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Research Paper

Phytochemical constituents of the aerial parts of *J. horizontalis* Moench and evaluation of its cytotoxic activity

N.S. Ghaly[1], S.A. Mina[*2], N. Younis[3]
[1]Chemistry of Natural Compounds Department, Research Center, Dokki, Giza, Egypt.
*[2]Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Cairo, Egypt.
[3]Pharmacognosy Department, Faculty of Pharmacy, El Ahram Canadian University
*Corresponding Author
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ABSTRACT

The Cytotoxic activity of the methanol extract of the aerial parts of *Juniperus horizontalis* Moench (Family-*Cupressaceae*) cultivated in Egypt was tested against three cancer cell lines: human prostate cancer cells (PC3), human colon cancer cells (HCT 116) and breast cancer cells (MCF7) using the mitochondrial dependent reduction MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) in vitro assay, the total extract showed promising activity against both (MCF7) and (HCT 116) comparable to that of the positive control Doxorubicin. The phytochemical analysis of the total methanolic extract lead to the isolation and structure elucidation of three compounds identified as 3',4',5' trimethoxy cinnamyl alcohol (**1**), corchoionoside C (**2**) and the biflavone podocarpusflavona A (**3**) .

Key words: *Juniperus horizontalis* Moench; MTT assay; Cancer.

INTRODUCTION

The search for a natural treatment for cancer was a major target for numerous scientists to avoid severe and destructive side effects of chemical and radiological treatments (Newman et al., 2007). The economic importance of genus juniperus (Family- *Cupressaceae*) is mainly attributed to its various phytochemical constituents as lignans (David et al., 1980), coumarins, Flavonoids (Gilles et al., 1996), phenylpropanoid (Gilles et al., 1997), volatile oils (Ehsani et al., 2012),etc. Several juniperus species were tested for various medicinal effects such as hypoglycemic, hypolipidemic (Jung et al., 2008), antihepatotoxic (Aboul-Ela et al., 2005), antidiarrheial (Qnais et al., 2005), antimicrobial activity against both Gram-positive and Gram-negative bacteria (Abdenour et al., 2012), antioxidant and anti-inflammatory (Marija et al., 2011) in addition to its antiproliferative activity against cancer cell lines namely HeLa, A-549 and MCF-7 (Moujir et al.,2010), and as anticancer agent (Laila et al., 2010). It is traditionally known about Genus juniperus its use as a strong urinary tract disinfectant, herbal remedy for diabetes (McCabe et al., 2005) and as a female contraceptive (Tilford 1997). This previous evidence on the ability of genus junipers to affect cancer cells encouraged us in the present study to

test the total methanolic extract of the aerial parts of *J. horizontalis* Moench. against three types of human cancer cell lines, human prostate cancer cells (PC3), human colon cancer cells (HCT 116) and breast cancer cells (MCF7) using MTT in vitro assay, both LC_{50} and LC_{90} were determined for each cell type using Doxorubicin as positive control, it is worth mentioning that it is the first record to study Cytotoxic activity for the aerial parts of *J. horizontalis* Moench. In addition to a phytochemical investigation of the methanolic extract using different chromatographic techniques.

MATERIALS AND METHODS

Plant material: Samples of non flowering aerial parts of *J. horizontalis* Moench. (Family- *Cupressaceae*) was collected during May 2013 from international garden of Cairo, Egypt. Identification of the plant material was verified by Dr. Therese Labib senior head of specialist for plant identification. A voucher specimen (JH-38, *J. horizontalis* Moench.) was deposited in the herbarium of pharmacognosy department, Faculty of Pharmacy, Helwan University.

