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RESEARCH ARTICLE

ANTIPROLIFERATIVE EFFECT OF EXTRACTS AND FLAVONOIDS OF *Juniperus Phoenicea* L. GROWING IN EGYPT

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ABSTRACT

Objective: To Investigate the Cytotoxic effect of the plant *Juniperus phoenicea* L. growing in Egypt. **Main outcome measures**: SRB and MTT assays are used to measure the antiproliferative activity of crude and successive extracts as well as the isolated flavonoids. Also, *in vivo* cytogenetic assays were carried out. **Results**: The antiproliferative effect of leaves crude extract of *Juniperus phoenicea* L. done by SRB assay showed high activity against lung carcinoma (H460), liver tumor (HEPG2) and breast carcinoma (MCF7) cell lines (IC50= 0.6, 0.9 and 4.9 µg/mL, respectively). Consecutively, among the successive extracts of leaves, the ethyl acetate fraction was the most active against (H460) (IC50=7.29 µg/mL). On the other hand, the antiproliferative effect of berries crude extract of *J. phoenicea* done by MTT assay showed 21% lethality at conc. 100 ppm against HEPG2. Phytochemical investigation of *J. phoenicea* resulted in the isolation and identification of 4 flavonoids; isoetin-7-O-β-glucoside which was isolated for the first time from berries extract, isoscutellarein, amentoflavone and agathisflavone which was isolated for the first time from leaves and berries extracts. Investigating the antiproliferative activity of the isolated flavonoids by MTT assay revealed that agathisflavone recorded high activity against HEPG2, colon carcinoma (HC112) and (H460) (IC50 =3.5, 53.1 and 59.9 µg/mL, respectively). *In vivo* cytogenetic assays (Micronucleus and Chromosome aberrations tests) were carried out on agathisflavone showed that no significant differences between treated and negative control groups were observed in the Micronucleus level in polychromatic erythrocytes and in chromosome aberrations in both tests, respectively, which indicates the safety of agathisflavone on normal cells. **Conclusion**: *Juniperus phoenicea* leaves exhibited more efficient antiproliferative activity than berries. Also, the biflavone; agathisflavone, isolated for the first time from leaves and berries showed the potent antiproliferative effect on cancer cell lines and is safe for normal cells of mice under the experimental conditions.

Keywords: *Juniperus phoenicea*, flavonoids, antiproliferative activity, Micronucleus, Chromosome aberrations.

INTRODUCTION

Cancer is the second leading cause of death, in developing countries, after heart diseases [1]. As numbers of undesired side effects sometimes occur during chemotherapy, natural therapies, such as the use of plant-derived products in cancer treatment, may reduce such adverse effects. Currently, a few plant products are being used to treat cancer [2].

Juniperus phoenicea L. is a plant belonging to family Cupressaceae, the Phoenicean Juniper or Arâr [3] is the juniper growing in Egypt in Sinai near the Red Sea (Yelleg, Halal and Maghara mountains) in rocky ridges, Mediterranean region, extending to Central Arabia [4]. In our previous studies, phytochemical screening of leaves and berries of the plant revealed that they are rich in essential oil, carbohydrates, glycosides, sterols, triterpenes, and flavonoids [5]. Biological investigations of crude and successive extracts of leaves and berries demonstrated that the plant possesses significant diuretic [6], hepatoprotective, anti-inflammatory and antioxidant activities [7]. Also, the antiproliferative effect of the essential oil of leaves and berries was determined where they showed high cytotoxicity against all tested cell lines [8].

The current research work comply the study of the *in vitro* cytotoxicity or the antiproliferative effect of the crude and successive extracts of *Juniperus phoenicea* L. growing in Egypt. The research also was continued to investigate the flavonoid contents present in leaves and berries and test the antiproliferative effect and mutagenicity of the isolated compounds.

MATERIAL AND METHODS

Plant material

Fresh aerial parts of *J. phoenicea* L. family Cupressaceae were collected from north Sinai, Maghara Mountain, Egypt and were kindly identified by Prof. Dr. Nabeel El- Hadidi, Botany department, faculty of Science, Cairo University. The plant was dried, ground and a voucher specimen (no. 21882) was kept at Pharmacognosy department, National Research Centre, Cairo, Egypt.

Preparation of Plant extracts for biological activity

100 g. of air dried powdered leaves and berries of *J. phoenicea* were exhaustively extracted by reflux with 80% ethanol to form the crude extracts. Another 100 g. of leaves were exhaustively and successively extracted in Soxhlet apparatus using petroleum ether, chloroform, ethyl acetate and methanol to form the successive extracts. All extracts were evaporated to dryness under vacuum at 40°C using rotatory evaporator (Heidolph, Germany).

The cancerous cell lines; lung carcinoma (H460, A549), liver carcinoma (HEPG2), breast carcinoma (MCF7), colon carcinoma (HCT116), brain tumor (U25l) and Cervix carcinoma (HELA) obtained from National Cancer Institute, Cairo, Egypt and Bioassay Cell Culture Laboratory, National Research Centre.

