with anti-inflammatory properties. The key chemical composition of walnut is juglone (5-hydroxy-1, 4-naphthoquinone), the toxic compound which is found only in green and fresh walnuts, but such property disappear in dried leaves. Other several phenolic compounds with antioxidant properties have been identified in *J. regia* leaves [10-13]. Walnut leaves are considered a source of healthcare compounds, and have been extensively used in traditional medicine for treatment of skin inflammations, hyperhidrosis and ulcers and for its anti-diarrheal, anti-helmintic, antiseptic and astringent properties [12-14].

The objective of the present study was to evaluate the anti-proliferative activity of the hexane extract of the leaves of *Juglans regia* against human prostate (PC3) cancer cells in vitro. We also studied the effect of the extract on cell cycle phase distribution as well as on the apoptosis induction using flow cytometry and inverted phase contrast microscopy.

EXPERIMENTAL

Plant material and extraction procedure

Juglans regia leaves were collected during July - August 2013 from a local site in Jianguo District, China. The plant material was identified by Professor Long Yuan-xi of the Huazhong Agricultural University, Wuhan, and a voucher specimen (no. DFST/HAU-0097654) was submitted to the University Herbarium. The plant material was confirmed by a well-known taxonomist. The leaves were thoroughly washed with tap water, shade-dried and then chopped into small pieces. Hexane (95 %) was used for hot extraction which was carried out for 4 h using a Soxhlet extraction apparatus. The extract was then concentrated under reduced pressure in a rotary evaporator at 45 ^{o}C and was then kept in a refrigerator at 4 ^{o}C prior to use.

Chemicals and reagents

Growth medium RPMI-1640 (Hangzhou Sijiqing Biological Products Co., Ltd, China), Minimum Essential Medium (MEM), Fetal Calf Serum (GIBCO), trypsin, penicillin, MTT, streptomycin, DMSO and phosphate buffer saline (PBS) were used in this study. MTT kit was obtained from Roche (USA). Annexin V-FITC-Propidium Iodide Apoptosis Detection Kit was purchased from Sigma (USA). All other chemicals and solvents used were of the highest purity grade. Cell culture plastic ware was bought from BD Falcon (USA).

Cell lines and cell viability assay

Human prostate carcinoma (PC3) cells were procured from the Shanghai Institute of Cell Resource Center of Life Science (Shanghai, China). All the cells were grown in a humidified 5 % CO_2 atmosphere at 37 ^{o}C in an incubator, and cultured in RPMI-1640 medium supplemented with 10 % heat-inactivated newborn calf serum, 100 IU/ml penicillin and 100μg/mL streptomycin. Inhibition of cell proliferation of the extract was measured by the MTT assay. Briefly, PC3 cells were plated in 96-well culture plates (1×10^5 cells/well) separately. After 24 h incubation, cells were treated with *Juglans regia* extract (5, 25, 50, 75 and 100 μg/mL; eight wells per concentration) for 24 h; MTT solution (5 mg/mL) was then added to each well. After 4 h incubation, the formazan precipitate was dissolved in dimethyl sulfoxide (100 μL) and then the absorbance was measured in an ELISA reader (Thermo Molecular Devices Co., Union City, USA) at 570 nm. Cell viability ratio was calculated as in Eq 1.

$$\text{Cell viability (\%)} = \{(Ac - At)/Ac\}100 \quad \ldots\ldots\ldots (1)$$

where Ac and At are the absorbance of control and treated cells, respectively.

Cytotoxicity was expressed as the concentration of the extract inhibiting cell growth by 50 % (IC_{50} value).

Cell cycle phase distribution analysis by flow cytometry

Human prostate carcinoma PC3 cells (1×10^6) were seeded in 60-mm dishes and subjected to various concentrations (5, 25, 50, 75 and 100

µg/mL) of the *Juglans regia* extract for 48 h. Floating and adherent cells were collected by trypsinization and washed twice with PBS. Cells were incubated in 70 % ethanol at -20 °C overnight, treated with 10 µg/mL RNase A, then stained with 2.0 µg/mL of propidium iodide. Finally the stained cells were analyzed and studied by flow cytometry at wavelength of 488 nm (FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest 3.3 software).

