OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2019.

Research Article

Cytogenetic Toxicity of *Juniperus procera* Extract with Silver Nanoparticles Against Carcinoma Colon (Caco2) Cell Line *in vitro*

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Abstract

Background and Objective: Medicinal plant species represent a large source of new compounds that help for the preparation of new drugs. In this research was evaluated the cytotoxic activity of leaves and fruits extract of *Juniperus procera*, commonly used in folk medicine in Saudi Arabia against Carcinoma colon (Caco2) cell lines. **Materials and Methods:** Caco2 cell lines were exposed to different concentrations of leaves and fruits extract of *J. procera* with silver nanoparticles (AgNPs). The MTT assay was used to determine the cytotoxic effect of all treatments. Morphology of Caco2 cell lines was monitored using an inverted microscope. Nuclei of Caco2 cell lines was counted using hemocytometer chamber. The DNA fragmentation of Caco2 cell lines was separated electrophoretically on a 2% agarose gel containing 1 μg mL^{-1} ethidium bromide and visualized under ultraviolet transillumination. **Results:** Toxicity percentage of leaves and fruits extracts of *J. procera* against Caco2 cells was dose dependent, with the concentrations. Fruits extract was more effective (IC_{50} 8.80 μg mL^{-1}) than leaves (IC_{50} 11.44 μg mL^{-1}). Mixing IC_{50} of AgNPs (47.32 ppm) with IC_{50} of leaves or IC_{50} of fruits showed strong positive anti-cancer activity where the toxicity reached to 65.39 and 74.44%, respectively. Number of nuclei in treated cell decreasing with increasing extract concentration and not detected at high concentration of leaves (500 μg mL^{-1}) and fruits extract (250 and 500 μg mL^{-1}). The DNA of treated cells with IC_{50} of fruit and leaves extract remained intact as in the controls and DNA smearing was not detected but DNA fragmentation was clear with IC_{50} of AgNPs treatment. **Conclusion:** Present investigation concluded that the obtained IC_{50} fruits or leaves *J. procera* extract against Caco2 cells meaning that the extracts have potential anti-cancer properties.

Key words: *Juniperus procera*, cytotoxicity, silver nanoparticles, carcinoma colon cells, anti-cancer properties

Received: January 16, 2019 Accepted: March 22, 2019 Published:

Citation: Magdah Ganash, 2019. Cytogenetic toxicity of *Juniperus procera* extract with silver nanoparticles against carcinoma colon (Caco2) cell line *in vitro*. Int. J. Pharmacol., CC: CC-CC.

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Competing Interest: The author has declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer is a pathological condition characterized by excessive cell growth and deriving from loss of control over the cell cycle and/or decreased apoptosis[1]. Colon cancer is one of the most common types of cancers worldwide. While chemotherapy is one of the most widely used therapeutic strategies against colon cancer, it also has some limitations, such as normal cell toxicity and gradually increasing resistance in cancer cells. The discovery of new drugs for use in alternative strategies in cancer treatment is therefore highly desirable. Plants are regarded as very promising from this perspective, since they represent substantial sources of substances with various therapeutic uses. Most anti-cancer drugs are today produced from plants[2,3].

All developing countries and Gulf countries, most of the population still depends on folk medicine to treat serious diseases including cancers and various types of inflammations. Nowadays, use of medicines from plant source increases significantly with conventional therapies. Hence, the plants are gaining more attention by the researchers to find out new and effective agents for different diseases[4]. Over 50% of drugs used in clinical trials for anti-cancer activity have been isolated from natural sources or are related to them. Hence, the search for natural products to be used in cancer therapy represents an area of great interest in which the plant kingdom has been the most important source, providing many anti-tumor agents with novel structures and unique mechanisms of action[5]. About 61% of new drugs developed between 1981 and 2002 were based on natural products and they have been very successful, especially in the areas of infectious disease and cancer[6]. The herbal medicines have a vital role in the prevention and treatment of cancer and they are also commonly accessible[7]. Natural extracts and biologically active compounds isolated from plant species used in traditional medicine could be resources for new drugs[8-11].