Method for *in vitro* antiproliferative effect

In vitro antiproliferative effect of the crude and successive extracts of leaves was done by SRB assay [9]. Data were statistically analyzed using the Student's t test [10]. The potency was compared with reference drug Cisplatin (Glaxo-Wellcome, Egypt). *In vitro* antiproliferative effect of the crude berries extract and the isolated compounds was done by MTT assay [11]; data were statistically analyzed using the independent t-test by SPSS 11 program. The potency was compared with reference drug Doxorubicin (DOX) (Pharmacia, Belgium).

Method for cytogenetic study

Male white Swiss mice, of similar age (aged 9–12 weeks ± 1 week) and weight (± 2 g) were used in the experiments. The animals were obtained from a closed random-bred colony at National Research Center and were housed in polycarbonate boxes with steel-wire tops (not more than five animals per cage)

and bedded with wood shavings. Ambient temperature was controlled at 22 ± 3 ºC with a relative humidity of 50 ± 15% and a 12 h light/ dark photoperiod. Food and water were provided *ad libitum*. Animals were sacrificed after treatment by cervical dislocation. Mice were i.p. treated with the sample at dose 100 mg / kg b.wt for 24 h. A negative, non-treated mice group was also examined (negative control). All experiments had followed the ethical animal care and approved by "The Medical Research Ethics Committee, National Research Centre".

I. Micronucleus test

The epiphyses of the treated and control group mice were cut and the bone marrow was flushed out by gentle flushing and aspiration with fetal calf serum [12]. The cell suspension was centrifuged at 1000 rpm for 10 min and the supernatant was discarded. A small drop of the resuspended cell pellet was spread onto clean glass slides and air-dried. The bone marrow smears were made in five replicates and fixed in absolute methanol for 10 min. and stained with May-Grünwald/ Giemsa at pH 6.8 [13]. Scoring the polychromatic erythrocytes and the percentage of micronucleated polychromatic erythrocytes (MNPCEs) was determined by analyzing the number of MN cells from 1000 PCEs per animal.

II. Chromosome aberrations

Mice were injected i.p. with colchicine 2–3 h before sacrifice (colchicine reduces the frequency of aberrations induced by centrifugation). Bone marrow preparations were made according to the technique described by [14]. A group of five mice was used for each treatment and 100 well-spread metaphases were analyzed per animal, scoring of different kinds of abnormalities. Gaps, breaks, fragments and deletions metaphases were recorded in bone marrow cells. Statistical analysis: the difference between treated and negative control groups was calculated using the t-test.

Method of Flavonoids isolation

Flavonoids isolation from berries extract

350 g. of the crude defatted 80% EtOH extract of *J. phoenicea* berries were subjected to column chromatography using polyamide 6 for column chromatography (CC) (Sigma-Aldrich Chemie GmbH, Germany) and eluted by stepwise gradient elution of water- methanol solvent system to obtain 2 main fractions;

Fraction 1: eluted from the polyamide column by 40% MeOH and was further purified by being applied on preparative paper chromatography (PPC) and eluted by butanol: acetic acid: water (BAW), 3:1:1. The band under investigation was cut into small pieces and macerated in 70% MeOH [15], the solution was filtered, concentrated under vacuum and was further purified by Sephadex LH-20 CC (Fluka Chemie AG, Switzerland) using 50% MeOH to yield compound F1.

Fraction 2: eluted from the polyamide column by 80-100% methanol which was further purified by Silica gel 60 for CC (Merck, Darmstadt, Germany) using CHCl3\ EtOAc\ MeOH as eluent, 2 main subfractions were obtained; subfr. 2/A eluted by 10% $CHCl_3$\ EtOAc was further purified by Sephadex CC. using MeOH as eluent to yield compound F2 and compound F3, and subfr. 2/B eluted by 40% CHCl3\ EtOAc which was further purified by Sephadex CC. using MeOH as eluent to yield compound F4.

Flavonoids isolation from the most biologically active extract of leaves:

150 g. of the ethyl acetate successive extract were applied on the top of a silica gel CC and eluted by stepwise gradient elution $CHCl_3$/ EtOAc to obtain one main fraction which was further purified by Sephadex LH-20 CC. using MeOH to yield 2 major compounds; F3 and F4.

All the purified compounds (F1, F2, F3 and F4) were subjected to UV spectral analysis, ^{1}H- and\or ^{13}C-NMR as well as ESI\MS determinations.

RESULTS

The structures of the isolated flavonoids were determined by conventional analytical methods and confirmed by MS and NMR spectral analysis. The spectroscopic UV data of these compounds were compared with the published data [15]. ^{1}H- and ^{13}C-NMR data of the isolated flavonoids are listed in the table (1).