Annexin V binding assay

We performed annexin V binding assay using flow cytometry to confirm apoptosis. The PC3 cells were treated with *Juglans regia* extract (5, 25, 50, 75 and 100 µg/mL) for 48 h. Subsequently, treated and untreated cells were harvested by trypsinization. Harvested cells were then incubated in annexin V-FITC (50 ng/mL) and propidium iodide (20 µg/mL), at room temperature for 30 min in the dark, and analysed using a FACS Calibur flow cytometer (BD Bioscience) taking a minimum 25,000 cells in each sample.

Live cell morphology assay of apoptosis

Apoptotic morphological characteristics of human prostate carcinoma cells were recorded with an inverted phase contrast microscope (IPCM) (Olympus, Japan) after treatment with various concentrations (5, 25, 50, 75 and 100 µg/mL) of the extract. Cells cultured with 0.5 % DMSO were used as the control.

Statistical analysis

All the data were analysed using analysis of variance (ANOVA), followed by Dunnett's test for pair-wise comparison. Mean values are presented with their standard deviation (SD). Statistical significance was defined as $p < 0.05$ for all tests.

RESULTS

Anti-proliferative activity of *Juglans regia* extract

Juglans regia leaf extract was evaluated for antiproliferative activity using the MTT assay against the human prostate carcinoma (PC3) cells for 24 h (Figure 1). The extract exhibited potent and dose-dependent cytotoxic activity against these cancer cells. The IC_{50} value of the extract was calculated to be 48.4 µg/mL. IC_{50} value gives an indication of the potency of a drug/agent. As shown in Figure 1, 100 µg/mL extract exhibited a very strong anticancer effect at which approximately only 15 % of the cells were alive and 85 % cells were damaged.

Effect of *Juglans regia* extract on cell cycle phase distribution

Treatment with different concentrations of the extract for 48 h induced an increase in the population of cells in the sub-G1 phase and a slight decrease in the G2/M phase (Figure 2).

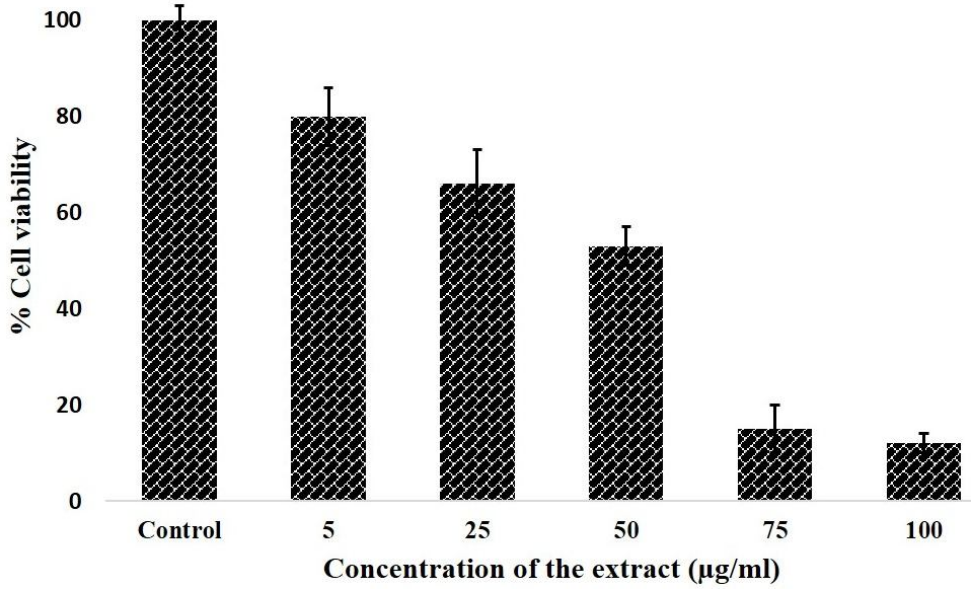

Figure 1: Anti-proliferative activity of the hexane extract of *Juglans regia* against prostate cancer cells *in vitro*

Approximately 2.1 % of the control cells were in the sub-G1 phase, while approximately 6.5, 23.5 and 35.2 % of the cells treated with 5, 50 and 100 µg/mL of the extract concentration were in this phase respectively.

