Journal of Medicinal Plants Research Vol. 5(20), pp. 5099-5103, 30 September, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Cytotoxic flavonoids from *Diplotaxis harra* (Forssk.) Boiss. growing in Sinai

Magdy M. D. Mohammed[1,2]*, Eman R. El-Sharkawy[3] and Azza A. Matloub[2]

[1]Institut für Umweltforschung (INFU), Technische Universität Dortmund, Otto-Hahn-Strasse 6, D-44221 Dortmund, Germany.
[2]Department of Pharmacognosy, National Research Center, Dokki-12311, Cairo, Egypt.
[3]Department of Ecology, Desert Research Center, Mathef El-Mataria, 15753, Egypt.

Accepted 22 June, 2011

As part of our ongoing collaborative effort to discover the anticancer activity of the phenolics isolated from terrestrial plant sources, the EtOH extract of the aerial parts of the Egyptian medicinal plant *Diplotaxis harra* (Forssk.) Boiss. was *in vitro* investigated for cytotoxicity against HCT116, HepG2 and MCF-7 cell lines, and resulted with IC_{50} = 4.65, 12.60 and 17.90 µg/ml, respectively. Doxorubicin (+ve control) showed *in vitro* cytotoxic activity with IC_{50} = 3.64, 4.57 and 2.97 µg/ml, respectively. The phenolic-rich fraction of the EtOH extract was subjected to further fractionation, which led to the isolation of five flavonoids; identified as quercetin, quercetin 3-*O*-*β*-glucoside, isorhamnetin 7-*O*-*β*-glucoside, apigenin 3-*O*-*β*-rhamnoside and kaempferol 3-*O*-*β*-glucoside, according to its' spectral data and comparison with the literature. Furthermore, the isolated flavonoids showed *in vitro* cytotoxicity against HCT116 cell line with IC_{50} = 20.1, 24.3, 22.8, 23.4 and 41.9 µg/ml as determined by NADH DIAPHORASE (MTT method).

Keywords: *Diplotaxis harra* (Forssk.) Boiss., cruciferae, flavonoid glycosides, cytotoxicity.

INTRODUCTION

It is well known that natural products have played an important role in the discovery of useful antitumor agents. Especially clinically relevant anticancer drugs; such as taxol, camptothecin, vinblastine and vincristine, which were discovered from higher plants. Nonetheless, as exemplified by the frequent morbidity and mortality associated with metastatic conditions, there is still clearly a need for the discovery of new agents with higher clinical efficacy (Sang-Kook et al., 2003). Investigations into the cytotoxic activity of some medicinal plants have been carried out (Kudi et al., 1999; Ogundipe et al., 2000; Isaac and Chinwe, 2001; Geidam et al., 2007; Sanni, 2007) with a view to authenticate their folkloric use. Family Cruciferae (Brassicaceae) are called Cruciferous vegetables, considered as one of the largest families rich with valuable medicinal plants, it includes 338 genera and 3350 species that are distributed worldwide from which 53 genera and 107 species distributed in Egypt (Tackholm, 1974; Boulos, 1999). Plants of this family were used traditionally as antidiabetic, antibacterial, antifungal, anticancer, antirheumatic and showed a potent insecticidal effect (Rizk, 1986). Consumption of Cruciferous vegetables has been associated with a reduced risk in the development of various types of cancer, this has been attributed to the bioactive hydrolysis products that are derived from these vegetables e.g. flavonoids. In the last decade, salad species consumption has become increasingly important worldwide, encouraged from the positive link between eating fresh raw materials and absorption of health-promoting phytochemicals. One of the Cruciferous vegetables is *Diplotaxis harra* (Forssk.) Boiss. commonly known as 'wall rocket'. It was widely used in the Mediterranean diet and well-studied as source of healthy phytochemicals (Melchini and Traka, 2010). *D. harra* (Forssk.) Boiss. is a desert medicinal plant growing in Sinai, it is locally known as Harra (wall rocket), it was used in the Egyptian folkloric medicine for the treatment of various diseases e.g. anti-inflammatory, antibacterial,

*Corresponding author. E-mail: melhenawy111@gmail.com. Tel: +202-33371718. Fax: +202-33370931.

antifungal and anti-tumor (Hartwell, 1982). Hashem and Saleh (1999) reported the isolation of steroids together with non-methylated fatty acids from *D. harra* (Forssk.) Boiss. which inhibited fungi, yeast, and Gram-negative and Gram-positive bacteria. In line with the folkloric antitumor uses of *D. harra* (Forssk.) Boiss. Our group started the phytochemical investigation as well as the cytotoxicity of *D. harra* (Forssk.) Boiss.