Table (1): ^{1}H- NMR and ^{13}C-NMR data of the isolated flavonoids from leaves and berries of *J. phoenicea*.

Atom No.	δ ^{1}H (ppm) (*J* in Hz)						δ ^{13}C (ppm)		
	F1	F2		F3		F4	F3		F4
		Moiety I	Moiety II	Moiety I	Moiety II		Moiety I	Moiety II	
2	---	---	---	---	---	---	164	164	163.6
3	6.59 (s)	6.73 (br. s)	6.73 (br. s)	6.65 (s)	6.68 (s)	6.74 (s)	102.5	102.5	102.8
4	---	---	---	---	---	---	182	182	182.4
5	---	---	---	---	---	---	157	157	161.3
6	6.17 (d, *J*= 2.5)	5.9 (s)	6.1 (d, *J*= 2.5)	Linkage to moiety II	6.14 (s)	6.39 (s)	103.7	99.6	99.7
7	---	---	---	---	---	---	163	163	155
8	6.54 (d, *J*= 2.5)	Linkage to moiety II	6.6 (d, *J*= 2.5)	6.42 (s, 1H)	Linkage to moiety I	---	94.6	99.6	128.2
9	---	---	---	---	---	---	157	157	155
10	---	---	---	---	---	---	103.7	103.7	103.5
1`	---	---	---	---	---	---	122.5	122.5	121.7
2`	---	7.77 (d, *J*= 8.6)	7.83 (dd, *J*= 8.6, 2.5)	7.84 (d, *J*= 8)	7.9 (d, *J*= 8, 2H)	7.44 (d, *J*= 8.6	128.6	128.4	128.2
3`	7.2 (s)	6.66 (d, *J*= 8.6)	Linkage to moiety I	6.89 (d, *J*= 8)	6.98 (d, *J*= 8)	6.68 (d, *J*= 8.6)	116.2	115.8	116.2
4`	---	---	---	---	---	---	161.3	161.3	161.2
5`	---	6.66 (d, *J*= 8.6)	6.8 (d, *J*= 8.6)	6.89 (d, *J*= 8, 2H)	6.98 (d, *J*= 8, 2H)	6.68 (d, *J*= 8.6)	116.2	115.8	116.2
6`	7.44 (s)	7.77 (d, *J*= 8.6)	7.83 (dd, *J*= 8.6, 2.5)	7.84 (d, *J*= 8)	7.9 (d, *J*= 8)	7.44 (d, *J*= 8.6)	128.6	128.4	128.2
(Sugar proton1``)	4.9 (d, *J*=6.6)	---	---	---	---	---	---	---	
(Sugar protons 2``-6``)	3.1– 4.0	---	---	---	---	---	---	---	

Compound F1

Yellow powder (10 mg), Rf= 0.21 and 0.6 on PC. in solvent systems BAW 3:1:1, 15% acetic acid, respectively, dark purple spot on PC under UV light and changed to yellow upon exposure to ammonia vapor. UV: 342, 270 (MeOH), 408,276 (NaOMe), 420, 362, 270 (AlCl3), 357, 277 (AlCl3 / HCl), 398, 263 (NaOAc), 387, 270 (NaOAc/ H3BO3). (ESI-MS): m/z 465 [M+-H] (100%), m/z 301 (30%), [M+]. Complete acid hydrolysis yielded glucose in the aqueous phase, identified by spotting against authentic sugars on PC. Whatmann 1 MM. using aniline phthalate reagent. Compound F1 was expected to be flavone glycoside on the basis of its chromatographic and spectroscopic properties. The ^{1}H-NMR data in $(CD3)_2O$\ D2O showed 2 doublet signals at δ= 6.17 and 6.54 ppm for H-6 and H-8 (*J*= 2.5 Hz), respectively, 1 singlet signal at δ= 6.59 ppm for H-3, and 2 singlet signals at δ= 7.2 and 7.44 ppm for H-3` and H-6`, respectively, indicating a substituted H-2`. These data were compared with those available in the literature [16]. A glucose moiety at C-7 has deduced from the anomeric proton signal at δ = 4.9 ppm (d, *J*= 6.6 Hz). The structure was confirmed by determination of negative electrospray ionization mass spectrometry (ESI-MS) m/z 465 [M+H] (100%), Which is the M.wt. of 5,2',4',5'-tetrahydroxy-7-glucosyl flavone ($C_{21}H_{20}O_{12}$), and m/z 301 (30%), [M+] of the aglycone. Therefore, compound F1 was identified as isoetin-7-*O*-*β*-glucoside (5,2',4',5'-tetrahydroxy-7-glucosyl flavone).

It was reported that isoetin is a rare flavone, has been identified as a yellow pigment and is frequently accompanied by apigenin, luteolin and scutellarein derivatives in the plant [17]. This is the first report for isoetin-7-*O*-*β*-glucoside isolation from the berries of *J. phoenicea*.