Junipers are long lived trees which sometimes live up to 2000 years. It is belong to the Cupressaceae family. Economic importance of genus *Juniperus* was attributed to its various phytochemical constituents as coumarins and flavonoids[12], phenylpropanoid[13] and essential oils[14]. *Junipers* species are known for their potential as a source for two important chemical products, the anti-cancer drug synthetic precursor, podophyllotoxin and essential oils[15]. The medicinal uses of *Juniperus* spp. are widespread in many countries such as Saudi Arabia, Bosnia, Lebanon and Turkey and according to folk medicine was used for treating skin and respiratory tract diseases[16], urinary problems, rheumatism and gall bladder stones[17]. *Juniperus procera* is used in the southern part of Saudi Arabia as a traditional remedy for tuberculosis and jaundice[18]. Three alpha-hinokiol (3) and 3 alpha-hydroxymannol (9) isolated from *Juniperus przewalskii*, exhibited effective anti-tumor activities to cervical carcinoma (HeLa) and human ovarian carcinoma (HO-8910) cell lines[19]. *Juniperus chinensis*, commonly known as Chinese juniper, is a native and widely used ornamental plant in East Asian countries. The *J. chinensis* and plants of the same genus exhibit many bio-activities, such as anti-microbial, anti-fungal, anti-viral, anti-insect, anti-fertility, vasorelaxing and anti-tumor activities[20]. Several anti-parasitic, nematicidal, anti-microbial, anti-mycobacterial and hepatoprotective compounds were isolated from bark, leaves and berries of *J. procera* including abieta-7, 13-diene, ferruginol, isocupressic acid, (+)-Z-communic acid, (+)-totarol, 4-epi-abietol and sugiol[21].

Several studies indicated that deoxypodophyllotoxin isolated from *Juniperus communis* and from bark of *J. procera*[22] significantly induced cell apoptosis of breast cancer cells[15] and non-small cell lung cancer cells[23]. Previous studies reported anti-cancer activity of *J. phoenicea*[24,25]. Native Americans used *J. communis* berries as an appetite suppressant and in the treatment of diabetes[26], antioxidant[27], antimicrobial[28] activity. *Juniperus excelsa* berries extract and its fractions showed good and moderate levels of tumor inhibition[29]. All scientific reports reflect the unique properties silver nanoparticles (AgNPs) possess that find myriad applications such as antibacterial, antifungal and anti-cancer drugs, very good antioxidants, treatment of diabetes-related complications and wound healing activities[30-34]. The anti-tumor properties of AgNPs may be a cost-effective alternative in the treatment of cancer[35]. The purpose of this study was therefore to determine the cyto and genotoxic effect of *J. procera* leaves and fruits extract against colon cancer cell lines, one of the most common forms of cancer worldwide, with assessment the cytotoxicity of AgNPs combined with *J. procera* extracts.

MATERIALS AND METHODS

Location and total time duration of research work: Location of research work in King Abdulaziz University, Jeddah, Saudi Arabia. Work planed begin at November, 2018 and continued to March, 2019.

Collection and preparation of plant extracts: The *J. procera* aerial parts (leaves and fruits) were collected in November, 2018 from Fifa mountains, Jizan Region, southwest Saudi

Arabia. The plant was identified according to Migahid[36] and Chaudhary[37]. The fresh leaves and fruits (100 g for each it) of *J. procera* were air dried at room temperature under shade and ground into powder using an electric grinder. About 10 g from dried powder of leaves and fruits were incubated with 50 mL ethanol with shaking overnight, the extract was filtrated using filter paper and then drayed and the dry weight measured as 1.25 and 0.82 g for leaves and fruits, respectively.

Silver nanoparticles (AgNPs) used: The AgNPs (chemically synthesized <100 nm) were obtained from Sigma-Aldrich.

Cell line: The Caco2 cells supplied by the America Type Culture Collection (ATCC, USA) were used (Organism, homo sapiens human, tissue, colon, cell type, epithelial, culture properties, adherent, disease, colorectal adenocarcinoma, ATCC, ATB-37).