Materials and apparatus: Silica gel G_{60} (E-Merck) for Column chromatography and Sephadex LH-20 (AB Uppsala, Sweden) were used. ^{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. Analytical precoated TLC silica gel 60 GF_{254} and Preparative TLC silica gel G for TLC (E-Merck) were used. A Laminar flow cabinet biosafety class II level (Baker, SG403INT and 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 dried aerial parts of the plant (1.2Kg) was finely powdered and extracted with hexane twice at room temperature. The marc was then extracted with methanol and allowed to stand at room temperature with occasional shacking. The extraction process was repeated twice with the same solvent and the combined extract was evaporated under reduced pressure at a temperature not exceeding $40^{0}C$ to give a green residue (136.6g). A part of the residue (104.6g) obtained from the methanolic extract was subjected to silica gel column chromatography. The column was eluted by CH_2Cl_2 and then with CH_2Cl_2-MeOH in the direction of increasing polarity up to 20% MeOH in CH_2Cl_2. A total of 50 fractions (100ml each) were collected. The fractions were monitored by TLC using the solvent system CH_2Cl_2-MeOH (5:1), examined under UV followed by spraying with $FeCl_3$ or spraying with 20% sulphuric acid in ethanol. The fractions eluted with (92:8) CH_2Cl_2-MeOH were found to be similar, so they were combined together and they were found to contain a mixture of compounds (1-3) after TLC examination. The combined fractions were repeatedly chromatographed on silica gel TLC plates eluted with CH_2Cl_2-MeOH (5:1). Repeated purification of each compound on Sephadex LH-20 column eluted with MeOH, afforded compounds **1** (67.0mg), **2** (65.0mg) and **3** (80mg).10g of the alcoholic extract of the aerial part of *J. horizontalis* were reserved for biological study.

General method for acid hydrolysis: The glycoside (2mg) in 3ml 2N HCL and 3ml methanol was heated at 100^{o}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 D-glucose by PC using solvent system BAW (4:1:5, upper 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 (Mossman, 1983). 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 37ºC 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. horizontalis* Moench. to give a final concentration of (100-50-25-12.5 and 1µg/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 37ºC under 5% CO2. After 48 hours of incubation, the medium was aspirated, 40µl 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 37°C. Doxorubicin was used as a positive control (Thabrew et al., 1997). DMSO is the vehicle used for dissolution of plants extracts and its final concentrations on the cells was less than 0.2%. 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.

The percentage of change in viability was calculated according to the formula:

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

- X: Absorbance of extract, NC: Absorbance of negative control

RESULTS

Cytotoxic activity results for the cytotoxic analysis are shown in (**Table 1**) with the LC50 and LC90 determined for each cell type against three cancer cell lines: (PC3), (HCT 116) and (MCF7) using Doxorubicin as positive control.

Table-1: LC values of *J. horizontalis* against human prostate cancer cells (PC3), human colon cancer cells (HCT 116) and Breast cancer cells (MCF7).

	MeOH extract of *J. horizontalis* Moench.		
	PC3	**HCT 116**	**MCF7**
LC_{50} (ppm)	32.7	36.7	17.7
LC_{90} (ppm)	62.19	75.4	53
LC_{50} (ppm) of Doxorubicin	23.8	37.6	23.8

Chemistry: ^{1}H and ^{13}C NMR data of compounds **1**, **2** and **3** are shown in (**Table 2, 3 and 4** respectively)**.** Structure elucidation and identification was done Using various spectroscopic analysis in addition to comparing data to published literature. The three

compounds were identified as 3',4',5' trimethoxy cinnamyl alcohol (**1**), corchoionoside C (**2**) and the biflavone podocarpusflavona A (**3**).

Table-2: The ^{1}H NMR and ^{13}C NMR spectrum of compound 1 in acetone-d_6.

NO	13C ppm	1H ppm	NO	13C ppm	1H ppm
1	62.01	4.33(dd, J=5.6,1.4 Hz)	**4'**	138.2	-----
2	131.8	6.2 (dt, J=5.8,15.8 Hz)	**5'**	153.6	-----
3	131.8	6.48 (d, J=15.8 Hz)	**6'**	103.9	6.63 (s)
1'	132.6	-----	**3' OMe**	55.5	3.71(s)
2'	103.9	6.63 (s)	**4' OMe**	61.0	3.58 (s)
3'	153.6	-----	**5' OMe**	55.5	3.71(s)