Compound F2

Yellow powder (15 mg), R_f= 0.91 and 0.16 on PC. in solvent systems BAW 3:1:1, 15% acetic acid, respectively, dark purple spot UV light, changed to yellow upon exposure to ammonia vapor; UV: 337, 270 (MeOH), 386, 277 (NaOMe), 384, 353, 278 (AlCl3), 384, 353, 278 (AlCl3 / HCl), 368, 276 (NaOAc), 354, 275 (NaOAc/ H3BO3). The ^{1}H-NMR data in DMSO-d_6 of compound F2 revealed the presence of two apigenin moieties; the spectrum showed 1 singlet signal at δ= 5.9 for H-6, 2 doublet signals at δ= 6.1 and 6.63 ppm for H-6``` and H-8```, respectively, and absence of 1 signals H-8. The presence of one AA` BB` system of H-3`, H-5` and H-2`, H-6` appeared at δ= 6.66 and 7.77 ppm (each 2H, d, *J*= 8.6), respectively, and one ABC system of H-5`` appeared at δ= 6.8 ppm (1H, d, *J*= 8.6 due to ortho coupling to H-6``), H-2``at δ= 8.3 ppm (1H, d, *J*= 2.5 due to meta coupling to H-6``), H-6`` at δ= 7.8 ppm (1H, dd, *J*= 8.6, 2.5 due to ortho and meta coupling to H-5``, 2``, respectively), indicating the absence of H-3``. So, it could be concluded that the two moieties are linked by C-C interflavonoid linkage at C-3`` and C-8, which suggests amentoflavone structure. (ESI-MS): m/z 538 (which is the M.wt. of 2 apigenin moieties; $C_{30}H_{18}O_{10}$). Therefore, compound F2 was identified as amentoflavone. To our knowledge, this is the first report for amentoflavone isolation from the berries of *J. phoenicea* growing in Egypt. It was previously isolated from the leaves of the same plant [18, 19].

Compound F3

Yellow powder (86 mg) R_f= 0.89 and 0.13 on PC. In BAW 3:1:1, 15% acetic acid, respectively, a dark purple spot on TLC silica gel under UV light and changed to yellow upon exposure to ammonia vapor indicating a flavonoid with free 5-OH and 4'-OH groups. UV; 335, 270 (MeOH), 396,306 sh, 276 (NaOMe), 387, 353, 301sh,280 ($AlCl_3$), 386, 353 , 300 sh, 280 ($AlCl_3$ / HCl), 360, 303, 275 (NaOAc), 346, 303, 272 (NaOAc\ H_3BO_3), The ^{1}H-NMR data of compound F3 revealed the presence of two apigenin moieties, the spectrum showed 2 doublet signals at δ = 7.9 and 7.84 for H-2`, 6`and H-2``, 6`` and 2 doublet signals at δ= 6.89 and 6.98 for H-3`, 5`and H- 3``,5``(i.e. 2 AA`BB` systems), 2 singlet signals at δ= 6.65 and 6.68 for 3 and 3```, two singlet signals at δ= 6.14 and 6.42 for H-6``` and H-8, respectively, and absence of 2 signals of H-6 and H-8``` indicating a C-C interflavonoid linkage at C-6-C-8```. In addition to a single signal at 6.5 which disappeared on the addition of trifluoroacetic acid (TFA) to the sample, and re-measure the ^{1}H-NMR data, which indicated an OH- group signal. ^{13}C-NMR data of compound F3 showed that the signals for the unsubstituted carbon atoms I-8 and II-6 appeared at the expected values 94.6 and 99.6 ppm, respectively, while I-6 and II-8 had resonances at 103.6 and 99.6 ppm, respectively. The downfield shifts experienced by the 2 latter carbon atoms were due to the substitution effect of C-C interflavonoid linkage. HMBC correlations of the compound F3 were also elaborated (Fig. 1.3). The structure was confirmed by determination of negative electrospray ionization mass spectrometry (ESI-MS) (Fig. 35), *m/z* 538 [M^+], the M.wt. of bi-apigenin. Therefore, compound F3 was identified as agathisflavone; (6, 8```-Biapigenin; 8-[5, 7-dihydroxy-2-(4-hydroxyphenyl)-4-oxo-chromen-6-yl]-5,7-dihydroxy-2-(4-hydroxyphenyl) chromen-4-one. All data were compared with those available in the literature [20, 21].

