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Original Article

Cytotoxic activity of phenolic constituents from *Echinochloa crus-galli* against four human cancer cell lines

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ABSTRACT

Echinochloa crus-galli (L.) P. Beauv., Poaceae, grains are used as a feed for birds and millet for humans. The sulforhodamine B assay was used to assess its cytotoxicity against four human cancer cell lines. The ethanolic extract (70%) proved to be most active against HCT-116 and HELA cell lines (IC_{50} = 11.2 ± 0.11 and 12.0 ± 0.11 μg/ml, respectively). On the other hand, the chloroform and ethyl acetate fractions exhibited their highest activities against HCT-116 cell lines. The chloroform and ethyl acetate fractions were subjected to several chromatographic separations to render pure phenolic compounds (**1**–**8**). Compounds **1**–**8** were identified as: 5,7-dihydroxy-3′,4′,5′-trimethoxy flavone, 5,7,4′-trihydroxy-3′,5′-dimethoxy flavone (tricin), quercetin, flavone, apigenin-8-*C*-sophoroside, 2-methoxy-4-hydroxy cinnamic acid, *p*-coumaric acid and quercetin-3-*O*-glucoside. All the isolated phenolic compounds exhibited various significant activities against the four human carcinoma where the methoxylated flavones (**1** and **2**) were the most active, in a way comparable to the anticancer drug Doxorubicin®. Thus, these methoxylated flavonoids may be considered as lead compounds for the treatment of cancer, which supports previous claims of *E. crus-galli* traditional use. This is the first report of the occurrence of these phenolic compounds in *E. crus-galli*.

Introduction

Cancer is a leading cause of death in economically developed countries and the second leading cause of death in developed countries. Lung, stomach, liver, colon and breast cancer cause the most cancer deaths each year. Breast cancer in females, and lung and prostate cancer in males are the most frequently diagnosed cancers and the leading causes of cancer death for each sex. About 30% of cancer deaths are due to the five leading behavioral and dietary risks; high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco use, and alcohol use (Jemal et al., 2011).

Plant derived compounds have played a major role in the development of several useful cytotoxic agents *viz.* vinblastine, vincristine, and paclitaxel (Taxol®). Other promising new agents are in clinical development stage, including flavopiridol and combretastatin, which clarifies the urge for screening native flora in search for new bioactive phytochemical compounds (Reddy et al., 2003; Cragg and Newman, 2005).

Echinochloa crus-galli (L.) P. Beauv., Poaceae, is a problematic summer weed found in rice fields and known as Barnyard grass. The grains are known as fodder for livestock and as millet for people in many Asian countries (Boulos and El Hadidi, 1984). It is known traditionally to reduce body weight, blood sugar, treat hypertension and help to detoxify liver and kidney. It is also used for carbuncles, hemorrhage, sores, spleen trouble, wounds and cancer ('t Mannetje and Jones, 1992). It was previously reported that the 70% hydroalcoholic extract of the grains of *E. crus-galli* showed significant antidiabetic activity in normal and alloxan induced diabetic rats (Devi et al., 2012), and that the methanol and aqueous extracts exhibited significant antioxidant activities (Ho et al., 2012; Mehta and Vadia, 2014). Several phenolic compounds; flavones, flavone glycosides, caffeoyl quinic acid derivatives were isolated from other *Echinochloa* species such as *E. utilis*, *E. frumentacea* and *E. colona* (Watanabe, 1999; Kim et al., 2008; Lazaro, 2009; Gomaa and Abd Elgawad, 2012; Hegab et al., 2013).

This study was carried out in order to prove the ethnopharmacological use of *E. crus-galli* grains as a remedy for cancer ('t Mannetje and Jones, 1992), and to specify the compounds responsible for its cytotoxic activity against four human cancer cell lines; MCF-7 (breast carcinoma), HCT-116 (colon carcinoma), HELA (cervical carcinoma) and HEPG-2 (liver carcinoma). The use of such

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relatively cheap millets in the Egyptian's daily diet could contribute to the prevention of cancer in the overgrowing number of cancer patients.

