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

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Summary

As a 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* (Forsk.) Boiss. was *in vitro* investigated for its 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 MTT method.

Key words: *Diplotaxis harra (Forsk.) Boiss., Cruciferae, flavonoid glycosides, cytotoxicity*

INTRODUCTION

Traditional medicine is undoubtedly the oldest form of medicine and probably evolved simultaneously with the evolution of human beings [1, 2] or even much earlier. Its ability to stand the test of time over the millennia to its current situation could not be disconnected from its important contribution to the maintenance of health. In fact the primary health care of about 80% of the world's population depends on the use of medicinal plants derived from traditional medicine [3]. The World Health Organization (WHO) stated that 74% of these plants have modern indications that correlate with their traditional, cultural and sometimes ancient uses [1]. In Africa more people seek medical attention from traditional medical practitioners than from medical doctors. Medicinal plants serve as a main source of medicine to rural poor communities that do not have access to modern medical services. About 25% of conventional drugs are derived from plants that have been used traditionally [4]. Investigations into the cytotoxic activity of some medicinal plants have been carried out [5-9] with a view to authenticate their folkloric use.

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 which is derived from these vegetables, e.g. flavonoids. *Diplotaxis harra* (Forsk.) Boiss. (Wall Rocket), is widely used in the Mediterranean diet, and well-studied as source of healthy phytochemicals [10]. 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.

Diplotaxis harra (Forsk.) Boiss. is a desert medicinal plant growing in Sinai, it is locally known as Harra (Wall Rocket), it was used in the Egyptian folk medicine as anti-inflammatory, anti-bacterial, anti-fungal and anti-tumor. It belongs to the Cruciferae family, which is one of the largest families rich in valuable medicinal plants. It includes 338 genera and 3350 species that are distributed worldwide, from which 53 genera and 107 species are distributed in Egypt [11, 12]. Plants of this family were used traditionally as anti-diabetic, anti-bacterial, anti-fungal, anti-cancer, anti-rheumatic and showed a potent insecticidal effect [13]. Hashem and Saleh reported the isolation of steroids and non-methylated fatty acids from *Diplotaxis harra* (Forsk.) Boiss. which inhibited the growth of fungi, yeast, as well as Gram-negative and Gram-positive bacteria [14], in line with the folk medicinal uses of *Diplotaxis harra* (Forsk.) Boiss. as anticancer, our group started the phytochemical investigation as well as the cytotoxicity evaluation of *Diplotaxis harra* (Forsk.) Boiss.

MATERIAL AND METHODS

Plant material

The aerial parts of *Diplotaxis harra* (Forsk.) Boiss. were collected from South and North regions of Sinai in 2006. The plant samples were kindly identified by Prof.

Dr. Ahmed Morsy - Botany Department, Desert Research Center, Egypt. 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 into fine powder, packed in tightly closed containers and stored for further phytochemical studies.

General experimental conditions

Thin layer chromatography (Silica gel G-60 F_{254} Merck). Silica gel (G-60, particle size 70-230 mesh) were used for column chromatography. Paper chromatography (Whattman No. 3). 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 before and after exposure to ammonia vapor or by spraying with aluminium chloride [15]. NMR spectra (1D, 2D COSY, HMQC & HMBC) were measured at 600.17 and 150.91 MHz for ^{1}H and ^{13}C-NMR, respectively, using a pulse sequence supplied from JEOL ECA 600 spectrometer. All solvents used were of analytical grade.

Extraction and isolation

One kg of the aerial parts of *Diplotaxis harra* (Forsk.) Boiss. was collected from two regions South and North Sinai during summer and winter, then it was subjected for Soxhlet extraction, starting with a defatting process using pet. ether (60–80ºC) to remove all of the lipoidal matter, followed by 95% ethanol, which afforded 150 g dried fraction. The EtOH fraction was diluted with water (300 mL), filtered, and then successively fractionated with $CHCl_3$, EtOAc, and *n*-butanol to obtain 10, 18.5 and 30 g dry fractions, respectively. The obtained fractions were chromatographically compared using TLC with different solvent systems (I-IV), the obtained results suggesting *n*-butanol fraction for further fractionation.

30 g of the *n*-butanol fraction was subjected to preparative TLC and PC (solvent system II) to afford five main bands corresponding to the flavonoidal compounds, theses bands were completely separated and extracted with MeOH–H_2O (1:1). The obtained compounds were further purified using HPLC RP-C_{18} (Agilant 1200 series) equipped with Diode Array Detector (DAD). Solvent Systems were: (A) 0.05% HCOOH in H_2O (B) 100% acetonitrile. Elution profile was; isocraticaly 100% (A) from 0–5 min., and gradient elution to 50% (B) from 5–45 min., finally from 50% (B) to 100% (A) in 10 min., then isocraticaly for 5 min. This process resulted in the isolation of five main flavonoidal compounds (**1-5**) which were subsequently purified on Sephadex LH-20.

Cytotoxic assay procedures

Human tumor cell lines

Authentic culture, HCT116, Hep-G2 and MCF-7 cells were obtained frozen under liquid nitrogen (–180ºC) from the American Type Culture Collection. The tumor cell lines were maintained by serial subculturing 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 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 was performed in the National Cancer Institute, according to the SRB assay method [16]. Adriamycin® (Doxorubicin) 10 mg vials (Pharmacia, Sweden) was used as a reference drug.