MATERIALS AND METHODS

Plant material

The aerial parts of *D. harra* (Forssk.) Boiss. were collected from South regions of Sinai during the year 2006. The plant samples were kindly identified by Prof. Dr. Ahmed Morsy - Botany Department - Desert Research Center. A voucher specimen (No. drcc20/774) of the plant materials were kept in the Herbarium of the Desert Research Center. Plant materials were air-dried in shade, grounded to fine powder, packed in tightly closed containers and stored for further phytochemical studies.

General experimental conditions

Thin layer chromatography (TLC) (Silica gel G-60 F_{254} Merck). Column chromatography (Silica gel G-60, 70-230 mesh). Paper chromatography (Whatman No. 3). Nuclear Magnetic Resonance (NMR) spectra (1D, 2D COSY (COSY: Correlation spectroscopy), Heteronuclear Multiple Quantum Coherence (HMQC) and Heteronuclear Multiple Bond Correlation (HMBC) were measured at 600.17 and 150.91 MHz for ^{1}H- and ^{13}C-NMR respectively, with a JEOL ECA 600 spectrometer. Solvent systems; I- chloroform – methanol (9:1), II- ethyl acetate – methanol – water (30:5:4), III- ethyl acetate – methanol – acetic acid – water (65:15:10:10) and IV- butanol – acetic acid – water (4:1:5 upper layer) were used. Visualization of chromatograms was achieved under UV (254 and 365 nm) before and after exposure to ammonia vapor or by spraying with aluminum chloride [8]. All solvents used were of analytical grade.

Extraction and isolation

One kilogram of the aerial parts of *Diplotaxis harra* (Forssk.) Boiss. was collected from two regions south and north Sinai during summer and winter seasons, then it was subjected for Soxhlet extraction, starting with a defatting process using petroleum ether (60 to 80°C) to remove all the lipoidal matter, followed by 95% Ethanol to afford 150 g dry fraction. The dried EtOH fraction was diluted with water (300 ml), filtered, and then successively fractionated with $CHCl_3$, EtOAc, and *n*-butanol to afford 10, 18.5 and 30 g dry fractions, respectively. The obtained fractions were chromatographically compared with TLC using different solvent systems (I-IV), the obtained results suggesting *n*-butanol fraction for further fractionation.

Thirty gram of the *n*-butanol fraction were subjected to preparative TLC and PC (solvent system II) to afford five main bands corresponding to the flavonoidal compounds, these bands were completely separated and extracted with MeOH–H_2O (1:1) to afford five main fractions. These fractions were further purified using high performance liquid chromatography (HPLC) RP-C_{18} (Agilent 1200 series) equipped with Diode Array Detector (DAD). Solvents systems were: (A) 0.05% HCOOH in H_2O (B) 100% acetonitrile. Elution profile as follow; isocratic elution with 100% A (0 to 5 min), then gradient elution to 50% B (5 to 45 min), from 50% B to 100% A (45 to 50 min), finally isocratically with 100% A (50 to 55 min), monitoring at 254 nm with flow rate 3 ml/min, resulted in the isolation of five main flavonoidal compounds (1-5) which were further purified on Sephadex LH-20.

Cytotoxic assay procedures

Human tumor cell lines

Authentic cultures of HCT116 (Human colon carcinoma), Hep-G2 (Human hepatocellular liver carcinoma) and MCF-7 (Human breast carcinoma) cells were obtained in frozen state under liquid nitrogen (-180ºC) from the American Type Culture Collection. The tumor cell lines were maintained by serial sub-culturing in the National Cancer Institute, Cairo, Egypt.

Culture media

HCT116, Hep-G2 and MCF-7 cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% antibiotic-antimycotic mixture (10.000 U/ml K-penicillin, 10.000 µg/ml streptomycin sulphate and 25 µg/ml amphotericin B) and 1% L-glutamine (all purchased from Lonza, Belgium).

Assay method for cytotoxic activity

The cytotoxicity against HCT116, Hep-G2 and MCF-7 cells were tested in the National Cancer Institute, according to the SRB (Sulforhodamine B) assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method by Skehan et al. (1990). Adriamycin® (Doxorubicin) 10 mg vials (Pharmacia, Sweden) was used as the reference drug.