Cytotoxicity of plant extract (leaves and fruits) and AgNPs: Viable cells were measured by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay according to MTT kit (R and D Systems) manufacturer's instructions. Caco2 cells were inoculated in 96 well tissue culture plates at 1×10^5 cells mL^{-1} (100 µL well^{-1}) and incubated at 37°C for 24 h to develop a complete monolayer sheet. Growth medium was decanted from 96 well micro titer plates after confluent sheet of cells were formed, cell monolayer was washed twice with wash media. Two-fold dilutions of tested sample were made in RPMI medium with 2% serum (maintenance medium). Each dilution (0.1 mL) was tested in different wells leaving 3 wells as control, receiving only maintenance medium. Plate was incubated at 37°C and examined. Cells were checked for any physical signs of toxicity, e.g., partial or complete loss of the monolayer, rounding, shrinkage or cell granulation. The MTT solution was prepared (5 mg mL^{-1} in PBS) (BIO BASIC CANADA INC.,) then 20 µL MTT solution were added to each well. Place on a shaking table, 150 rpm for 5 min , to thoroughly mix the MTT into the media. Incubate (37°C, 5% CO_2) for 1-5 h to allow the MTT to be metabolized. Resuspend formazan (MTT metabolic product) in 200 µL DMSO. Place on a shaking table, 150 rpm for 5 min, to thoroughly mix the formazan into the solvent. Read optical density at 560 nm and subtract background at 620 nm. Optical density should be directly correlated with cell quantity. The OD of formazan formed in control cells was taken as 100% of viability and the positively stained cells with MTT are expressed as the percentage (%) compared to control. Log concentrations versus (%) cell viabilities were plotted with a logarithmic graph, which was then used to determine the IC_{50} values[38].

Morphological analysis: The morphology of cell was monitored using an inverted microscope. The Caco2 cells were checked for morphologic changes after 48 h exposure to range concentrations of plant extract or as compared to control and photographs were taken[39].

Combination assay of fruits and leaves extracts of *J. procera* with AgNPs: Combined assay of IC_{50} of fruits and leaves of *J. procera* with AgNPs (47.32 ppm) was evaluated to determine its toxicity on Caco2 cells using MTT assay as described previously[40].

Nuclei counting of treated Caco2 cells: The treated cells incubated in a mixture of citric acid and crystal violet that causes cells to lyse and the released nuclei to stain purple. Allow micro carriers from a culture sample (1 mL) to settle to the bottom of a centrifuge tube. Then the clear supernatant was removed by aspiration, 1 mL of crystal violet reagent was added and incubate at 37 at least 1 h. Introduce a sample into the hemocytometer chamber to count the purple-stained nuclei for whole cells as following: Volume of cell solution (mL)×Dilution factor in PBS blue (1:10)×Mean number of stained cells×10^4 (Conversion of 0.1 mm^3 to mL)[40].

DNA fragmentation: The culture medium of treated cells by IC_{50} of fruits (8.80 µg mL^{-1}) and leaves (11.44 µg Ml^{-1}) of *J. procera* extract and AgNPs (47.32 ppm) was removed and centrifuged at 3000 rpm for 5 min to collect detached cells. Adherent cells were lysed with a hypotonic lysis buffer (10 mM Tris-HCl, pH 8.0) containing EDTA (10 mM) and Triton X-100 (0.5%) and then pooled with pellets made of detached cells. RNA was digested using RNase (0.1 mg mL^{-1} at 37°C for 1 h) followed by proteinase K treatment for 2 h at 50°C. The DNA was extracted with a mixture of phenol, chloroform and isoamyl alcohol (25:24:1). The DNA was precipitated by adding an equal volume of isopropyl alcohol, stored overnight at 20°C and centrifuged at 12,000 rpm for 15 min at 4°C. The pellet was air-dried, resuspended in 20 µL Tris acetate EDTA buffer supplemented with 2 µL of sample buffer (0.25% bromphenol blue, 30% glyceric acid) and electrophoretically separated on a 2% agarose gel containing 1 µg mL^{-1} ethidium bromide and visualized under ultraviolet transillumination[41].

Statistical analysis: The results are reported as mean±standard error S.E. of three independent replicates. Statistical analysis of data were carried out by computer using SPSS ver. 22.0 software.