HCT116, Hep-G2 and MCF-7 cells were plated in 96-multiwell plates ($5x10^4$–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 of alcoholic extract and isolated compounds (5.0, 12.5, 25, 50 μg/ml) were added and the plates were incubated for 24, 48 and 72 hrs 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 hrs 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 the 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 (tab. 1) of the EtOH extract of the aerial parts of *Diplotaxis harra* (Forsk.) Boiss. was evaluated against HCT116, HepG2 and MCF-7 cell lines in comparison with Doxorubicin (control) which was resulted with IC_{50}=4.65, 12.60 and 17.90 μg/ml for the EtOH extract, and with IC_{50}=3.64, 4.57 and 2.97 μg/ml for Doxorubicin (tab. 1). The obtained results revealed a promising cytotoxicity of the EtOH extract against HCT116 with IC_{50}=4.65 μg/ml, which prompted our group to follow

up the fractionation process to isolate the active constituent(s). The EtOH extract was fractionated into three main fractions $CHCl_3$, EtOAc, and *n*-butanol, chromatographic comparison 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 showed *in vitro* cytotoxicity against HCT116 cell line with IC_{50}=20.1, 24.3, 22.8, 23.4 and 41.9 μg/ml respectively (Table 2), as determined by MTT method.

Table 1.

The cytotoxicity of the EtOH extract of *Diplotaxis harra* (Forsk.) 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

Table 2.

The cytotoxic activity of the isolated compounds (**1-5**) from *n*-butanol fraction of *Diplotaxis harra* (Forsk.) 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 *

Conc. (μg/ml)	% of inhibition cells ±SEM					
	compounds					doxorubicin
	1	2	3	4	5	
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

It appears that the cytotoxicity of the crude EtOH extract against HCT116 was more potent than that of the isolated flavonoids whichmay be due to 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 [17]. The weak cytotoxicity of the isolated flavonoids, may be attributed to the presence of sugar moieties and polyhydroxylation which reduce the hydrophobicity of flavonoids thereby making difficult flavonoid's entry into the cell [18]. Sometimes, this effect might decrease their efficacy. This can be clearly appeared in our study 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 (tab. 2). At 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 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 [19].

Several investigations showed that flavonoids inhibit tumour cell growth via the cessation of aerobic glycolysis by blocking membrane Na^+, K^+-ATPase of tumour cells [20], flavonoids appeared to be compounds of low toxicity and some of them apparently have antiproliferative activity against human tumour cells [21]. The chemopreventive activity of flavonoids may results from their ability to

inhibit phase I and induce phase II 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 [22]. Thus, some of the past studies support the antitumor cell effects of flavonoids being caused via DNA damage to tumour cells [20]. The limited understanding about a possible structure-activity relationship of flavonoids as anticancer agents is possibly due to the few or not appropriated flavonoids used in the previous studies.

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CYTOTOKSYCZNOŚĆ FLAWONOIDÓW POCHODZĄCYCH Z ROŚLINY *DIPLOTAXIS HARRA* (FORSK.) BOISS. ROSNĄCEJ NA PÓŁWYSPIE SYNAJ

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Streszczenie

W ramach badań nad poszukiwaniem nowych związków fenolowych o aktywności przeciwnowotworowej pochodzących z roślin lądowych badaniom w kierunku cytotoksyczności w warunkach *in vitro* w poddano wyciąg alkoholowy z nadziemnych części egipskiej rośliny leczniczej *Diplotaxis harra* (Forsk.) Boiss. W wyniku przeprowadzonych badań cytotoksyczności z wykorzystaniem linii komórkowych HCT116, HepG2 oraz MCF-7 otrzymano odpowiednio następujące wartości: IC_{50}=4,65, 12,60 oraz 17,90 μg/ml. Doksorubicyna (kontrola pozytywna) w warunkach *in vitro* wykazała w stosunku do badanych linii komórkowych następującą aktywność cytotoksyczną: odpowiednio IC_{50}=6,64, 4,57 oraz 2,97 μg/ml. Dalsze rozdzielanie frakcji wyciągu etanolowego, bogatej w związki fenolowe, doprowadziło do wyizolowania pięciu flawonoidów: kwercetyny, 3-*O*-β-glukozydu kwercetyny, 7-*O*-β-glukozydu izoramnetyny, 3-*O*-β-ramnozydu apigeniny oraz 3-*O*-β-glukozydu kemferolu (zidentyfikowanych według danych spektralnych oraz na podstawie porównania z danymi pochodzącymi z literatury przedmiotu). Ponadto wyizolowane flawonoidy wykazywały *in vitro* cytotoksyczność określoną przy użyciu metody MTT w stosunku do linii HCT116 na poziomie IC_{50} =20,1, 24,3, 22,8, 23,4 oraz 41,9 μg/ml.

Słowa kluczowe: *Diplotaxis harra (Forsk.) Boiss., Cruciferae, glikozydy flawonidowe, cytotoksyczność*