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EHRETIA WALLICHIANA HOOK.F. AND THOMSON EX GAMBLE REDUCES THE RISK OF BREAST CANCER

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ABSTRACT

Globally researchers are agreed that diet is the single greatest contributor to human cancer, and maybe associated with 35-70% of the incidence of the disease. Although various carcinogens are present in foods, their effects are minor compared with dietary components that inhibit the cancer process. The crude extract (80% ethanol extract) of leaves of *Ehretia wallichiana* Hook. f. & Thomson ex Gamble family Borginaceae was examined for their cytotoxic activity against different cell lines. It showed higher activity against breast cancer (IC50=10 µg/mL). Petroleum ether, chloroform and ethyl acetate extracts (successive to each other), showed high activity against breast cell line. Four phenolic compounds were isolated from *Ehretia wallichiana*. Two of them are flavonoids: quercetin 3-*O*-*β*-(6"-galloyl glucopyranoside) and quercetin-3-*O*-*β*-glucopyranoside. The third is a precursor of coumarin 5-hydroxy-6-methoxybenzofuran-3-carbaldehyde and the fourth is *p*- methoxybenzoic acid (*p*-anisic acid). They were identified by their spectroscopic data.

Keywords. *Ehretia wallichiana*, phenolics, anticancer activity.

INTRODUCTION

The plants of the genus *Ehretia* Linn. is composed of about 50 species. They are mainly distributed in tropical Asia and Africa. *Ehretia* Linn. species are used in traditional Chinese herbal medicines as ethno pharmaceuticals. For example, in folklore it is used for the treatment of various ailments such as inflammation, cough, itches, swellings, pain, diarrhea, dysentery, fever, cachexia and syphilis (Iqbal *et al*., 2005).

The compounds identified in the genus mainly belong to the classes of phenolic acids, flavonoids, benzoquinones, cyanoglucosides and fatty acids. The chemical constituents of some species of *Ehretia* have been reported to be long chain aliphatic unsaturated acids (Kleiman *et al.,* 1964), baurenol (Anjaneyulu *et al.*, 1965) and allantoin (Koyama*,* 1953; Agarwal *et al*., 1980). Species of the genus *Ehretia* were reported to contain pyrrolizidine alkaloids (Suri *et al*., 1980), nitrile glucosides and rosmarinic acid with histamine-inhibitory activity (Simpol *et al*., 1994). Also, dimeric prenylbenzoquinones with antiallergic activity (Yamamura *et al*., 1995) and quinonoid xanthene with antisnake venom activity (Selvanayagam *et al*., 1996).

MATERIALS AND METHODS

Plant materials

Fresh non flowering aerial parts of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble, family Boraginaceae, was collected from Giza Zoo in May 2009, Cairo, Egypt. Voucher specimen of the plant was identified by plant taxonomist Dr. M. El-Gibaly and Mrs. Trease Labib.

Total ethanol extract

Crude extract was prepared by percolating 100g dry powder of the plant with 80% ethanol until exhaustion. The filtered percolate was concentrated under reduced pressure at 40°C. It yielded a result of 22.5% w/w of solvent free extract.

Preparation of successive extracts

One kg of the air-dried powdered non flowering aerial parts of the plant was successively extracted with solvents of increasing polarities. Which were: petroleum ether, chloroform, ethyl acetate and 95% ethanol in a Soxhlet apparatus. After a complete extraction, these extracts were evaporated to dryness reduced pressure at 40°C yielding 4.48, 4.35, 1.14 and 4.85% w/w of solvent free extracts.

Isolation of flavonoids I and II

The ethanol successive extract was subjected to PC examination for the detection of flavonoids using Whatmann No. 1 sheets for developing with the two solvent systems (a) *n*-butanol - acetic acid - water (4:1: 5 v/v organic upper layer) and (b) acetic acid - water (15:85 v/v). The chromatograms were examined under UV light before and after exposure to ammonia vapour and sprayed with $AlCl_3$ solution. Spots were detected in each extract, as their R_f values in systems (a) & (b) and colors were

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being recorded. These compounds were isolated by preparative paper chromatography (PPC) on Whatmann 3MM, using solvent system (a), then purified by repeated PPC using solvent system (b). Final purification was performed on Sephadex LH-20 column and elution with methanol.

Isolation of compounds III and IV

The powdered air-dried non flowering aerial part of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble (500g) was extracted with 80% ethanol in a Soxhlet apparatus. The extract was concentrated and treated with an equal volume of 10% KOH solution at room temperature for one hour to liberate potassium salt of coumarin. The alkaline alcoholic extract was diluted with water and extracted with ether. The aqueous layer was acidified with dilute HCl, refluxed for 1.5 hours, cooled and extracted with ether, whereby the ethereal extract was evaporated to dryness. The ether extract fraction was dissolved in ethanol and subjected to TLC using silica gel G60, F_{254} precoated plates, developed with benzene: ethyl acetate (8:2) and sprayed with I_2/KI reagent. Two compounds showed blue color under UV, one of which was intensified by spraying with I_2/KI reagent. Both were purified by PLC using the same solvent system and recovered from silica gel by chloroform, evaporated under reduced pressure at 40°C and then subjected to spectral analysis (MS, UV, ^{1}HNMR).