RESULTS

Cytotoxicity of *J. procera* extract and AgNPs: Leaves and fruits extracts of *J. procera* were tested against Caco2 colorectal carcinoma cells to determine their ability to inhibit cancer cell growth (Table 1). Toxicity (%) of leaves and fruits extracts of *J. procera* against Caco2 cells were dose dependent, with the concentrations. Toxicity (%) of leaves and fruits extracts of *J. procera* was similar up to 125 µg mL^{-1}. However, fruits extract was more effective because its IC_{50} (8.80 µg mL^{-1}) was less than leaves (11.44 µg mL^{-1}) extracts. The IC_{50} of leaves and fruits extracts of *J. procera* indicated the strongest active against Caco2. Further evaluation of AgNPs outlined that lowest level of cytotoxic activity against Caco2 cells at concentration less than 62.5 µg mL^{-1} with the IC_{50} values of 47.32 µg mL^{-1} but more than 62.5 µg mL^{-1} concentration, it showed highest toxicity (Table 1).

Combined effect of IC_{50} of AgNPs with *J. procera* extract on Caco2 cell line: The synergistic potential of AgNPs IC_{50} together with the IC_{50} of leaves and fruits extracts were tested against Caco2 cells (Table 2). Mixing AgNPs IC_{50} (47.32 ppm) with leaves IC_{50} (11.44 µg mL^{-1}) or fruits IC_{50} (8.80 µg mL^{-1}) showed strong positive anti-cancer activity where the toxicity reached to 65.397 and 74.44%, respectively. But unfortunately 1/10 IC_{50} of leaves or fruits antagonize the activity of AgNPs against Caco2 cells where the toxicity was 47.46 and 40.64%, respectively. Nuclei not detected at high concentration of leaves (500 µg mL^{-1}) and fruits (250 and 500 µg mL^{-1}) extract. At high concentration (250 and 500 ppm) of AgNPs, nuclei was detected (Table 3).

Effect of *J. procera* extract and AgNPs on morphological features of Caco2 cell line: Morphological features of Caco2 cells treated with different concentration of leaves extract of *J. procera* were reported (Fig. 1) and compared with the untreated cells. At 250 and 500 µg mL^{-1} concentrations of leaves extracts of *J. procera* the treated cells showed remarkable difference with the control. Also, morphological changes of Caco2 cells treated with fruits extract of *J. procera* were reported (Fig. 2), where destructuration of cells were clear at high concentration up to IC_{50} (8.80 µg mL^{-1}) of fruits extract. From the current results, the cells treated with fruits extract was more affected than treated with leaves extract, where the cells appeared less uniform with the loss of membrane integrity, rounding, shrinkage, however still intact at 31.25 and 62.50 µg mL^{-1}.

On the other hand, Identifiable morphological features of apoptosis were observed in the treated cells with AgNPs (Fig. 3), morphological changes were clear in a concentration-dependent manner in case AgNPs treatment.

Table 1: Cytotoxicity of *J. procera* extract (leaves and fruits) and AgNPs against Caco2 cells

Concentration (µg mL^{-1})	Leaves			Fruits			AgNPs		
	Viability (%)	Toxicity (%)	S.E.	Viability (%)	Toxicity (%)	S.E.	Viability (%)	S.E.	Toxicity (%)
500	6.633	93.367	0.00058	4.932	95.068	0.00033	6.803	0.00088	93.197
250	6.293	93.707	0.00033	5.102	94.898	0.00116	12.585	0.00353	87.415
125	5.952	94.048	0.00167	4.592	95.408	0.00058	15.476	0.00176	84.524
62.5	11.054	88.946	0.00186	6.293	93.707	0.00120	40.136	0.00176	59.863
31.25	20.918	79.081	0.00379	15.306	84.693	0.00416	53.401	0.00536	46.599
15.62	38.776	61.224	0.00458	23.809	76.190	0.00784	82.313	0.00318	17.687
7.81	49.489	50.510	0.00208	39.795	60.204	0.00379	97.959	0.00500	2.041
3.9	88.605	11.395	0.00328	82.143	17.857	0.00458	99.489	0.00452	0.510
0.00	100.000	0.000	0.00	100.000	0.000	0.00	100.000	0.00	0.000
IC_{50}	11.440			8.80			47.320		