HCT116, Hep-G2 and MCF-7 cells were plated in 96-multiwell plates (5×10^4–10^5 cells/well in a fresh media) for 24 h before treatment with the tested sample to allow attachment of cells to the wall of the plate. Then, 200 µl aliquot of serial dilution with DMSO (100%) of alcoholic extract and isolated compound (5.0, 12.5, 25, 50 µg/ml) were added and the plates were incubated for 24, 48 and 72 h at 37°C in a humidified incubator containing 5% CO_2 in air. Control cells were treated with vehicle alone. Four wells were prepared for each individual dose. Following 24, 48 and 72 h treatment, cells were fixed, washed and stained with Sulforhodamine B stain (Sigma, USA). Colour intensity was measured in an ELISA reader spectrophotometer (Tecan Group Ltd.-Sunrise, Germany).

Statistical analysis

All values were expressed as the mean of percentage of inhibition cells of the three replicates for each treatment. Data were subjected to SPSS (ver.8.0). $P<0.05$ was regarded as significant.

RESULTS AND DISCUSSION

The *in vitro* cytotoxicity (Table 1) of the EtOH extract of the aerial parts of *D. harra* (Forssk.) Boiss. was evaluated against HCT116, HepG2 and MCF-7 cell lines in comparison with Doxorubicin (control). The results

Table 1. The cytotoxicity of the EtOH extract of *D. harra* (Forssk.) Boiss., and Doxorubicin against human cell lines.

Human cell lines	% of inhibition cells ± SEM					
	HCT116		Hep-G2		MCF-7	
Conc.(µg/ml)	EtOH extract	Doxorubicin	EtOH extract	Doxorubicin	EtOH extract	Doxorubicin
5	53.44±0.02*, ** (78.8)***	67.80±0.05*	13.62±0.06*, **(20.63)***	66.00±0.02*	11.74±0.03*, **(14.53)***	80.80±0.03*
12.5	61.91±0.04*, ** (83.21)***	74.40±0.01*	49.47±0.04*, **(70.77)***	69.90±0.01*	31.79±0.01*, **(38.38)***	82.80±0.02*
25	77.07±0.01*, **(92.96)***	82.90±0.03*	85.78±0.05*, **(107.76)***	79.60±0.01*	72.48±0.05*, **(89.04)***	81.40±0.05*
50	77.81±0.01*, ** (95.47)***	81.50±0.01*	82.52±0.02*, **(97.77)***	84.40±0.03*	80.13±0.03*(100.29)***	79.90±0.06*
IC_{50} (µg/ml)	4.65	3.74	12.6	4.57	17.9	2.97

Each value represents the mean of percentage of inhibition cells of three replicates ± SEM (Standard error of mean), *Significantly different from control value at $p<0.05$ according to paired-samples *t*- test, ** Significantly different from control value at $p<0.005$ according to paired- samples *t*- test, *** Relative inhibition of crude EtOH extract related to Doxorubicin on the growth of different human cell lines.

revealed a promising cytotoxicity of the EtOH extract, with IC_{50} = 4.65, 12.60 and 17.90 µg/ml, while Doxorubicin showed IC_{50} = 3.64, 4.57 and 2.97 µg/ml (Table 1). The obtained results revealed a promising *in vitro* cytotoxicity of the EtOH extract against HCT116 with IC_{50} = 4.65 µg/ml, this activity was suspected to be due to the phenolics content. This prompted us to follow up the fractionation process to isolate the active constituent(s). In this regard, the EtOH extract was fractionated into three main fractions $CHCl_3$, EtOAc, and *n*-butanol. Chromatographic comparison of the three fractions suggested *n*-butanol fraction with major phenolics content. Further purification using RP-HPLC led to the isolation of five flavonoidal glycosides identified as; quercetin (1), quercetin 3-*O*-*β*-glucoside (2), isorhamnetin 7-*O*-*β*-glucoside (3), apigenin 3-*O*-*β*-rhamnoside (4) and kaempferol 3-*O*-*β*-glucoside (5), according to their spectral data and comparison with the literature. Furthermore, the isolated flavonoids were tested for their *in vitro* cytotoxicity against HCT116 cell line in comparison with Doxorubicin (IC_{50} = 3.64 µg/ml), and resulted with IC_{50} = 20.1, 24.3, 22.8, 23.4 and 41.9 µg/ml for the isolated flavonoids (1-5), respectively, as determined by MTT method (Table 2).