To our knowledge, this is the first report for agathisflavone isolation from leaves and berries of the species *phoenicea.*

Compound F4

Yellow powder (6 g.). R_f were 0.87 and 0 on PC. in solvent systems BAW 3:1:1, 15% acetic acid, respectively, dark purple spot on PC and TLC under UV light and changed to yellow upon exposure to ammonia vapor and on spraying with AlCl3. UV; 328, 276 (MeOH), 392, 283 (NaOMe), 396, 353, 306sh, 281 (AlCl3), 396, 348, 306sh, 281 (AlCl3 / HCl), 380, 284 (NaOAc), 333, 280 (NaOAc / H3BO3), ^{1}H-NMR data of compound F4 showed four aromatic protons appearing as two doublets at δ= 7.44 ppm with (*J*= 8.6 Hz) and δ= 6.68 ppm with (*J*= 8.6 Hz) due to *ortho*-coupled protons, were assigned to H-2`, 6` and H-3`, 5`, respectively, a singlet signal at δ= 6.74 corresponding to H-3 characteristic to flavone structure and one proton singlet at δ= 6.28 assigned to H-6, which indicates a blocked C-8. These data were compared with those available in the literature [22] ^{13}C-NMR data of compound F4 revealed the presence of downfield C-8 and C-6 and upfield C-7 and C-9 relative to apigenin data which suggests the presence of electronegative element attached to C-8; a hydroxyl group [23]. The structure was confirmed by determination of negative electrospray ionization mass spectrometry (ESI-MS) *m/z* 286 [M^+] (which is the M.wt. of 8-hydroxy apigenin; $C_{15}H_{10}O_6$) Therefore, compound F4 was identified as isoscutellarien.

To our knowledge, this is the first report for isoscutellarien isolation from leaves and berries of the species *phoenicea* growing in Egypt. It was previously isolated from the berries of the same plant growing in Libya (24).

The structures of the isolated compounds are shown in figure (1)

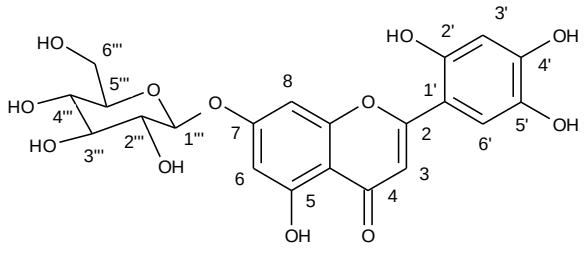

Fig (1.1) Isoetin-7-*O*-*β*-glucoside

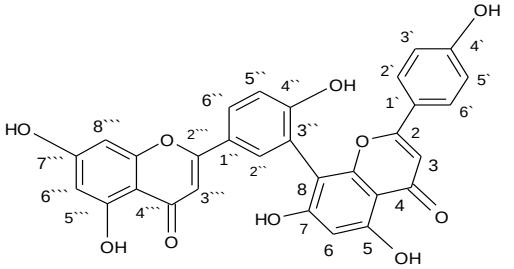

Fig (1.2) Amentoflavone

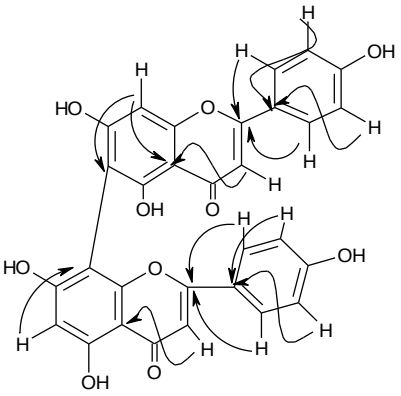

Fig (1.3) HMBC correlation of Agathisflavone

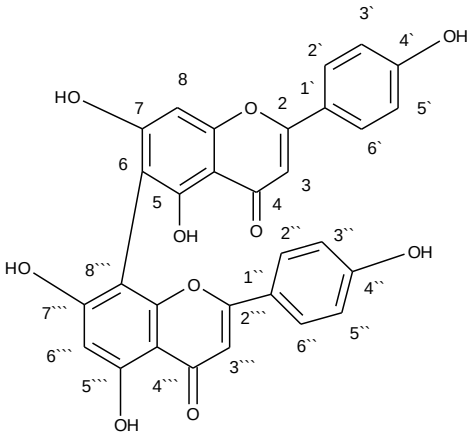

Fig (1.4) Agathisflavone

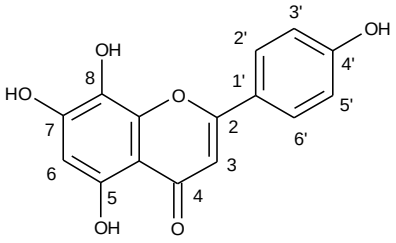

Fig (1.5) Isoscutellarien

Fig. 1: Chemical structures of the isolated flavonoids.

Antiproliferative effect of *J. phoenicea* leaves

Antiproliferative effect and IC_{50} of specific concentrations of the crude 80% ethanol extract of leaves tested by SRB assay showed that it was active against three out of the five tested cell lines; lung carcinoma (H460), liver carcinoma (HEPG2), breast carcinoma (MCF7), brain tumor (U25l) and Cervix carcinoma (HELA). The highest activity was recorded against lung carcinoma (IC_{50}= 0.6 µg/mL) as compared with cisplatin (IC_{50}= 4.77 µg/mL), followed by liver tumor cells (IC_{50} =0.9µg/mL), cisplatin (IC_{50}= 9.83 µg/mL), then breast carcinoma (4.9 µg/mL); cisplatin (IC_{50}= 0.6). It showed moderate activities against cervix carcinoma and brain tumor cells at the used concentrations where the IC_{50} was higher than10 µg/mL Results are shown in fig (2).