Materials and methods

Plant material

Grains of *Echinochloa crus-galli* (L.) P. Beauv., Poaceae, were collected between July and September, 2010 from plants grown in the Food Technology Research Institute, Faculty of Agriculture, Cairo University, Giza. They were identified by Prof. Dr. Osama El Kopacy, Professor of Botany, Faculty of Agriculture, Cairo University. A voucher specimen number 9010 was placed at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University.

General

Silica gel H (Merck, Darmstadt, Germany) was used for vacuum liquid chromatography (VLC), silica gel 60 (70-230 mesh ASTM; Fluka, Steinheim, Germany), sephadex LH-20 (Pharmacia, Stockholm, Sweden), polyamide and cellulose (Merck, Darmstadt, Germany) were used for column chromatography (CC). Thin-layer chromatography (TLC) was performed on silica gel GF_{254} pre-coated plates (Fluka) using the following solvent systems: S_1, chloroform:methanol (9:1, v/v); S_2, petroleum ether:ethyl acetate:formic acid (45:16:3.6, v/v/v); S_3, chloroform:acetone:formic acid (65:15:1.5, v/v/v); S_4, chloroform:acetone:formic acid (75:16.5:8.5 v/v/v); S_5, ethyl acetate:methanol:water (100:16.5:13.5, v/v/v); S_6, chloroform:methanol (8:2, v/v). The chromatograms were visualized under UV light (at 254 and 366 nm) before and after exposure to ammonia vapor and spraying with $AlCl_3$, as well as after spraying with *p*-anisaldehyde/sulphuric acid spray reagent. Melting points (uncorrected) were determined on a D. Electrothermal 9100 instrument (Labe-quip, Markham, Ontario, Canada). UV spectra were recorded using a Shimadzu UV 240 (P/N 204-58000) spectrophotometer (Kyoto, Japan). A Varian Mercury-VX-300 NMR instrument (Palo Alto, CA, USA) was used for ^{1}H NMR (300 MHz) and ^{13}C NMR (75 or 125 MHz). The NMR spectra were recorded in DMSO-d_6 and chemical shifts were given in δ (ppm) relative to tetramethylsilane as an internal standard.

Extraction, fractionation and isolation

The air-dried powdered grains of *E. crus-galli* (1 kg) were percolated with ethanol 70% till exhaustion to yield 115 g of the alcoholic extract (AlEx). The residue was suspended in distilled water and partitioned successively using n-hexane, chloroform, ethyl acetate, and *n*-butanol saturated with water giving the fractions HxFr, ClFr, EtFr, and BuFr, respectively. The fractions were separately concentrated under reduced pressure to yield 58 g, 7.6 g, 5.8 g and 4.3 g, respectively. The ClFr (5 g) was chromatographed over a VLC column (5 cm × 20 cm, silica gel H, 250 g). Gradient elution was carried out using *n*-hexane/methylene chloride mixtures, methylene chloride/ethyl acetate mixtures, and ethyl acetate/methanol mixtures. Fractions of 200 ml each were collected and monitored by TLC to yield seventeen fractions ($A_c - Q_c$). Fraction G_c (60% methylene chloride in ethyl acetate) was re-chromatographed over silica gel 60 column using methylene chloride in ethyl acetate (9:1, v/v) as an eluent to give compound **1** (22 mg, yellow powder, R_f 0.72 in S_2, m.p. 251–253 °C) and compound **2** (17 mg, yellow powder, R_f 0.70 in S_2, m.p. 249–251 °C). Fraction I_c (90% ethyl acetate in methanol) was purified using several sephadex LH-20 column to yield compound **3** (23 mg, yellow powder, R_f 0.60 in S_2, m.p. 310–312 °C). Fraction E_c (20% methylene chloride in ethyl acetate) was re-chromatographed on a silica gel 60 column, using *n*-hexane/ethyl acetate (8:2 v/v) as an eluent to give compound **4** (15 mg, white powder, R_f 0.91 in S_2, m.p. 349–351 °C). The EtFr (5 g) was chromatographed over polyamide column (250 g, 5 cm × 120 cm). Gradient elution was carried out with water, followed by increasing amount of methanol starting with 5% up to 90% methanol. Fractions of 250 ml each were collected and monitored by TLC to yield five main fractions ($A_{Et} - E_{Et}$). Fraction B_{Et} (40% methanol in water) was re-chromatographed over cellulose column (50 g, 3.5 cm × 120 cm) using 10% methanol in water as an eluent to give compound **5** (15 mg, yellowish brown powder, R_f 0.15 in S_4, m.p. 216–218 °C). Fraction C_{Et} (60% methanol in water) was purified several times over cellulose columns (50 g, 3.5 × 10 cm) using 15% methanol in water to yield compound **8** (12 mg, yellow powder, R_f 0.42 in S_4, m.p. 239–240 °C). Fraction D_{Et} (70% methanol in water) was purified using several sephadex LH-20 columns to yield compound **6** (11 mg, yellowish white powder, R_f 0.71in S_4, m.p. 168–170 °C) and compound **7** (15 mg, yellowish white powder, R_f 0.73in S_4, m.p. 169–171 °C).