Quantification of apoptotic cell death induced by *Juglans regia* extract

To verify if this increase in the sub-G1 population of human prostate cancer cells was due to apoptosis, PC3 cells were labeled with FITC-conjugated Annexin V and PI for flow cytometry analysis. The results of the flow cytometry study with Annexin V/FITC and PI showed that, within 48 h of incubation, approximately 9.5 %, 15.5 % and 26.3 % of the cells underwent early apoptosis after treatment with 5, 50 and 100 µg/mL of the extract respectively. Similarly, 5.2 %, 11.2 % and 18.9 % of the cells underwent late apoptosis after treatment with 5, 50 and 100 µg/mL of the extract respectively (Figure 3 and Figure 4).

Apoptosis induced by *Juglans regia* extract

The induction of apoptosis by *Juglans regia* extract was confirmed by studying apoptotic body formation using inverted phase contrast microscope. As shown in Figure 5, the formation of apoptotic bodies was observed 48 h post-treatment in *Juglans regia* extract-treated cells. The extract exhibited a dose-dependent effect on PC3 cells.

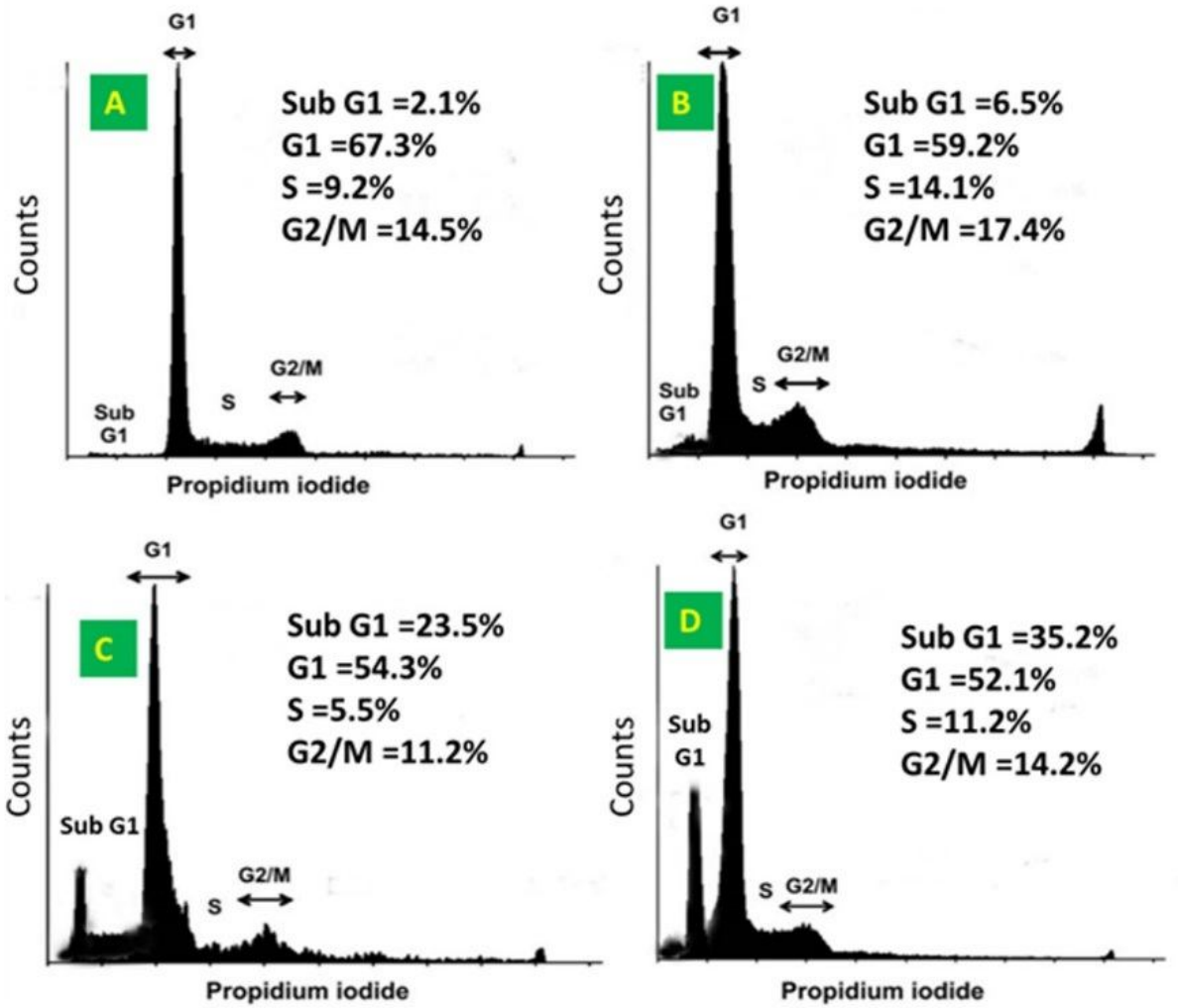

Figure 2: Cell cycle distribution of human prostate carcinoma (PC3) cells after treatment with 5.0 µg/ml (B), 50 µg/ml (C) and 100 µg/ml (D) of *Juglans regia* extract for 48 h. The cells were treated with RNase and propidium iodide and then analyzed by flow cytometry. (A) represents negative control (DMSO solvent)

