RESEARCH ARTICLE

Evaluation of cytotoxicity and Apoptotic effects of Simarouba glauca on the Prostate Cancer Cell Lines PC3

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Abstract

Objective: To investigate the anti-proliferative and pro-apoptotic activities of simarouba glauca on prostate cancer cell lines PC-3.

Method: This cross-sectional study was conducted at Iraqi centre for Genetic and Cancer Research from 15th January to 20th June 2021. The evaluation was designed through apoptotic and membrane depolarisation assays. Methanol extract at 0.29µg/ml, 29µg/ml and 290µg/ml doses were used, while rifampicin acted as the positive control and dimethyl sulfoxide acted as the negative control. Real time gene expression was also done to evaluate the levels of messenger ribonucleic acid expression. Data was analysed using SPSS 16.

Results: The methanol extract inhibited 50% of the PC-3 cells at a mean concentration of 35.24±0.23µg/mL compared to 15.31±1.83µg/mL for positive control. The extracts pushed PC3 cells significantly into early apoptosis compared to the positive control ($p<0.05$). The extracts greatly boosted the percentage of mitochondrial membrane potential depolarisation. Methanol extract was found to under-express both vascular endothelial growth factor A by 5-fold and arachidonate 5-lipoxygenase by 4-fold compared to positive control ($p<0.05$).

Conclusion: *Simarouba glauca* methanol extracts were found to differentially control proliferation and apoptosis in cancer cell lines PC-3**.**

Keywords: Methanol, Plant extracts, Gene expression, Prostatic neoplasms, Phytochemicals, Drug design.

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Introduction

One of the most prevalent cancers in the world, primarily affecting Western populations, is prostate cancer, which is thought to have affected 1.1 million men worldwide in 2012, making up over 15% of all cancer diagnoses in males.[1] About 70% of these occurrences took place in the more developed countries, which may be related to the widespread use of prostate-specific antigen (PSA) testing and subsequent biopsies in those parts of the world.[2] Nearly 100% of men diagnosed and treated for androgen-dependent prostate cancer will be clear of the illness 5 years post-diagnosis, which explains why the cure rate is so high.[3] However, when the condition worsens to an androgen-independent state, many men die as treatment ultimately fails.[4]

From a nutritional and medical standpoint, traditional herbal remedies are still highly significant for the majority of people. *Simarouba (S.) glauca* is among the herbs that are most frequently utilised in Asian countries.[5]

The Simaroubaceae family includes *S. glauca DC* (*S. glauca, SG*), also called laxmitaru and paradise tree.[6]

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Traditional medicine has made considerable use of *S. glauca* plant parts to cure malignancies.[7] For instance, it has been claimed that a decoction made from SG leaves is useful in treating a number of malignancies.[8] The pharmacological properties of SG include haemostatic, antipyretic, anthelminthic, antimalarial, antibacterial and anti-parasitic effects.[9] The fruit, seeds, pulp, and leaves of SG are thought to have stomachic, emmenagogue, antiviral, astringent, antibacterial and vermifuge properties.[10] In Guatemala, SG extracts are also used to cure digestive issues.[11] Food, fuel, fertiliser, wood and medicines are produced by plants from their bark, leaves, leaf litter, fruit pulp, roots, seeds, shells, stems and undesirable branches.[12] According to studies, SG water extract boosts skin moisture and hydration while encouraging keratinocyte division.[13]

The current study was planned to characterise the key mechanisms underlying the cytotoxic activity of *S. glauca*, and to evaluate its cytotoxic and pro-apoptotic effects.

Materials and Methods

This cross-sectional study was conducted at Iraqi centre for Genetic and Cancer research from 15th January to 20th June 2021 as no humans were directly involved in the study, permission was obtained to carry out the study in the laboratory of the Middle Technical University, Baghdad

Iraq. The simarouba stems were bought at the neighbourhood market, were well cleaned and allowed to air-dry for 7-10 days. The dried stems were then pulverised into fine powder and employed in the Soxhlet device for extraction with methanol, ethanol, chloroform, ethyl acetate and hot water. To effectively extract the phytoconstituents, approximately 20gm of the material was extracted with 300ml of the appropriate solvent at 45^0C for 24-30 hours. Following lyophilisation, the extracts were reconstituted in dimethyl sulfoxide (DMSO) 20mg/mL.