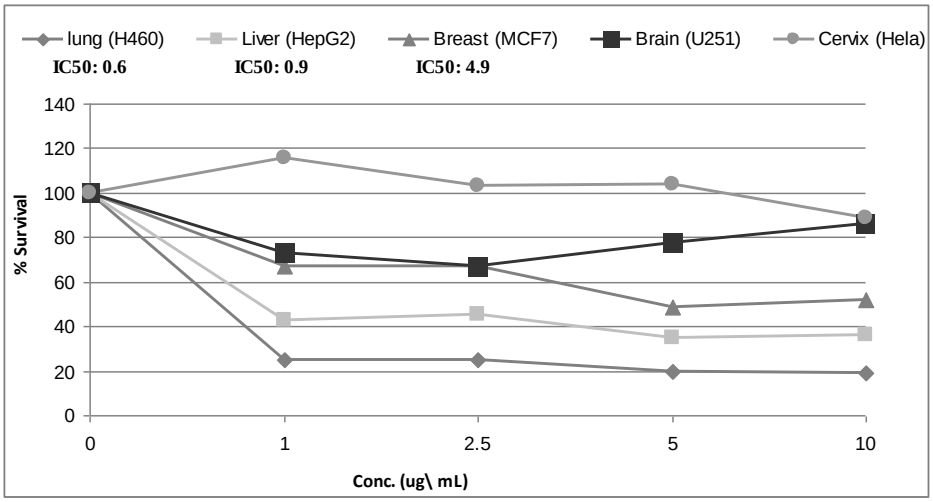

Fig. 2: In vitro antiproliferative effect of the crude leaf extract against different cell lines.

Antiproliferative effect of the successive extracts of *J. phoenicea* leaves:

As the crude alcoholic extract showed a significant antiproliferative effect against the liver tumor, lung carcinoma, and breast carcinoma cell lines; potential antiproliferative effect of the successive extracts of *J. phoenicea* leaves was carried out against these cell lines. Ethyl acetate extract was the most active one against lung carcinoma cell line where IC_{50} was 7.29 µg/mL and had weak antiproliferative effect against the other tumor cell lines tested at the chosen concentrations. Also, the other extracts had no antiproliferative effect against the human tumor cell lines tested at the chosen concentrations. Results are shown in figures (3, 4, 5).

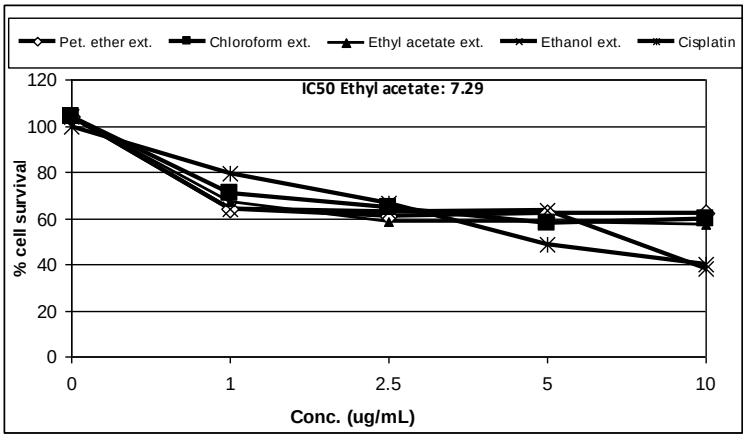

Fig. 3: Antiproliferative effect of successive leaf extracts against lung carcinoma cell line compared to cisplatin.

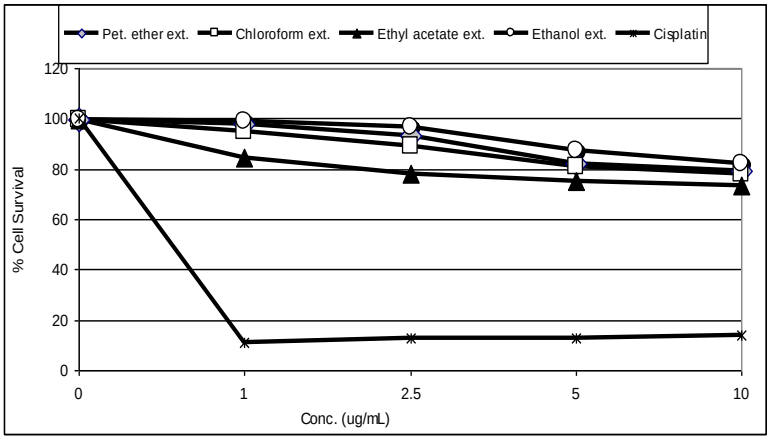

Fig. 4: Antiproliferative effect of successive leaf extracts against breast carcinoma cell line compared to cisplatin.