Table 2: Cytotoxicity of combined IC_{50} of *J. procera* extract (leaves and fruits) with IC_{50} AgNPs against Caco2 cells

Treatments	Concentration (µg mL^{-1})	Viability (%)	Toxicity (%)	Standard error
IC_{50} Leaves+IC_{50} AgNPs	11.440+47.32	34.60	65.40	0.00715
IC_{50} Fruits+IC_{50} AgNPs	8.800+47.32	25.56	74.44	0.00284
1/10 IC_{50} Leaves+IC_{50} AgNPs	1.144+47.32	52.54	47.46	0.00437
1/10 IC_{50} Fruits+IC_{50} AgNPs	0.880+47.32	59.37	40.64	0.00252
Control	0.00	100.00	0.00	0.00770

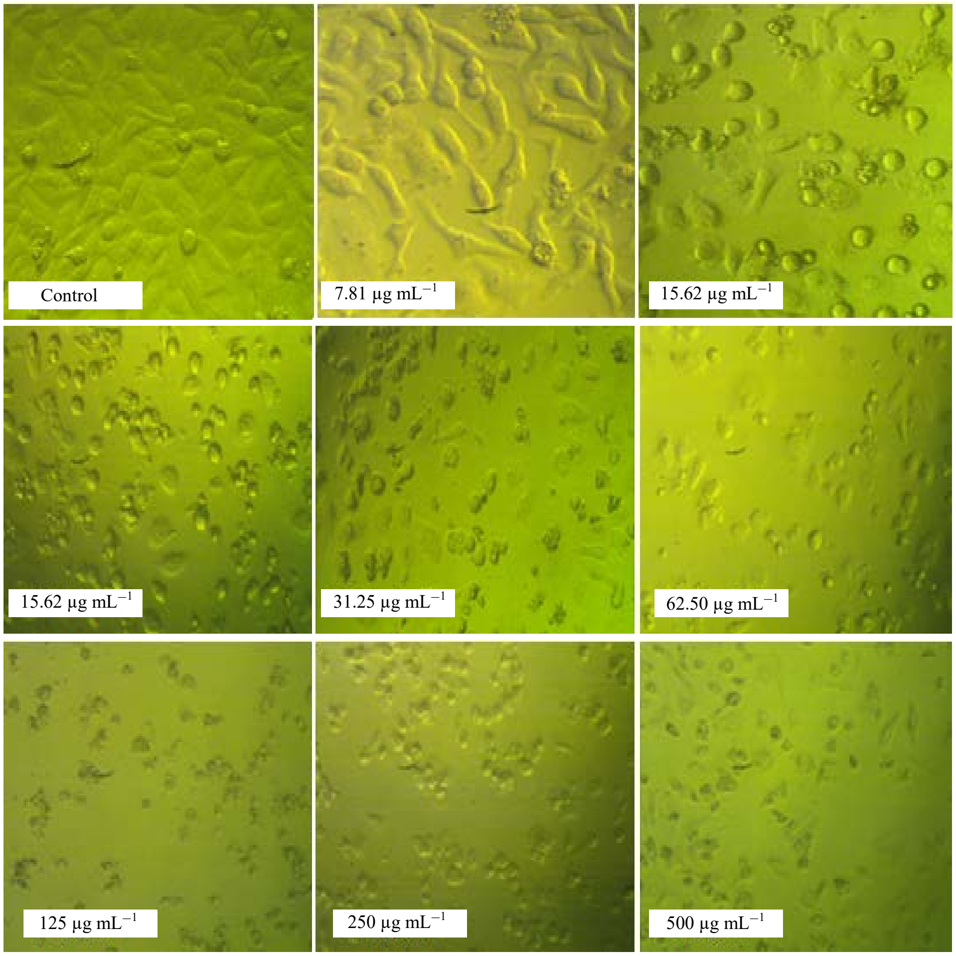

Fig. 1: Morphological features of Caco2 cells treated with different concentration of leaves extract of *J. procera*

Table 3: Nuclei counting of Caco2 cells lines treated with leaves, fruits extracts of *J. procera* and AgNPs

