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In vitro Cytotoxic Activity and Phytochemical Analysis of the Aerial Parts of *J. communis* L. Cultivated in Egypt

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Abstract

In the present study screening of the cytotoxic activity of *J. communis* L. family (Cupressaceae) cultivated in Egypt was performed using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) in vitro assay against three cancer cell lines, human prostate cancer cells (PC3), human colon cancer cells (HCT 116) and breast cancer cells (MCF7). Both LC_{50} and LC_{90} were determined for each cell type using Doxorubicin as positive control, the highest activity with the safest margin of use was recorded for the total methanolic extract against human breast cancer cell line (MCF7). In addition to a phytochemical analysis of the total methanolic extract of the aerial parts which led to the isolation and structure elucidation of two flavonoids, amentoflavone (**1**) and quercetin-3-o-α-L-rhamnopyranoside (**2**) separated for the first time from this specie.

Keywords: cancer, flavonoids, Juniperus.

INTRODUCTION

Cancer treatment is one of the most investigated subjects in recent days and natural plants have provided many of the effective anticancer agents currently used. Over 50% of the drugs used in clinical trials for anticancer are of natural origin or related to them [1]. Economic importance of genus *Juniperus* was attributed to its various phytochemical constituents as lignans [2], coumarins and flavonoids [3], phenylpropanoid [4] and essential oils [5]. Junipers species are known for their potential as a source for two important chemical products, the anticancer drug synthetic precursor, podophyllotoxin and essential oils [6,7,8]. In previous studies, several biological activities were reported for *Juniperus communis* L. including antioxidant [9,10,11] , for hair growth promoting in alopecia patients [12], antinociceptive and antiinflammatory activities [13] and several studies on antimicrobial activity against both gram positive and gram negative bacteria [14,15,16,17]. Also it was reported to posses antidiabetic and antihyperlipidemic activities [18] , anticataleptic and neuroprotective activity in Parkinson disease [19], *Juniperus communis* berries used in traditional medicine as a strong urinary tract disinfectant [20] and as a female contraceptive [21]. Being such an abundantly distributed plant all over the world may be it is worth studying for an important medicinal use in treatment of such dangerous diseases.

MATERIALS AND METHODS

Plant material:

Samples of non flowering aerial parts of *Juniperus communis* L. family (Cupressaceae) were collected during April 2013 from international garden of Cairo, Egypt. The systematic identification of the plants material was verified by Dr. Therese Labib specialist for plant identification. A voucher specimen (JC-37 *J. communis* L.) was deposited in the herbarium of pharmacognosy department, Faculty of Pharmacy, Helwan University.

Materials and apparatus:

^{1}H and ^{13}C NMR were recorded on a Varian Mercury instrument (^{1}H-, 500MHZ, ^{13}C-, 75 MHz). TMS was used as internal standard, solvent acetone. Analytical precoated TLC silica gel 60 GF_{254} and Preparative TLC silica gel G for TLC (E-Merck). Silica gel G_{60} (E-Merck) for Column chromatography and Sephadex LH-20 (AB Uppsala, Sweden). Rota-vapor (Buchi, G. Switzerland). A Laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). A water jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). microplate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA)

Materials for biological assay:

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), RPMI 1640 medium (for PC3 and HCT116) and DMEM for (MCF 7), 1% antibiotic-antimycotic mixture (10.000 µg/ml potassium penicillin, 10.000µg/ml Streptomycin sulfate, 25µg/ml Amphotericin B), 1% L-glutamine, Sodium dodecyl sulphate (SDS), Doxorubicin. Three human cell lines: human prostate cancer cells (PC3), human colon cancer cells (HCT 116) and Breast cancer cells (MCF7)**.** This study followed principles in the Declaration of Helsinki.