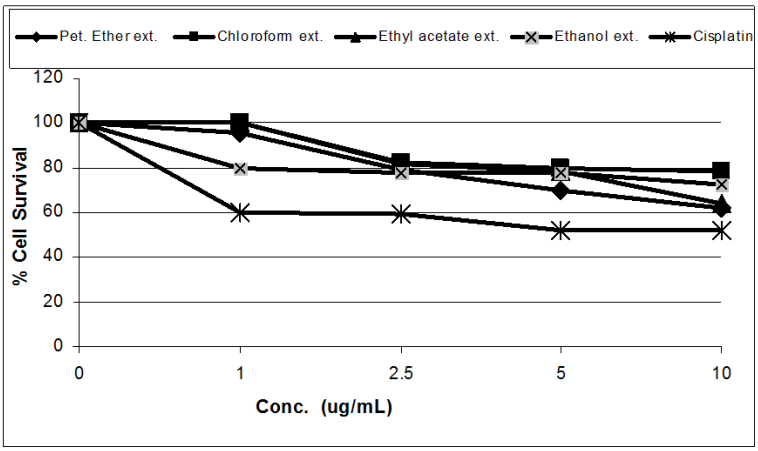

Fig. 5: Antiproliferative effect of successive leaf extracts against liver tumor cell line compared to cisplatin.

Antiproliferative effect of crude extract of *J. phoenicea* berries

Antiproliferative effect and IC_{50} of specific concentrations of the crude 80% ethanol extract of berries were tested against lung carcinoma A549, HEPG2, colon carcinoma cell line HCT116 and MCF7 using MTT assay. It showed 21% lethality at cons. 100 ppm against HEPG2 and didn't show any antiproliferative effect against the other tested cell lines at the chosen concentrations.

Antiproliferative effect of Some Isolated Flavonoids:

Potential antiproliferative effects of agathisflavone, isoscutellarien and amentoflavone were tested against A549, HEPG2, MCF7 and HCT116 by MTT assay. The highest activity of agathisflavone was recorded against liver tumor cells (IC_{50} =3.5µg/mL) as compared with standard DOX. (IC_{50}= 1.5 µg/mL) Followed by colon carcinoma (IC_{50}= 53.1 µg/mL), DOX. (IC_{50}= 2.3 µg/mL) Then lung carcinoma (IC_{50}= 59.9 µg/mL), DOX. (IC_{50}= 1.14 µg/mL), while it gave moderate activity against MCF7 (breast carcinoma cell line) where it showed only 62% lethality at conc. 100 ppm. Results are shown in figure (6).

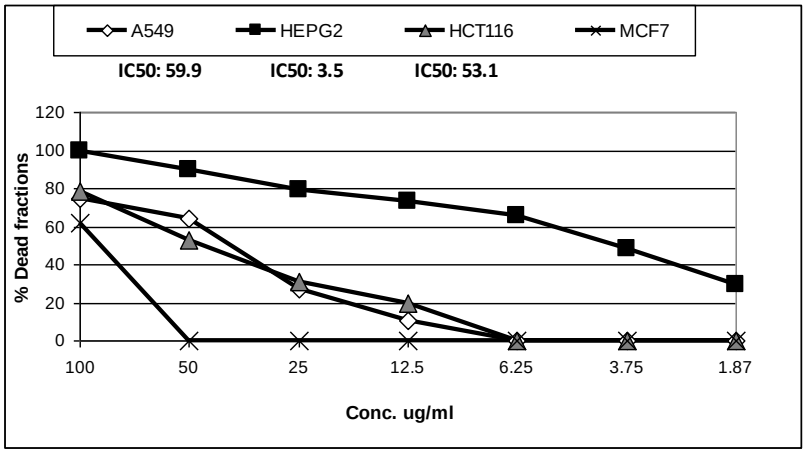

Fig. 6: Antiproliferative effect of agathisflavone against the tested cell lines.

On the other hand, isoscutellarien and amentoflavone didn't show any antiproliferative effect against the tested cell lines (A549, HEPG2, HCT116, and MCF7) at concentration 100µg/mL

***In vivo* genotoxicity for the biflavone agathisflavone (cytogenetic assay)**

As the biflavone compound, agathisflavone isolated from leaves and berries of *J. phoenicea* showed a potent antiproliferative effect against cancer cell lines, *in vivo* genotoxicity tests were done for that compound to show its effect on normal cells.

Micronucleus test

No elevation of MN level in PCE was observed after i.p. injection of mice with agathisflavone in comparison to the control group, results are shown in Table (2).