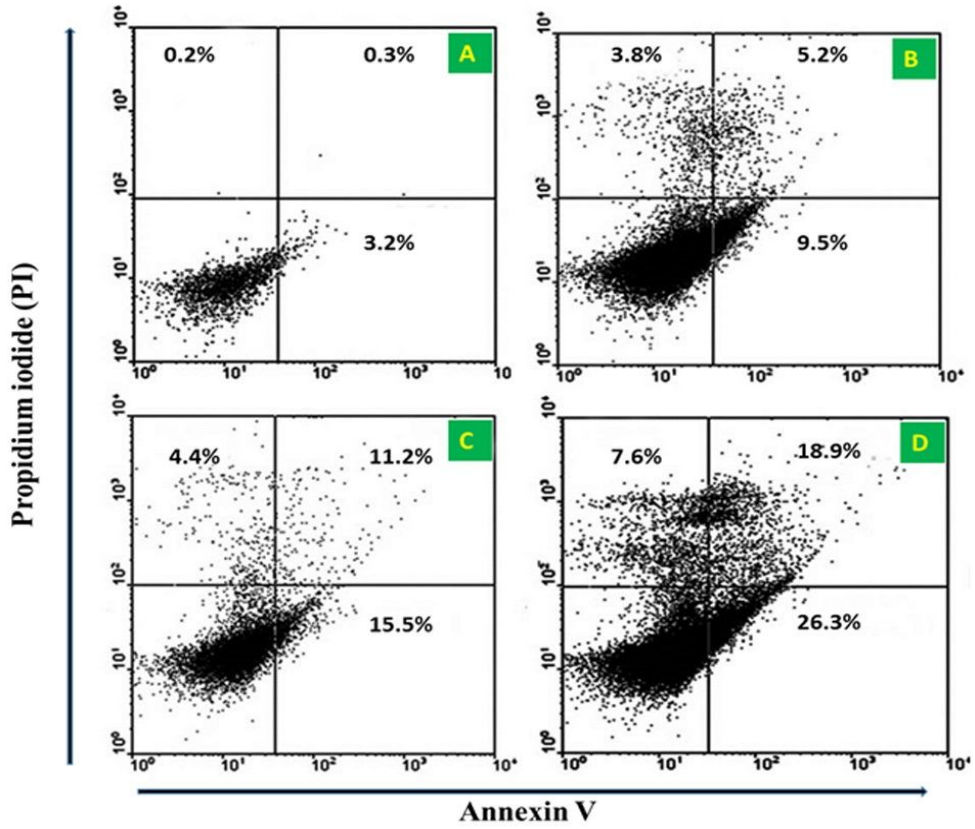

Figure 3: Dot plot of human prostate carcinoma (PC3) cells after exposure to 5, 50 and 100 µg/ml concentration of *Juglans regia* extract for 48 h and flow cytometry analysis with Annexin V-FITC versus PI. The divisions of the plots distinguish necrotic cells (Annexin V-/PI+, left upper quadrant) from early apoptotic cells (Annexin V+/PI-, right lower quadrant) and late apoptotic cells (Annexin