Dulbecco's modified eagle medium (DMEM) supplemented with levo (L-) Glutamine was used to develop PC-3 cell lines. In order to stop bacterial development, 10% heat-inactivated foetal bovine serum (FBS) (HI Media) and 1% penicillin 10,000U/ml-streptomycin 10,000mg/ml) (HI Media) were added. All cells were incubated at 37°C (5% carbon dioxide [CO2]). For cellular-based tests, plant extracts and fraction dilution, the prepared media was used to seed and culture the cells on a 96-well plate.

The PC-3 cell lines were raised in culture flasks at 37°C and 5% CO2 using DMEM. To ascertain the cytotoxicity effect of the extracts, sulforhodamine B (SRB) assay was used.[14] In 96-well plate (Falcon), the cells were seeded at 7.5x103 cells/mL and incubated for 24 hours at 37^0C and 5% CO2. After that, 100µL of the extract and camptothecin (Catalogue No: 7689-03-4) at 100, 75, 50, 30, 20, 10, 5, 2.5, 1.0 and 0.5µg/ml concentrations were added in place of the previous DMEM medium. Following 48 hours of incubation at 37^0C, 100µL of the extract and camptothecin at the different concentrations were added in place of the previous DMEM medium. Subsequently, 48 hours were spent incubating at 37^0C. Then, 50µL of cold trichloroacetic acid 50% was added, and the treated and untreated cells were fixed at 4^0C for 1 hour. The plates were air-dried after being cleaned with distilled water and dyed with 0.2% SRB for 20-30 min at room temperature. To get rid of the unbound colour, 1% acetic acid was used to wash the plates. Each well received 200µL of 10mM Tris-base after being allowed to air-dry. For 20 minutes, the plates were shaken, and the absorbance was recorded. After 20 minutes of shaking in the enzyme-linked immunosorbent assay (ELISA) photometer, the absorbance was measured at 492nm with a reference wavelength at 620nm. The impact on cell growth was calculated[15] by the formula: GI% = 1 - [OD (492 - 620) sample / OD (492 - 620) blank]) x 100. The GI% meant the percentage of inhibition in growth, and OD meant the optical density. IC50, or half maximal inhibitory concentration, values were also determined using linear-regression analysis.

PC-3 cells were cultured in DMEM with the addition of 10% foetal calf serum (FCS) and 1% antibiotics streptomycin. Briefly stated, 24 hours after the cells ($2x10^6$ cells/well) were implanted, methanol extract at various doses 0.29µg/ml, 29µg/ml and 290µg/ml was applied. Rifampicin was employed as positive control, whereas DMSO was used as negative control. The cells were taken out and utilised to measure the amount of apoptotic activity using annexin apoptosis kit (Genetic Bioscience) following 48 hours of incubation. The annexin V-fluorescein isothiocyanate/propidium iodide (FITC/PI) test was used in determining the proportion of apoptotic cells.[15]

Using the flow cytometry technique and the right fluorescent probe, the shift in mitochondrial transmembrane potential (1%m) caused by active phytochemical constituents on PC-3 was identified.[16] PC-3 was seeded at density of $1x10^6$ cells/ml and incubated for 48 hours in a 6-well plate. Methanol extracts and carbonyl cyanide chlorophenylhydrazone (CCCP) positive control were added following incubation, and the mixture was then incubated for a further 6 hours at 37°C with 5% CO2. After 15 minutes at 37°C of 2mM JC-1 (5,5,6,6'-tetrachloro-1,1',3,3' tetraethylbenzimi-dazoylcarbocyanine iodide) labelling on the treated cells, they were rinsed with warm PBS (phosphate buffered saline; pH7.4). On a flow cytometer equipped with 488nm, 530nm and 585nm emission filters, the cells were examined. Cells treated with 10mM of CCCP served as positive controls.