Table 2: Micronucleus (MN) level in polychromatic erythrocyte (PCE) in mice treated with agathisflavone for 24 hrs.

Group	Number of animals	No. of PCE cells	No of MN in PCE cells	% of MN in PCE cells ± S.E
Control	5	5000	37	0.74 ± 0.41
Agathisflavone treated	5	5000	48	0.96 ± 0.48

Chromosome aberrations

No significant difference between treated and negative control group in chromosome aberrations induced in bone marrow cells after i.p. treatment with agathisflavone. Results are shown in Table (3)

Table 3: Mean percentage of chromosome aberrations in mice treated with agathisflavone for 24 hrs.

Group	Number of metaphases	Number of abnormal metaphases	Chromosome aberrations (Mean % ± S.E) without gaps	Types of abnormal metaphases			
				Gaps	Fragment	break	Deletion
Control (5 animals)	500	8	1.60 ± 0.35	2	4	2	2
Agathisflavone treated (5 animals)	500	12	2.40 ± 0.41	3	5	5	2

These results showed that the compound examined is safe to genetic material as the percentage of chromosomal aberrations induced in cells was statistically not significant in comparing to the negative control group. It is worth mentioning that there is no available data about the mutagenic activity of this compound and this study represents the first data about the mutagenic activity of the biflavones agathisflavone.

DISCUSSION

The pharmacological evaluation of extracts and substances from plants is an established method for the identification of lead compounds which can lead to the development of novel and safe medicinal agents. Isolation and identification of some potent anti-tumor compounds from medicinal plants have motivated researchers to screen different parts of plant species for the determination of anti-tumor effects. From these viewpoints, biological and phytochemical investigation of leaves and berries extracts of *Juniperus phoenicea* L. growing in Egypt was carried out in an attempt to discover potent compounds from the plant.

The biological investigation, the antiproliferative effect and IC_{50} of crude and successive extracts of leaves and berries of *Juniperus phoenicea* as well as the isolated flavonoids were accomplished. Results revealed that the crude 80% alcoholic extract of leaves was more potent than the successive extracts on liver and breast cell lines. This may be due to synergistic effects of compounds present in the crude extract. The significant antiproliferative effect of the ethyl acetate fraction could possibly be attributed to its flavonoid content. While the potency of berries crude extract as a cytotoxic agent was weak.

The phytochemical investigation led to the isolation and identification of 4 flavonoids from berries; isoetin-7-*O*-*β*-glucoside which was isolated for the first time from berries extract, the flavone isoscutellarein, and 2 biflavones; amentoflavone and agathisflavone which was isolated for the first time berries extracts. Isoscutellarein and agathisflavone were also isolated from the most potent cytotoxic fraction; ethyl acetate successive extract from leaves.

Consecutively; potential cytotoxicity was measured to the isolated flavonoids and revealed that the biflavone agathisflavone was the most active one. Cytogenetic assays were done *in vivo* to this compound to ensure its safety on normal cells. Results showed that no significant difference between treated and negative control group was observed in the MN level in PCE and in chromosome aberrations in both tests, respectively. These results showed that the compound examined is safe to genetic material as the percentage of chromosomal aberrations induced in cells was statistically not significant in comparing to the negative control group.

The genetic material of most living organisms is DNA. Human beings, like all living organisms, are constantly subjected to

agents in the world's environment which can break the DNA (UV light for instance). Fortunately, we all possess mechanisms in our cells which repair DNA that breaks. If this did not happen, we would quickly become ill. A breakdown in one's DNA repair system is known to be linked to a number of cancers and perhaps a range of illnesses.

Micronuclei are cytoplasmic chromatin-containing bodies formed when chromosomes lag during anaphase and fail to become incorporated into daughter cell nuclei during cell division. Because genetic damage results in chromosomal abnormalities lead to micronucleus formation, the micronucleus assay has been widely used to screen for chemicals that cause these types of damage. The assay entails scoring the number of micronuclei; the higher the number, the more incidence of the chemical to cause cytogenetic damage. The polychromatic erythrocyte is an immature erythrocyte, in an intermediate stage of development that still contains ribosomes and therefore can be distinguished from the mature, normochromatic erythrocytes, that lacks ribosomes, by stains selective for ribosomes [25, 26, 27].

The micronucleus test (m.t.) *in vivo* is a method devised primarily for screening chemicals for chromosome-breaking effects [28].

Chromosomal aberration or chromosomal mutation is a number of structural changes in chromosomes due to cell exposure to a genotoxic material. These changes result from abnormal divisions within the chromosomes, generally accompanied by the reunification of the resulting segments in combinations other than those existing in the original chromosomes. Such changes include the loss (deletion) or gain (duplication) of a segment of the chromosome, or the exchange (translocation) of a segment of the chromosome with another segment, or a chromosome segment is deleted, turned through 180 °C, and reinserted at the same position on the chromosome (inversion) [29].

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