Extraction and isolation:

The air-dried coarsely powdered aerial parts of *J. communis* L. (1.0 kg) were extracted with methanol (3L×2) at room temperature. The combined methanolic extract was evaporated to dryness under reduced pressure to yield a residue of (35g).The residue was suspended in water (500ml) and successively partitioned with hexane (500mlx2), methylene chloride (500mlx2), ethyl acetate (700mlx3), and finally butanol saturated with water (700mlx3). A part of the ethyl acetate fraction (3g) was chromatographed on silica gel column starting elution with 100% CH_2Cl_2 and gradient increase in polarity was done using methanol up to 20%. A total of 60 fractions (100ml

each) were collected. The fractions were monitored by TLC using solvent system CH_2Cl_2-MeOH (7:1) and examined under UV followed by spraying with $FeCl_3$ reagent. Fractions eluted with CH_2Cl_2-MeOH (88:12) were found to be similar and the combined fractions were repeatedly chromatographed on preparative silica gel TLC plates eluted with CH_2Cl_2-MeOH (7:1). Repeated purification of each compound on Sephadex LH-20 column, eluted with methanol, afforded compounds **1** (20mg) and **2** (35mg). 10g of the alcoholic extract of the aerial part of *J. communis* L. were reserved for biological study.

General method for acid hydrolysis:

Each glycoside (2mg) in 3ml 2N HCL and 3ml methanol, was heated at 100°C for 2 hours. The mixture was left to cool, diluted with water and extracted twice with ethyl acetate. From the ethyl acetate layer, the aglycone was detected by TLC. The aqueous layer was repeatedly diluted with methanol and evaporated to dryness. The residue was investigated to detect the sugar L-rhamnose by PC using solvent system BAW (4:1:5, top layer).

Cytotoxic activity (MTT assay):

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to purple formazan [22]. All procedures were done in a sterile area using a Laminar flow cabinet , Cells were batch cultured for 10 days, then seeded at concentration of $10x10^3$ cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 ºC for 24 h under 5% CO_2 using a water jacketed Carbon dioxide incubator , Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of the total alcoholic extract of aerial part of *J. communis* L. to give a final concentration of (100-50-25-12.5 and 1 ug/ml). Cells were suspended in DMEM medium,1% antibiotic-antimycotic mixture (10,000 U/ml Potassium Penicillin, 10,000µg/ml Streptomycin Sulfate and 25µg/ml Amphotericin B) and 1% L-glutamine in 96-well flat bottom microplate at 37 ºC under 5% CO2. After 48 hours of incubation, the medium was aspirated, 40 µl MTT salt (2.5µg/ml) were added to each well and incubated for further four hours at 37ºC under 5% CO2. To stop the reaction and dissolve the formed crystals, 150µL of 10% Sodium dodecyl sulphate (SDS) in deionized water was added to each well and incubated overnight at 37ºC. Doxorubicin was used as a positive control [23]. The absorbance was then measured using a microplate multi-well reader at 595nm and a reference wavelength of 620nm. Statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plants extracts and its final concentrations on the cells was less than 0.2%. The percentage of change in viability was calculated according to the formula:

$$\left(1 - \frac{X}{NC}\right) \times 100$$

X: Absorbance of extract

NC: Absorbance of negative control

RESULTS

Cytotoxic activity

Results of the cytotoxic analysis are shown in Table (1), Figure (1) with the LC_{50} and LC_{90} determined for each cell type using Doxorubicin as positive control.

Table 1: LC90 of *J. communis* L extract against human prostate cancer cells (PC3), human colon cancer cells (HCT 116) and Breast cancer cells (MCF7).