	Number of nuclei		
Concentration (μg mL^{-1})	Leaves	Fruits	AgNPs
500	0	0	175000
250	25000	0	150000
125	50000	25000	475000
62.50	25000	50000	800000
31.25	75000	100000	1175000
15.62	275000	225000	1550000
7.81	500000	550000	1425000
3.90	1475000	1200000	1400000
0.00	1400000	1400000	1400000

Effect of *J. procera* extract and AgNPs on DNA fragmentation of Caco2 cell line: The DNA fragmentation assay was performed on Caco2 cells to elucidate the mechanism of cells death. As shown in Fig. 4, treatment with IC$_{50}$ of fruit and leaves extract of *J. procera* (8.80 and 11.44 μg mL^{-1}) remained intact as in the controls and DNA smearing was not detected. In cells treated with IC$_{50}$ of AgNPs light smearing was observed.

DISCUSSION

The leaves and fruits extracts of *J. procera* displayed patented the inhibitory activity against Caco2 cells. Generally, *Juniperus* species are a good bet in the development of new drugs with natural compounds, it was reported that phytochemical analysis *J. procera* leaves indicated the presence of diterpenes, alkaloids and

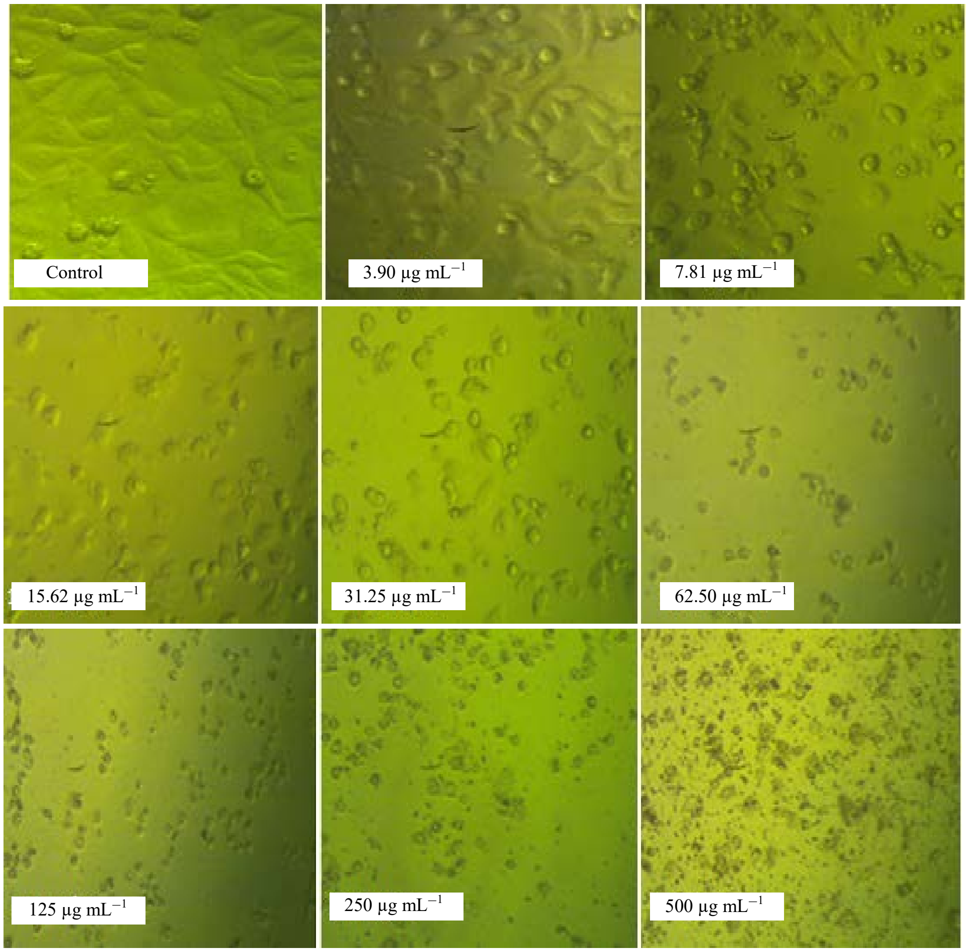

Fig. 2: Morphological features of Caco2 cells treated with different concentration of fruits extract of *J. procera*