It appeared that the *in vitro* cytotoxicity of the crude EtOH extract against HCT116 was more potent (IC_{50} = 4.65 µg/ml) than that of the isolated flavonoids (IC_{50} = 20.1, 24.3, 22.8, 23.4 and 41.9 µg/ml (1-5) respectively), this may be due to what is called 'the synergistic effect'. Normally, plant extracts are multi-composed mixtures that can be subdivided into main active substances and concomitant compounds. Concomitant compounds are called co-effectors because they can change the physicochemical properties of the main active substances and therefore influence the biopharmaceutical parameters, e.g. solubility and bioavailability. Concomitant compounds affect the physical and chemical stability of plant extracts (Eder and Mehnert, 1998). The weak cytotoxicity of the isolated flavonoids may be attributed to the presence of sugar moieties and polihydroxylation, which reduce the hydrophobicity of flavonoids thereby making difficult flavonoid's entry into the cell (Mohammed et al., 2009). Sometimes, this effect might decrease their efficacy. This can be clearly appeared with the *in vitro* cytotoxicity against HCT116 cell line, as the IC_{50} of quercetin (1) was 20.1 µg/ml, while, for the isolated glycosides; quercetin 3-*O*-*β*- glucoside (2), isorhamnetin 7-*O*-*β*-glucoside (3), apigenin 3-*O*-*β*-rhamnoside (4) and kaempferol 3-*O*-*β*-glucoside (5), were 24.3, 22.8, 23.4 and 41.9 µg/ml respectively (Table 2). Other times, an increase of hydrophobicity is necessary to obtain a good interaction between the flavonoid and a determined target implicated in cancer. The presence of C-2 and C-3 double bond as well as the C-4 oxo group is required for maximal biological activity of flavonoids. Both of the aromatic substituents and the keto-enol functionality can serve as targets for future structure activity relationship (SAR) studies of flavonoids (Wu et al., 2003). Several investigations showed that flavonoids inhibit

Table 2. The cytotoxic activity of the isolated compounds (1-5) from *n*-butanol fraction of *Diplotaxis harra* (Forssk.) Boiss., against HCT116.

Conc. (µg/ml)	% of inhibition cells ± SEM					
	Compounds					Doxorubicin
	1	2	3	4	5	
5	28.46± 0.08 * ** (41.97)***	6.70±0.03 * ** (9.88)***	3.34±0.06 ** (4.92)***	5.00±0.14 ** (7.37)***	15.13±0.08 * ** (22.32)***	67.80±0.05 *
12.5	29.84±0.02 * ** (38.78)***	29.77±0.11 * ** (40.01)***	28.55±0.03 * ** (38.37)***	28.00±0.05 * ** (37.63)***	27.84±0.02 * ** (37.42)***	74.4±0.01 *
25	63.00±0.04 * ** (76.00)***	50.79±0.07 * ** (61.27)***	54.15±0.04 * ** (65.32)***	52.86±0.13 * ** (63.76)***	29.55±0.12 * ** (35.66)***	82.90±0.03 *
50	67.43±0.03 * ** (82.74)***	61.01±0.07 * ** (74.97)***	55.29±0.04 * ** (67.84)***	67.72±0.04 * ** (83.09)***	59.75±0.01 * ** (73.31)***	81.5±0.01 *
IC_{50} (µg/ml)	20.1	24.3	22.8	23.4	41.9	3.74

Each value represents the mean of percentage of inhibition cells of three replicates ± SEM (Standard error of mean),* Significantly different from control value at $p<0.05$ according to paired- samples *t*- test, ** Significantly different from doxorubicin value at $p<0.05$ according to paired- samples *t*- test,*** Relative inhibition of isolated compounds (1-5) related to Doxorubicin on the growth of HCT116 cell line.

tumour cell growth through the cessation of aerobic glycolysis by blocking membrane Na^+, K^+-ATPase of tumour cells (Suolina et al., 1975), flavonoids appeared to be compounds of low toxicity and some of them apparently have antiproliferative activity against human tumour cells (Hirano et al., 1994). The chemopreventive activity of flavonoids may results from their ability to inhibit phase I and induce phase II of the carcinogen metabolizing enzymes that initiate carcinogenesis. They also inhibit the promotion stage of carcinogenesis by inhibiting oxygen radical-forming enzymes or enzymes that contribute to DNA synthesis or act as ATP mimics and inhibit protein kinases that contribute to proliferative signal transduction.