	MCF7	HCT 116	PC3
LC_{90} (ppm)	54	86.95	85.55

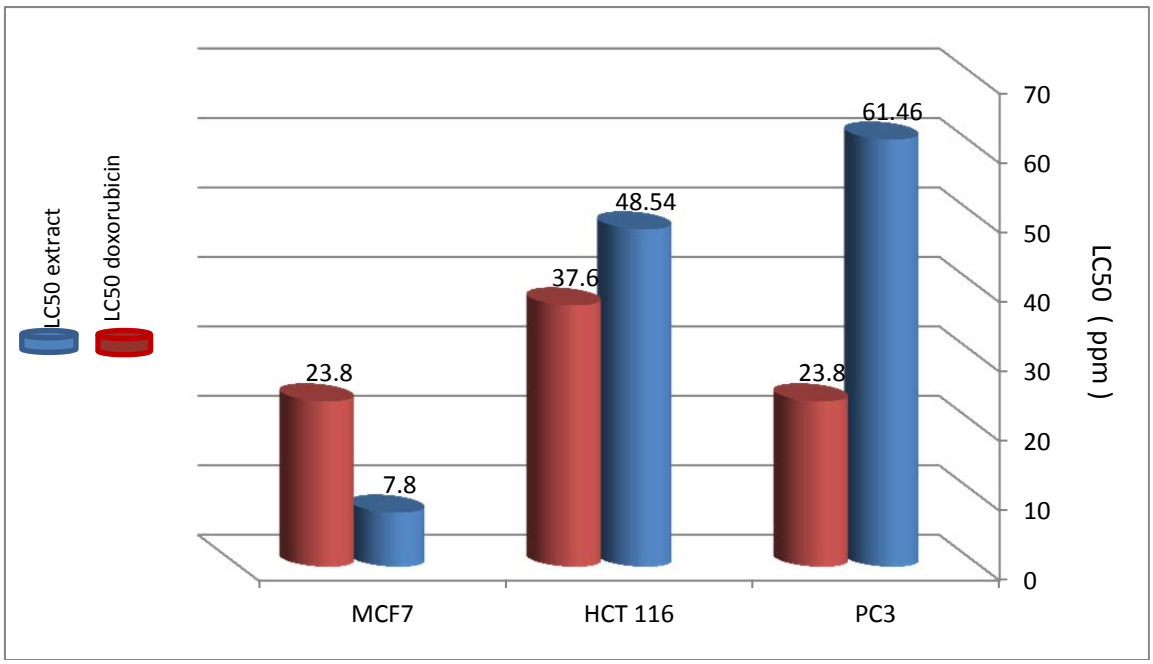

Figure 1: LC_{50} of *J. communis* L against human prostate cancer cells (PC3), human colon cancer cells (HCT 116) and Breast cancer cells (MCF7)

Chemistry

Two flavonoids were separated for the first time from the total methanolic extract of the aerial parts of *J. communis* L. using different chromatographic techniques, structure elucidation and identification was done Using various spectroscopic analysis in addition to comparing data to published literature.

Amentoflavone ***(1):*** (Figure 2) was obtained as yellow powder (20 mg) soluble in acetone, m.p=300 ºC, ^{1}H NMR (500 MHz, acetone-d6) δ: 7.70 (2H, d, J=9 Hz, H-2‴, H-6‴), 6.62 (2H, d, J=9 Hz, H-3‴,H-5‴), 8.32 (1H , d, J=9 Hz, 5'), 7.80 (1H, dd, J=2, 9 Hz, H-6'), 6.66 (1H, d, J=9 Hz, H-2'), 6.15 (1H, d, J=2Hz, H-6), 6.28 (1H, d, J=2Hz, H-8), 6.50 (1H, s, H-3) , 6.61(1H, s, H-3"), 6.10 (1H, s, H-6"). ^{13}CNMR (500 MHz, acetone-d_6) δ: 178.5 (C-4), 157.9 (C-4'), 157.7 (C-2), 165.5 (C-2"), 102.1 (C-3, C-3"), 162.3 (C-5), 160.4 (C-5"), 98.8 (C-6), 101.4 (C-6"), 164.4 (C-7), 162.9 (C-7"), 93.7 (C-8), 106.4 (C-8"), 157.2 (C-9), 159.7 (C-9"), 104.9 (C-10), 102.5 (C-10"), 121.5 (C-1'), 121.0 (C-1‴), 131.6 (C-2'), 128.1 (C-2‴), 124.2 (C-3'), 119.8 (C-5'), 115.5 (C-3‴), 176.1 (C-4"), 160.5 (C-4‴), 115.4 (C-5‴), 131.6 (C-6'), 128.1 (C-6‴)