flavonoids in extract. These constituents play an important role as anti-tumor activity. The IC$_{50}$ obtained in current study on Caco2 cell using *J. procera* extract of fruits or leaves meaning that the extracts have potential anti-cancer properties[42]. Topcu *et al.*[43] showed weakly active (IC$_{50}$ = 17.7 μg mL^{-1}) of *J. excels* leaves extract against A2780 (human ovarian cancer cell line). In recent study, the anti-proliferative activity of *J. communis* berry extracts against Caco2 carcinoma cell line was noted, with IC$_{50}$ value[44] 500 μg mL^{-1}. In previous studies it was investigated that the anti-cancer activities of *J. procera* leaves extract against hepatocellular carcinoma (Hep G-2) cells lines with IC$_{50}$ value 131.9 μg mL^{-1}. The differences in IC$_{50}$ may be due to species of plant, target cells, types of extracted solvent and ingredient of plant but all these factors don't eliminate effectiveness of *J. procera* against cancer cells[42]. Similar findings were reported by Nabi *et al.*[29], where the anti-tumor activities of *J. excelsa* extract and its fractions showed good levels of tumor inhibition 86.6% inhibition. AgNPs also showed anti-cancer activity against Caco2 cells (Table 1). Several studies reported that, good anti-cancer activity of AgNPs against HCT-116 colon cancer cells[45] and LoVo cells line[46]. Synergistic potential of AgNPs IC$_{50}$ with leaves extract IC$_{50}$ or of fruits extract IC$_{50}$ were reported in the current study against cancer cells suggested that a better interaction of the mixture with tested cells. Generally, synergistic abilities of leaves and fruits extract of *J. procera* with AgNPs may reduce the use of AgNPs and therefore, reduce the side effects of metal.

Number of nuclei in treated cell decreasing with increasing extracts concentration. The toxic effect of treatments and its concentration reflected by the number of nuclei and suggestive for the apoptotic activities of treatments. Actually, the cytotoxic effect observed in current study is probably due to the presence of Hinokiol (3,12-dihydroxy-abieta-8,11,13-trien) whose presence in