PC-3 cells were treated with active methanol extract and positive control at IC50 to induce apoptosis in order to study deoxyribonucleic acid (DNA) fragmentation. A DNA purification kit was used to extract the DNA In accordance with the manufacturer's recommendations, (Hi Media). Following quantification, 2µg of DNA sample were loaded for electrophoresis on a 0.8% agarose gel, and the gel was stained with ethidium bromide 10g/mL before being photographed under ultraviolet (UV) light.[17]

After exposing the PC-3 cells 5x105 cells/well to the plant extract and DMSO 1% negative control for 96 hours, they were used for ribonucleic acid (RNA) extraction. The chloroform extract was taken into consideration for the expression studies since it demonstrated the highest rate of decrease. RNA was extracted from confluent cells both treated and control using the RNeasy Mini Kit (Qiagen 74104),[18] according to the manufacturer's guidelines. The cells were trypsinised, spun down (6000rpm) for 5 minutes, and then dissolved in 560µl of AVL buffer along with 560µl of cooled ethanol. Following mixing, the mixture was centrifuged for approximately 1 minute at 8000rpm. The column was thoroughly cleaned by using 700µl of wash buffer before being incubated with AVE

buffer. The RNA obtained was stored at 20°C after being eluted. The RNA was used to synthesise the complementary DNA (cDNA) after being subjected to a qualitative UV spectrophotometer analysis (260/280).

Next, cDNA synthesis was performed with SuperScript TMII Reverse Transcriptase, 200U/l using an reverse transcription polymerase chain reaction (RT-PCR) kit (HI Media) in accordance with the manufacturer's instructions. The amount of RNA utilised in the reaction was 1.31µl, and the concentration that was obtained was 1.68µg/µl. Random primers were added along with 1µl of Reverse Transcriptase enzyme, and maintained for 10 minutes at 25°C. The cDNA obtained was saved and used for quantitative PCR (qPCR) after 45 minutes of incubation at 70°C.

Primers (Sigma-Aldrich) were designed using primer3 software and employed in the real-time test (Table). The real-time amplification was performed utilising iQTM SYBR Green (HI Media), and 1µl of the RT products and 600nM primers were utilised to carry out the reaction, which required 12.5µl in total.[19] Each reaction was carried out twice, along with the corresponding negative controls. In the Corbett Research cycler, qPCR was performed on both control and treatment samples (Bio-Rad). In the amplification investigation, 600nM of vascular endothelial growth factor A (VEGFA) (forward: 5′ TGCTCTACTTCCCCAAATCACT 3′ and reverse: 5′ CTCTCTGACCCCGTCTCTCT 3′) and arachidonate 5-lipoxygenase (ALOX5)- (forward: 5′ AAGCGATGGAGAACCTGTTCA 3′ and reverse: 5′ GTCTTCCTGCCAGTGATTCATG 3′) were employed. The programme was run for roughly 35 cycles at 94°C for 60 seconds, 65°C for 45 seconds, with extension at 72°C for 50 seconds using 1.31µl of the RNA products. For a comparison of the mRNA expression, the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5′ GCTGAGTACGTCGTGGAGTC 3′ and reverse: 5′ CCCATTCCCCAGCTCTCATA 3′; 455bp) was amplified alongside the gene members. The Ct method was used for comparative analysis.[20] The $\Delta\Delta^{Ct}$ method was used for comparison analysis and the Ct values were normalised to the housekeeping GAPDH gene.

Values were calculated using mean and standard deviation with each experiment performed in triplicate. Data was analysed using SPSS 16, and $p<0.05$ was taken as significant for all comparisons.