V+/PI+, right upper quadrant). The plots in the figure are representative of 5 independent experiments

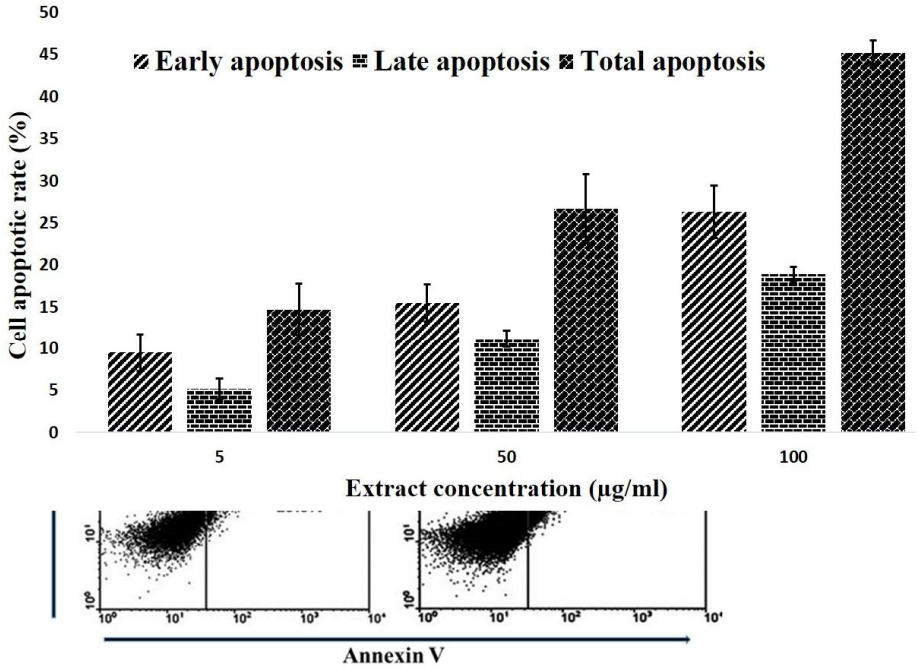

Figure 4: Percentages of cells in apoptosis at each *Juglans regia* extract concentration. Cells in the bottom and top right quadrants of Figure 3 were summed to obtain the total number of apoptotic cells. Findings are presented as the mean of three similar experiments ± standard deviation