Assessment of cytotoxic activity

The cytotoxicities of the AlEx, the four fractions (HxFr, ClFr, EtFr and BuFr) and the eight isolated compounds from ClFr, and EtFr were assessed using the sulforhodamine B assay (Skehan et al., 1990) against the four human cancer cell lines; colon (HCT-116), cervical (HELA), liver (HEPG-2) and breast (MCF-7) adenocarcinoma using Doxorubicin® as a reference standard. Active fractions and compounds were assessed against normal human fibroblast cell lines (HFB4). Dose-dependent activities were studied for all samples using concentrations from 5 to 50 μg/ml, and the IC_{50} values (concentration which reduced survival to 50%) were calculated. Three separate experiments, each with three replicates, were performed for each sample.

Results and discussion

The cytotoxic effects of the extracts of *E. crus-galli* grains at concentrations up to 50 μg/ml and 48 h of exposure showed that the AlEx exhibited IC_{50} values of 12.0 ± 0.11, 11.2 ± 0.11, 18.9 ± 0.12, and 14.2 ± 0.11 μg/ml against HELA, HCT-116, MCF-7, and HEPG-2 cells, respectively (Table 1). According to the US NCI plant screening program a crude extract is considered to possess an *in vitro* cytotoxic activity if its IC_{50} value is less than 20 μg/ml, following an incubation period of 48 and 72 h (Boik,

Table 1
Cytotoxic activities of alcoholic extract, fractions and the isolated compounds of *Echinochloa crus-galli* (IC_{50} values are given in μg/ml).

Tested sample	IC_{50} ± SD (μg/ml)			
	HELA	HCT-116	MCF-7	HEPG-2
AlEx	12.0 ± 0.11	11.2 ± 0.11	18.9 ± 0.12	14.2 ± 0.11
HxFr	29.5 ± 0.01	20.5 ± 0.03	17.1 ± 0.01	15.5 ± 0.04
ClFr	27.3 ± 0.21	17.1 ± 0.01	21.7 ± 0.01	23.9 ± 0.02
EtFr	21.3 ± 0.01	3.8 ± 0.01	16.8 ± 0.01	22.4 ± 0.04
BuFr	18.5 ± 0.01	4.2 ± 0.03	13.5 ± 0.01	14.3 ± 0.03
1	4.5 ± 0.03	18.5 ± 0.04	11.5 ± 0.01	4.5 ± 0.03
2	2.8 ± 0.01	3.6 ± 0.03	6.6 ± 0.03	2.4 ± 0.02
3	13.8 ± 0.01	20.4 ± 0.02	12.7 ± 0.02	11.3 ± 0.02
4	11.0 ± 0.03	27.0 ± 0.01	14.0 ± 0.02	9.3 ± 0.03
5	11.8 ± 0.03	14.5 ± 0.04	6.3 ± 0.01	7.4 ± 0.03
6	5.1 ± 0.01	2.7 ± 0.03	12.4 ± 0.03	17.3 ± 0.02
7	6.5 ± 0.01	4.5 ± 0.02	15.6 ± 0.02	13.5 ± 0.02
8	11.5 ± 0.03	13.5 ± 0.01	5.3 ± 0.02	11.3 ± 0.03
Doxorubicin	4.5 ± 0.03	4.5 ± 0.53	4.3 ± 0.03	4.2 ± 0.03