Table -3:^{1}H NMR and ^{13}C NMR spectrum of compound 2 in CD_3OD

NO	^{13}C NMR	^{1}H NMR	NO	^{13}C NMR	^{1}H NMR
1	41.3	-----	**11**	18.17	1.84 (d,J=1.2 Hz)
2	49.4	2.07(1H, brd, J=16.5 Hz, 2α-H); 2.52 (1H, brd, J=16.8 Hz,2β-H)	**12**	22.8	0.94(s)
3	199.9	-----	**13**	23.3	0.91(s)
4	125.7	5.76 (s)	**1'**	99.9	4.18 (d, J=7.7 Hz)
5	166.0	-----	**2'**	73.6	3.02-3.55 (m)
6	78.6	-----	**3'**	76.8	
7	132.4	5.85(d, J=15.5Hz)	**4'**	70.3	
8	132.3	5.63 (dd, J= 7.5,15.6 Hz)	**5'**	77.0	
9	73.9	4.5	**6'**	62.9	3.53(dd, J=6.0,12.0 Hz) ; 3.75(dd, J=2.2, 12.0 Hz)
10	22.80	1.2 (d, J=6.4 Hz)			

Table-4:^{1}H NMR and ^{13}C NMR spectrum of compound 3 in acetone-d_6.

No	^{13}C NMR	^{1}H NMR	No	^{13}C NMR	^{1}H NMR
2	165.4		**2"**	165.5	7.6 (d, J=8 Hz)
3	101.8	6.54 (s)	**3"**	102.0	6.34 (s)
4	182.1		**4"**	182.2	
5	162.0		**5"**	160.0	
6	98.7	6.04 (d, J=2.0Hz)	**6"**	101.8	6.31 (s)
7	165.02		**7"**	162.1	
8	94.0	6.1 (d, J=2.0Hz)	**8"**	102.2	
9	158.0		**9"**	155.5	
10	102.2		**10"**	108.0	
1'	120.0		**1'"**	123.9	
2'	131.6	8.2 (d, J=2.0 Hz)	**2'"**	127.9	7.67(d, J=8.8Hz)
3'	123.9		**3'"**	114.0	6.44 (d, J=8.8 Hz)
4'	158.0		**4"'**	162.1	
5'	120.0	7.04 (d, J=8.7 Hz)	**5'"**	114.0	6.44 (d, J=8.8 Hz)
6'	130.5	7.80 (dd, J=2, 8.7 Hz)	**6'"**	127.86	7.67 (d, J=8.8 Hz)
			4' OCH3	54.7	3.65(s)

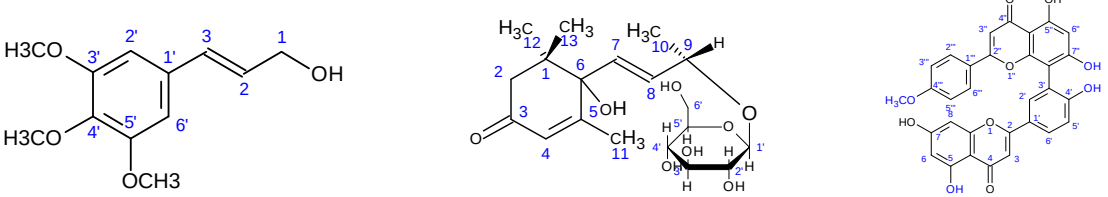
DISCUSSION

Cytotoxic activity: The methanolic extract of *J. horizontalis* aerial parts showed promising activity against human colon cancer cells (HCT 116) and breast cancer cells (MCF7), its LC_{50} in both cases are less than that of Doxorubicin used as positive

control. These results are in conjunction with the previously reported studies on other members belonging to genus Juniperus (El-Sawi et al., 2008; Moujir et al., 2010).