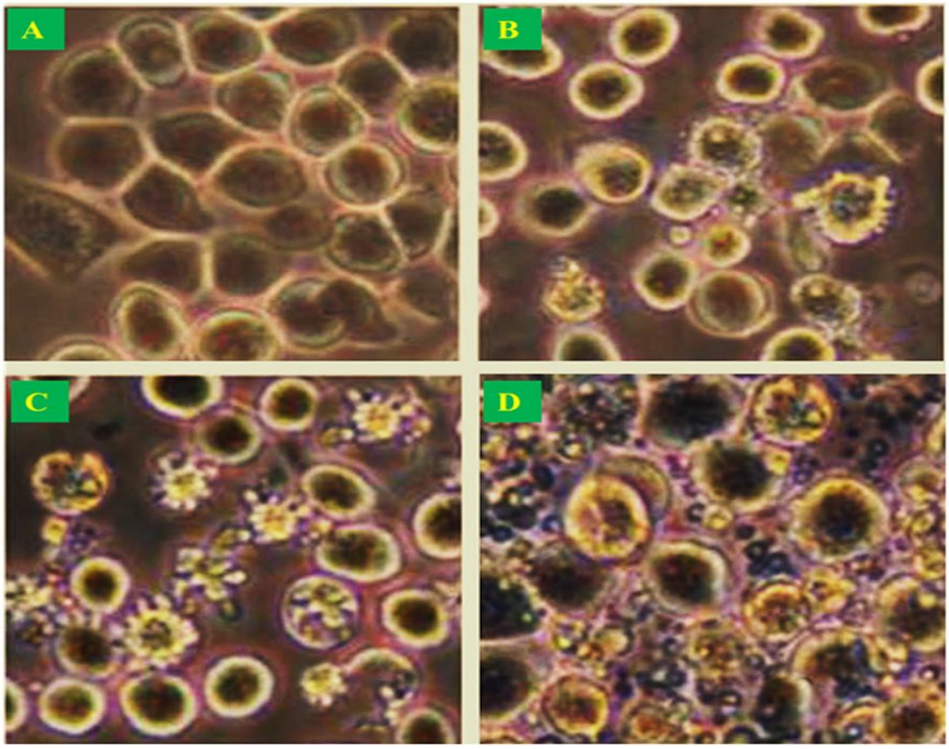

Figure 5: *Juglans regia* extract (B, C and D represent 5, 50 and 100 µg/ml concentration of the extract respectively while A represents negative control) induced cell apoptosis in human prostate carcinoma (PC3) cells is marked by apoptotic body formation and apoptotic DNA degradation. Apoptotic body formation was observed 48 h onwards after treatment with the extract

DISCUSSION

Prostate cancer (PC) is the second most wide-spread malignancy and the second leading cause of cancer-related deaths among men in many Western countries including USA [1]. Clinically, PC is generally diagnosed in men over 50 years of age; with increasing life expectancy the prevalence of PC is likely to increase worldwide. PC usually develops from androgen-dependent to androgen-independent stage, making anti-androgen therapy unproductive, thus leading to a rise in metastatic potential [1,15]. Suppression of cancer development frequently involves modulation of signal transduction pathways, resulting in alterations in gene expression, arrest in cell cycle progression or apoptosis. Apoptotic cell death represents a universal and intricately efficient suicide pathway, considered as a perfect method for elimination of damaged cells [16]. Recently, the apoptosis signaling systems have been shown to provide encouraging targets for the development of novel anticancer agents [17]. Several plant-derived bioactive compounds are known to be chemopreventive agents inducing apoptosis in a number of experimental models of carcinogenesis. Thus induction of apoptosis is considered as a possible mechanism of chemo preventive agents [18-20].

In this study, the *Juglans regia* hexane extract was evaluated for its anticancer activity against human prostate PC3 cancer cells. The extract showed a dose-dependent inhibition of the cancer cells with 100 µg/mL of the extract producing the most potent growth inhibition. In order to further study the mechanism of the extract, we evaluated effect of the extract on cell cycle analysis, apoptosis induction using Annexin V binding assay as well as inverted phase contrast microscopy. The results revealed that the *Juglans regia* extract increased the fraction of sub-G1 cells in a dose-dependent manner along with a slight decrease in G2/M fraction of cells. The apoptosis assay revealed that the number of cells with both late and early apoptosis increases greatly and dose-dependently after extract treatment.