Also, they may prevent tumor development by inducing tumor cell apoptosis by inhibiting DNA topoisomerase II and p53 down regulation or by causing mitochondrial toxicity, which initiates mitochondrial apoptosis (Galati and O'Brien, 2004). Thus, some of the past studies support the antitumor cell effects of flavonoids being caused via DNA damage to tumour cells (Suolina et al., 1975).

ACKNOWLEDGEMENT

The authors are grateful to the Institut für Umweltforschung (INFU), Technische Universität Dortmund, Otto-Hahn-Strasse 6, D-44221 Dortmund, Germany, for supporting with the facilities of NMR analysis.

REFERENCES

Boulos L (1999). Flora of Egypt Check-list. Al Hadara Publishing Co., Cairo, Egypt, pp. 419-420.

Eder M, Mehnert W (1998). The importance of concomitant compounds in plant extracts. Pharm., 53(5): 285-293.

Galati G, O'Brien PJ (2004). Potential toxicity of flavonoids and other dietary phenolics: Significance for their chemopreventive and anticancer properties. Free Radic. Biol. Med., 37: 287-303.

Geidam YA, Ambali AG, Onyeyili PA (2007). Preliminary phytochemical and antibacterial evaluation of crude aqueous extract of *Psidium guajava* leaf. J. Appl. Sci., 7(4): 511-514.

Hartwell JL (1982). Plants Used Against Cancer. A Survey. Quarterman Inc., USA, p. 164.

Hashem FA, Saleh MM (1999). Antimicrobial components of some Cruciferae plants (*Diplotaxis harra* Forssk. and *Erucaria microcarpa* Boiss). Phytother. Res., 13(4): 329-332.

Hirano T, Gotoh M, Oka K (1994). Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells. Life Sci., 55: 1061-1069.

Isaac OO, Chinwe JA (2001). The phytochemical analysis and antibacterial screening of extract of *Tetrecarpidum conophorum*. J. Chem. Soc. Nig., 26(1): 53-55.

Kudi AC, Umoh JU, Eduvie LO, Gefu J (1999). Screening of some Nigerian medicinal plants for antibacterial activity. J. Ethnopharmacol., 67: 225-228.

Melchini A, Traka MH (2010). Biological profile of erucin: A new promising anticancer agent from Cruciferous vegetables. Toxins, 2: 593-612

Mohammed MMD, Christensen LP, Ibrahim NA, Awad NE, Zeid IF, Pedersen EB, Jensen KB, Colla PL (2009). Anti-HIV-1 activities of the extracts from the medicinal plant *Linum grandiflorum* Desf. Med. Aroma. Plant Sci. Biotechnol., 3: 37-41.

Ogundipe OO, Moody JO, Fakeye TO, Ladipo OB (2000). Antimicrobial activity of *Mallotus oppositifolium* extracts. Afr. J. Med. Sci., 29: 281-283.

Rizk AM (1986). The Phytochemistry of the Flora of Qatar. Scientific and Applied Research Center, University of Qatar, Qatar, p. 582.

Sang-Kook L, Kyung-Ae N, Yoen-Hoi H (2003). Cytotoxic activity and G2/M cell cycle arrest mediated by antofine, a phenanthroindolizidine alkaloid isolated from *Cynanchum paniculatum*. Planta Med., 69(1): 21-25.

Sanni S (2007). Pharmacological and Toxicological Effects of *Ocimum basilicum* L. water extracts in rats. PhD Thesis. Usman Dan-Fodio University, Sokoto, pp. 98-119.

Skehan P, Storeng R, Scudiero D, Monks A, Mcmahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. J. Natl. Cancer Inst.., 82: 1107-1112.

Suolina EM, Buchsbaum RN, Racker E (1975). The effect of flavonoids on aerobic glycolysis and growth of tumor cells. Cancer Res., 35: 1865-1872.

Tackholm V (1974). Students Flora of Egypt (2nd Ed.). The Cooperative Printing Co., Cairo, Egypt, **p**p. 170-171.

Wu JH, Wang XH, Yi YH, Lee KH (2003). A potent anti-HIV chalcone and flavonoids from genus Desmos. Bioorg. Med. Chem. Lett., 13: 1813-1815.