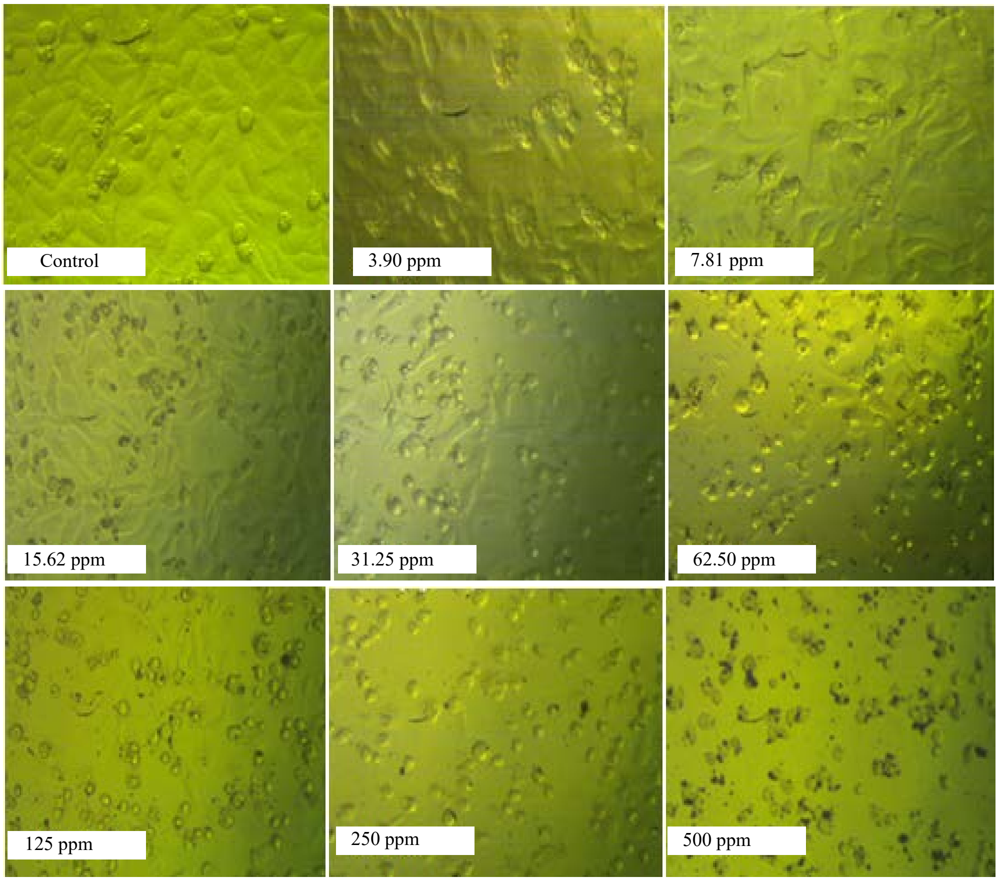

Fig. 3: Morphological features of Caco2 cells treated with different concentration of AgNPs

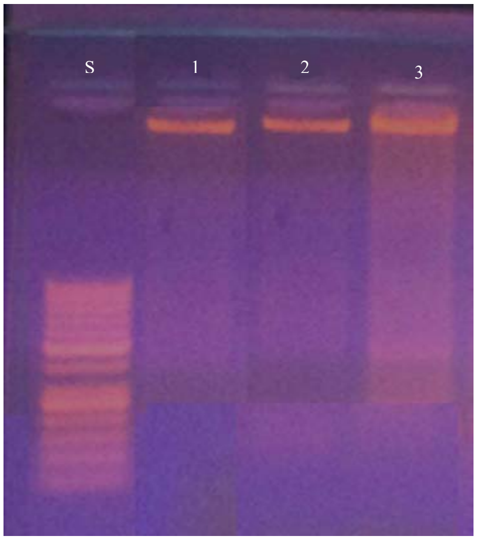

Fig. 4: Effect of leaves (Lane 1) and fruit (Lane 2) extract of *J. procera* and AgNPs (Lane 3) on DNA fragmentation in Caco2 cells. DNA standard 100 bp (Lane St.)

the *J. procera* has been reported by Wang *et al.*[19]. A very recent study detected that sugiol (2-hydroxy-abieta-8,11,13-triene-7-one) as a constituent in *J. procera* reduced the cell viability of human pancreatic cancer cells (Mia-PaCa2)[47]. Therefore, the results indicated that apoptosis may be induced by the use of these two plant extracts but further studies should be performed to confirm this conclusion. Compared to control cells, morphology of treated Caco2 cells were changed particularly at high concentrations strongly suggesting that cell death is occurring in the wells treated with the plant extract and AgNPs. George *et al.*[48] detected morphological alterations in the Caco2 cells exposed to *Rubusfairholmianus* root extract. Therefore, the results obtained from this study confirm that AgNPs induced cell death via apoptosis. The data obtained by Krishnaraj *et al.*[49] exhibited cytotoxic effects of AgNPs on MDA-MB-231, human breast cancer cells and the apoptotic features were confirmed through DNA fragmentation assays.

CONCLUSION

These findings support and extend previous studies examining the anti-cancer effects of *J. procera* extracts against cancer cell. The *J. procera* fruits extract was more anti-cancer effective than leaves extract. The IC_{50} of *J. procera* extracts of leaves or fruits induced the cytotoxic effects of AgNPs, therefore *J. procera* extract may minimize the concentration of AgNPs used in cancer treatment. The DNA fragmentation assay confirmed that AgNPs induced Caco2 cells death via apoptosis. The results indicated that *J. procera* was a promising anti-cancer agent for Caco2 cells. The performed experiments add scientific evidence to conduct further studies.

SIGNIFICANCE STATEMENT

Public pressure to increase the use of natural therapeutic agent in treatment of diseases has increased in the recent years. Therefore; this study discovered the *J. procera* extract (leaves and fruits) for cancer treatment *in vitro*. This study will help the researchers to uncover the natural compounds from medicinal traditional plants that many researchers were not able to explore and helpful information in cancer therapy.

ACKNOWLEDGMENT

Thankful to King Abdulaziz University, Jeddah, Saudi Arabia.

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