Results

By using the SRB test, it was determined whether *S. glauca* extracts had a cytotoxic effect on the development of PC-3 cancer cell lines. Aqueous and ethyl acetate exhibited low activity (30%) at the same concentration of 100µg/ml compared to the methanol extract, which had high activities on human PC-3 cell lines (Figure 1). Comparing the chloroform (68±0.61) and ethanol (65±0.85) extracts to the positive control and the methanol extract (92±0.11) at 100µg/ml showed that the activity was similar and the action was dosage-dependent ($p<0.05$). When compared to positive controls, the methanol extract on PC-3 killed 50% of the tumour cells at a concentration of 35.24±0.23µg/mL. This was highly significant when compared to positive control (15.31±1.83µg/mL) ($p<0.05$).

The results confirmed a significant increase ($p<0.05$) in PC-3 cells in the early apoptosis on treatment with S. glauca methanol extract (20%) compared to the control cells (7%) (Figure 2). The results were significant compared to positive control (25%). The percent reduction of viable cells was 48% and 55%, respectively, for methanol extract and camptothecin ($p<0.05$).

The cells were incubated with varying concentrations of methanol extracts for 6 hours and showed significant ($p<0.05$) results compared to the control cells (Figure 3).

The primary events of drug-mediated apoptosis are mitochondrial alterations, including fluctuations in mitochondrial membrane potential (MMP) (1% m). Disruption of mitochondrial functionality, including changes to the MMP and the mitochondria's oxidation-reduction potential, is one of the defining characteristics of early stage in apoptosis.[21] Methanol extract in PC-3 cell lines greatly boosted the percentage of MMP depolarisation. This may indicate that mitochondrial membrane depolarisation was involved in the active plant extracts' induction of cell death in PC-3. The quantification was 11.59% and 17.89% for methanol and positive control, respectively, compared to 4.31% of negative control ($p<0.05$) (Figure 4).

From the defragmentation assay after 72 hours of treatment with active methanol extract, the PC-3-treated cells exhibited more shearing of DNA than the positive control ($p<0.05$) (Figure 5).

Methanol extract significantly inhibited the gene mRNA expression by 5-fold. VEGFA expression was decreased by 5-fold compared to positive control, while ALOX5 gene expression was reduced 4-fold compared to positive control (Figure 6). In both cases, methanol extract could significantly alter the expression levels compared to positive control after 6 hours of incubation. However, after 12 hours of treatment, the activity was less compared to positive control ($p<0.05$).

Table-1: The list of primers used.

Gene		Template strand	Length	Tm	GC%	Product length
VEGFA	FW	TGCTCTACTTCCCCAAATCACT	21	56.4	55	138
	RV	CTCTCTGACCCCGTCTCTCT	20	58.6	50	
ALOX5	FW	AAGCGATGGAGAACCTGTTCA	21	56.8	55	350
	RV	GTCTTCCTGCCAGTGATTCATG	20	56.8	50	
GAPDH	FW	GCTGAGTACGTCGTGGAGTC	20	60.18	60	455

FW: Forward primer, RV: Reverse primer, Tm: Melting temperature, VEGFA: Vascular endothelial growth factor A, ALOX5: Arachidonate 5-lipoxygenase, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

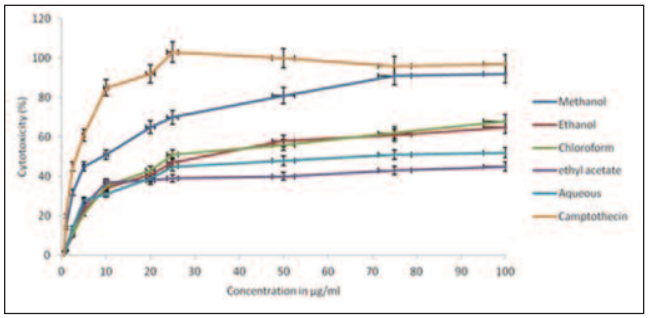

Figure 1: The cytotoxicity of the simarouba (S.) glauca extracts on PC3 cells at varying concentrations. All the values are average of triplicates and expressed as mean±standard deviation (SD). (p<0.05). Camptothecin was used as positive control.