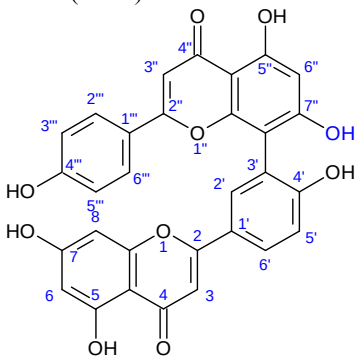

Figure 2: Amentoflavone

Quercetin-3-o-α-L-rhamnopyranoside ***(2):*** (Figure 3) was obtained as yellow powder (18 mg) soluble in acetone m.p. = 174ºC, ^{1}HNMR (500 MHz, acetone-d_6) δ: 12.68 (1H, s, OH-5), 7.48 (1H, d, J=2.3 Hz, H-2'), 7.35 (1H, dd, J=8.4 Hz, H-6'), 6.95 (1H, d, J=8.4 Hz, H-5'), 6.44 (1H, d, J=2.3 Hz, H-8), 6.23 (1H-d, J=2.3 Hz, H-6), 5.49 (1H, d, J=1.5 Hz, H-1" Rha), 3.37-3.40 (4H, H-2" –H5"), 0.88 (3H, d, J=5.6Hz, Me-6"). ^{13}CNMR (500 MHz, acetone-d_6) δ: 178.47 (C-4), 164.23 (C-7), 162.34 (C-5), 148.5 (C-4'), 157.13 (C-9), 157.6 (C-2), 145.06 (C-3'), 134.91 (C-3), 121.95 (C-1'), 121.73 (C-6'), 115.95 (C-2'), 115.37 (C-5'), 104.91 (C-10), 101.76 (C-1"), 98.74 (C-6), 93.72 (C-8), 72.13 (C-4"), 71.36 (C-2"), 70.7 (C-3"), 70.50 (C-5"), 16.94 (C-6").

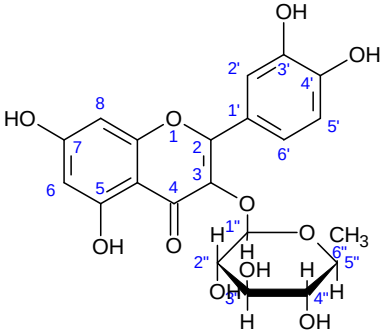

Figure 3: Quercitrin

DISCUSSION

Cytotoxic activity:

The total methanolic extract of *J. communis* L. showed the highest activity against human breast cancer cell line with the safest margin of use [8], its LC50 is less than half that of Doxorubicin used as positive control followed by its effect against human colon cancer cells (HCT 116) and the least affected was against human prostate cancer cells (PC3) as seen in the results shown in Table (1), Figure (1). These results are in conjunction with the previously reported studies on other members belonging to genus Juniperus including the leaves of *J. phoenicea* L., *J. excels, J. brevifolia* and *J. oxycedrus* [2, 24, 25, 26, 27].

Chemistry:

Structure elucidation and identification was done Using various spectroscopic analysis in addition to comparing data to published literature for Amentoflavone (**1)** [28, 29] which was previously reported from genus juniperus [30, 31] and quercetin-3-o-α-L-rhamnopyranoside (Quercitrin) (**2**) [32, 33, 34] which are separated for the first time from *J. communis* L.

CONCLUSION

This study provides a base for further investigation of the promising medicinal plant, *J. communis* L., as an anticancer drug. Promising results were obtained for the crude methanol extract of the aerial parts especially against human breast cancer cell line (MCF7). However further in vitro and in vivo studies are recommended for an in deep study for the determination of the mechanism of action and creation of more cell targeted cancer treatment.

DECLARATION OF INTEREST

The authors report no declarations of interest

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