CONCLUSION

Juglans regia leaf extract induces potent growth inhibitory effects against human prostate cancer cells (PC3) by inducing apoptosis and by altering the cell cycle phase distribution in these cells. Therefore, the extract may find application in curbing prostate cancer and is a potential

complementary and alternative medicinal treatment for this disease.

REFERENCES

1. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ. Cancer statistics, 2005. CA Cancer J Clin 2005; 55(1): 10–30.
2. Rini BI, Small EJ. Prostate cancer update. Curr Opin Oncol 2002; 14(3): 286–291.
3. Tsao AS, Kim ES, Hong WK. Chemoprevention of cancer. CA Cancer J Clin 2004; 54(3):150–180.
4. Jain MG, Hislop GT, Howe GR, Ghadirian P. Plant foods, antioxidants, and prostate cancer risk: findings from case-control studies in Canada. Nutr Cancer 1999; 34(2): 173–184.
5. Kiran YB, Devendranath Reddy C, Gunasekar D, Suresh Reddy C, Leon A, Barbosa LC. Synthesis and anticancer activity of new class of bisphosphonates/phosphanamidates. Eur J Med Chem 2008; 43(4): 885–892.
6. Cragg GM, Grothaus PG, Newman DJ. Impact of natural products on developing new anti-cancer agents. Chem Rev 2009; 109(7): 3012–3043.
7. Ferreira PM, Costa-Lotufo LV, Moraes MO, Barros FW, Martins AM, Cavalheiro AJ, Bolzani VS, Santos AG, Pessoa C. Folk uses and pharmacological properties of Casearia sylvestris: a medicinal review. An Acad Brás Cienc 2011; 83(14): 1373–1384.
8. Ghantous A, Gali-Muhtasib H, Vuorela H, Saliba NA, Darwiche N. What made sesquiterpene lactones reach cancer clinical trials? Drug Discov Today 2010; 15(15-16): 668–678.
9. Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Nat Prod 2012; 75(3): 311–335.
10. Mozafarian V. Walnut tree. Identification of medicinal and aromatic plants of Iran. Tehran, Iran: Farhang Moaser press 2012; 473–475.
11. Kaur K, Michael H, Arora S, Härkönen PL, Kumar S. Studies on correlation of antimutagenic and antiproliferative activities of Juglans regia L. J Environ Pathol Toxicol Oncol 2003; 22(1): 59–67.
12. Amaral J, Seabra RM, Andrade PB, Valentão P, Pereira JA, Ferreres F. Phenolic profile in the quality control of walnut (Juglans regia L.) leaves. Food Chem 2004; 88: 373–379.
13. Pereira JA, Oliveira I, Sousa A, Valentão P, Andrade PB, Ferreira I, Ferreres F, Bento A, Seabra RM, Estevinho L. Walnut (Juglans regia L.) leaves: phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. Food Chem Toxicol 2007; 45(11): 2287–2295.
14. Bruneton J. Pharmacognosie, phytochimie, plantesmédicinales. In: Technique et Documentation Lavoisier Paris 1999; 418–419 pp.
15. Rini BI, Small EJ. Prostate cancer update. Curr Opin Oncol 2002; 14(3): 286–291.
16. Singh RP, Dhanalakshmi S, Agarwal R. Phytochemicals as cell cycle modulators: a less toxic approach in halting human cancer. Cell Cycle 2002; 1(3): 156–161.
17. Hu W, Kavanagh JJ. Anticancer therapy targeting the apoptotic pathway. Lancet Oncol 2003; 4(12): 721–729.
18. Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. J Natl Cancer Inst 1997; 89(24): 1881–1886.
19. Lee E, Surh YJ. Induction of apoptosis in HL-60 cells by pungent vanilloids, [6]-gingerol and [6]-paradol. Cancer Lett 1998; 134(2): 163–168.
20. Taraphdar AK, Roy M, Bhattacharya RK. Natural products as inducers of apoptosis: implication for cancer therapy and prevention. Curr Sci 2001; 80: 1387–1396.