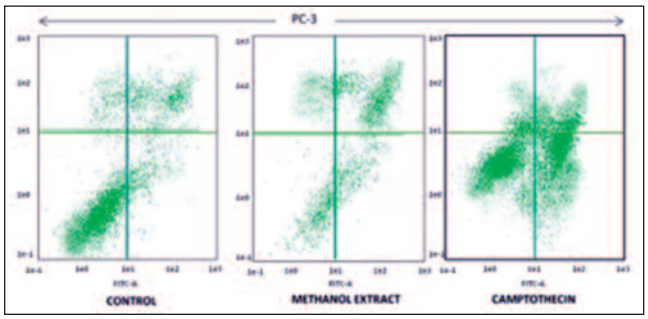

Figure 2: Dot plots of PC-3 (6hr treatment) with methanol extracts of simarouba (S.) glauca. The cells were treated with varying concentrations of methanol extracts for 6hr (p<0.05) compared to the control cells. Only results at 29µg/ml are shown. All the values are average of triplicates.

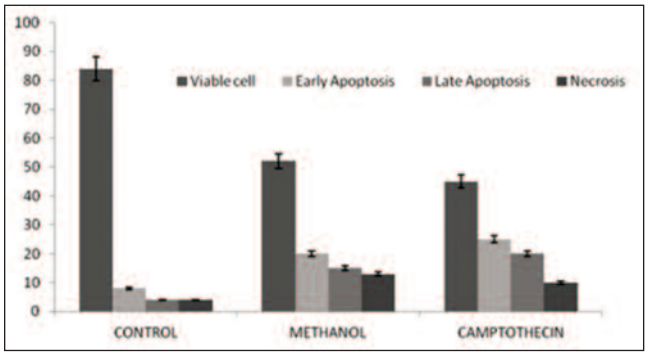

Figure 3: Cell distribution of PC-3 (6hr treatment) with methanol extracts of simarouba (S.) glauca. The cells were incubated with varying concentrations of methanol extracts for 6hr (p<0.05) compared to the control cells. Only results at 29µg/ml are shown. All the values are average of three independent experiments.

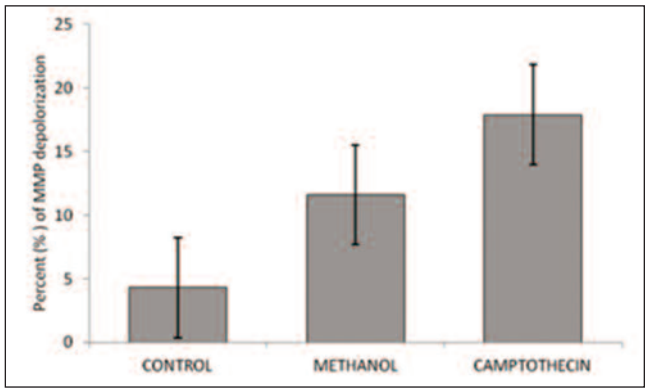

Figure 4: The depolarisation seen on PC3 prostate cancer cell lines as induced by methanol extracts of simarouba (S.) glauca. PC3 cell lines were incubated with IC50 of methanol extracts for 6hr. Data is represented mean ± standar error (SE) (n=3) (p<0.05).

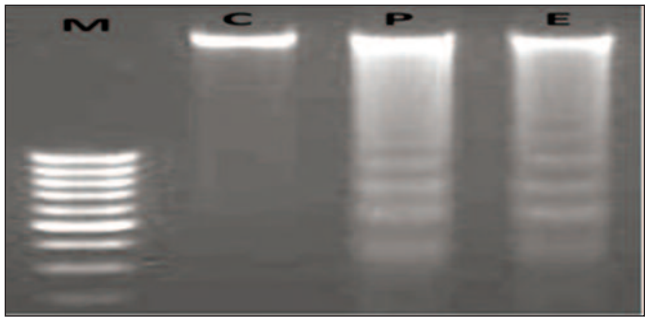

Figure 5: Agarose gel 0.8% showing the defragmentation assay of the active methanol extract. *C: Control, P: Positive control, E: Methanol extract, M: Molecular marker (1000bp).*