Chemistry:

Compound (1): It was obtained as white amorphous powder (67.0mg) soluble in acetone. ^{1}H NMR spectrum of compound 1 (acetone-d6) showed the characteristic singlet for 2H appearing at δ 6.63 (H- 2'& 6') with two olifenic protons at δ 6.48 and δ 6.2 (H-3 and H-2) and the calculated J value confirmed the trans configuration of this two olifenic protons, in addition to a peak at δ 4.33 (dd, J=5.6, 1.4 Hz) corresponding to 2 H at (H-1). The presence of three methoxy groups were deduced from the appearance of two singlets at δ 3.71 (6H), δ 3.58 (3H) in addition to 3 carbons at δ 55.52 (2C) and δ 61.03 (1C) ppm suggesting a trimethoxy cinnamyl moiety.^{13}C NMR spectrum and 2D HMQC correlation (**Table 2**) in conjunction with the UV absorption band appearing at 268 nm and comparing data to published literature (Gilles et al., 1996) lead to the identification of compound **1** to be : 3(3',4',5' trimethoxy –phenyl) prop-2-ene-1-ol also known as 3', 4', 5' trimethoxy cinnamyl alcohol (Figure 1a).



(**1a**) 3', 4', 5' trimethoxy cinnamyl alcohol (**1b**) corchoionoside C (**1c**) podocarpusflavona A

Figure-1: compounds 1, 2 & 3

Compound (2): It was obtained as white amorphous powder (65.0mg) soluble in methanol. ^{1}H NMR spectrum of compound **2** showed two singlets and two doublets appearing at δ 0.91 (s), 0.94 (s), 1.2 (d, J=6.4 Hz) and 1.84 (d, J=1.2 Hz) corresponding to four methyl groups, two tertiary methyls at δ 23.3(C-13), 22.8 (C-12) and two secondary methyls at δ 22.8 (C-10) and 18.17 (C-11) in ^{13}C NMR spectrum respectively , their attachments were confirmed by direct HMQC correlation. Also the appearance of a sharp singlet at δ 5.76 ppm corresponding to the enone proton correlated to (C-4) at δ 125.7 ppm and the two olifenic protons at δ 5.63 (dd, J=7.2, 15.6 Hz, H-8) and δ 5.85 (d, J=15.5 Hz, H-7) also confirmed by direct correlation to two sp2 carbons C-8 at δ 132.3 and C-7 at δ 132,4 respectively .In ^{13}C NMR the carbonyl carbon appeared at δ 199.8 in conformation with published data (Çalış et al., 2002). An anomeric proton at δ 4.18 ppm correlated to an anomeric carbon at δ 99.9 ppm in addition to a typical spin system for glucose moiety (**Table 3**) confirmed the glycosidic nature of **2**. Considering all the above data using both 1 & 2D spectral analysis in addition to acid hydrolysis and comparing our data to published one (Özgen et al., 2010) lead to the suggestion of compound **2** to be corchoionoside C (**Figure 1b**).

Compound (3): was obtained as amorphous yellow powder (80mg) (**Figure 1c**). It was the major component of the methanol extract of *J. horizontalis* and it was expected to be a phenolic compound as it showed a strong $FeCl_3$ reaction. Compound **3** was identified mainly by analysis of ^{1}H and ^{13}C NMR spectrum and HMQC experiment. ^{13}C NMR spectrum showed 31 signals with two carbonyl groups at δ 182.1 and 182.2 ppm. Among the aromatic carbons, one methoxy group was also identified at δ 54.7ppm. The presence of two carbonyls in addition to the interpretation of characteristic data in the ^{1}H NMR spectrum suggested a flavonoids dimer, which contain two flavones units in conjunction with reported typical peaks for

a biflavon nucleus (Suárez et al., 2003) previously isolated from *Podocalyx loranthoides* and *Caesalpinia pyramidalis* respectively. Thus **3** was characterized as 3', 8" biflavonoid and its structure was fully confirmed by comparing it to published data (Marcus et al., 2005) to be 4"'- methoxy amentoflavone known as podocarpusflavona A.

CONCLUSION

This study revealed for the first time marked cytotoxic activity for the methanol extract of the aerial parts of *J. horizontalis* especially against both breast cancer cells (MCF7) and human colon cancer cells (HCT 116) compared to the positive control Doxorubicin. It is worth mentioning that it is the first record for the isolation of the three separated phenolic compounds from *J. horizontalis*.

Conflict of interest: The author(s) confirm that this article content has no conflict of interest.

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