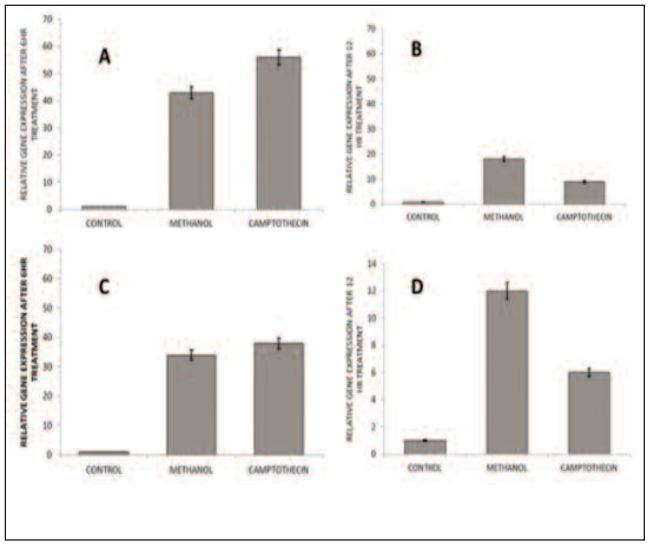

Figure 5: The biological activity of in vitro plant extracts through relative fold change expression. All values are average of three independent replicates and expressed as mean ± standard deviation (SD)(n=3) (p<0.05). A: Vascular endothelial growth factor A (VEGFA) expression at 6hr; B: VEGFA expression at 12hr; C: Arachidonate 5-lipoxygenase (ALOX5) expression at 6hr; D: ALOX5 expression at 12hr.

Discussion

The current study found that the methanol extract was highly significant in inhibiting the cell growth ($p<0.05$). For in vitro chemosensitivity testing, protein stain SRB was utilised. The SRB assay was more accurate and reproducible than tetrazolium (MTT) assay, but with a greater linearity.[22] As pre-clinical tests are required to determine the compatibility of materials, the findings are pertinent to medical products and devices used in dentistry. The methanolic extract showed significant cytotoxicity activity compared to the positive control. The findings are comparable to an earlier study[23] which used in vitro cytotoxicity assays, like MTT and SRB, to examine the cytotoxicity of methanol extracts of artocarpus heterophyllus. In the A549 cell line, the IC50 values of methanol extracts were 35.26µg/ml and 35.27µg/ml for MTT and SRB, respectively ($p>0.05$). Both tests were utilised in that study[23] to assess the cytotoxicity of a methanolic extract of *solanum nigrum*.

In the Middle East, elaeagnus angustifolia (EA) is used as a traditional medicine to treat a wide range of human illnesses.[24] Human oral and human epidermal growth factor receptor 2 (HER2)-positive breast cancer cells are inhibited in their ability to proliferate and invade when treated with EA flower extract. EA extract significantly and dose-dependently causes early and late apoptosis in both triple-negative breast cancer (TNBC) cell lines.[25]

Chromosome DNA was broken down into tiny internucleosomal fragments after treatment with dorema glabrum seed extract, which is a biochemical sign that a cell is going through apoptosis. Chromosome DNA began to break down into smaller fragments after exposure to the plant extract, which is a biochemical sign that a cell is going through apoptosis.[26]

Conclusion

S. glauca could slow down the development of human PC-3 cells, which makes *S. glauca* a potentially valuable natural product that needs to be studied further in search of novel anticancer drugs. In PC-3 cells, the three primary characteristics of cancer apoptosis activation, downregulation of gene members' expression, and DNA fragmentation were all overcome by methanol extracts.

Limitations: Only the effect on one cell line was studied. The effect on the normal cell line to act as a control was not examined so as to confirm the cytotoxicity.

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Conflict of Interest: None.

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