Apparatus

1- UV-Visible Spectrophotometer: UV–VIS double beam Jasco v-630.
2- Mass spectrometer: Thermo Scientific, ISQ Single Quadrupole Mass Spectrometer, an electron ionization system with ionization energy of 70eV.
3- NMR Spectrometers: Nuclear Magnetic Resonance spectrometers JEOL 500 MHz (for determination of ^{1}HNMR).

Characterization of compounds

Compound **I:** R_f values (PC): 0.52 (a), 0.32 (b). It appears as a dark spot under UV which turned yellow upon exposure to NH_3 and $AlCl_3$, on complete acid hydrolysis (2 N HCL, at 100°C for 5 hours) gave D-glucose (CoPC) in the aqueous phase, while gallic acid and quercetin were detected in the organic phase (CoPC, UV, and ^{1}HNMR spectral data).

UV:λ_{max} (MeOH): 257, 268 (sh), 356; NaOMe: 273, 325 sh, 410; $AlCl_3$: 272, 300 (sh), 413; $AlCl_3$/ HCl: 268, 295 sh, 385, 398; NaOAc: 266, 402 NaOAc/ H_3BO_3: 265, 402.

^{1}HNMR in $(CD_3)_2CO$ δ ppm 7.80 (d, *J*= 2 Hz, H-2') 7.50 (dd, *J*= 8 , 2 Hz, H- 6') 7.25 (d, *J*= 2 Hz, H-2 "and 6" of galloyl moiety) 6.90 (d, *J*= 8 Hz, H- 5'), 6.45 (d, *J*=2 Hz, H-8) 6.21 (d, *J*=2 Hz, H-6) 5.50 (d, *J*=7 Hz, H-1'"glucosyl) 5.27 (dd, *J*= 12.5 and 4.8 Hz, H- 6'"$_a$) 5.20 (dd *J*= 12.5 and 2.5 Hz, H-6'"$_b$) while glucosyl protons from 3.46- 3.60 ppm.

Compound **II:** R_f values (PC): 0.59 in (a), 0.36 in (b). It appears as a dark spot under UV, turned yellow orange upon exposure to NH_3 vapours and yellow upon exposure to $AlCl_3$. On complete acid hydrolysis it gave glucose (CoPC) and quercetin (CoPC, UV and ^{1}HNMR spectral data).

UV: λ_{max} (MeOH): 258, 270 sh, 300 sh, 360; NaOMe: 272, 328, 406; $AlCl_3$: 275, 300 sh, 330 sh, 430; $AlCl_3$/ HCl: 270, 299 sh, 365, 400; NaOAc: 269, 405; NaOAc/H_3BO_3: 263, 380.

^{1}HNMR (DMSO,d_6): δ ppm 7.53 (m, H- 2' and H- 6'), 6.80 (d, *J*= 8 Hz, H- 5'), 6.29 (d, *J*= 2 Hz, H- 8), 6.1 (d, *J*= 2 Hz, H- 6), 5.42 (d, *J*=7 Hz, H- 1'' glucosyl) while glucosyl protons (3.46- 3.80 ppm were hidden by OH groups).

Compound **III**: It was isolated as white powder at R_f0.6 in benzene: ethyl acetate, (8:2). It had violet color under UV turned to dark violet by I_2/ KI spray reagent.

UV: λ_{max} (MeOH): 274, 330.

EIMS: m/z 192 (M^+) corresponding to molecular formula $C_{10}H_8O_4$.

^{1}HNMR (DMSO- d_6): δ in ppm: 7.65 (s, H-7); 7.45 (s, H-4); 5.30 (s, H- 2); 3.60(s, OCH_3 at C-6).

Compound **IV:** It was isolated as white amorphous powder at R_f values (PC): 0.80 in (a) and 0.49 in (b). It appeared as a blue spot under UV and did not change after exposure to NH_3 vapours.

UV: λ_{max} (MeOH): 260 nm.

MS: m/z 152 (M^+ 10%), 115 (13%), 91 (22%), 77 (22%), 63 (45%), 55 (18%), 43 (40%), 27 (80%), 18 (80%) corresponding to molecular formula $C_8H_8O_3$.

^{1}H NMR(DMSO-d_6). δ ppm: 7.74 (d, *J*= 8.5 Hz, H- 2 and H-6); 6.78 (d, *J*= 8.5 Hz, H- 3 and H-5); 3.75 (s, OCH_3-4).

Investigation of cytotoxic activity of total ethanol extract of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble by SRB assay

Potential cytotoxicity of the total ethanolic extract of the non flowering aerial part of the plant was tested using the method of (Skehan *et al*., 1990) SRB assay as follows:

A. The total ethanol extract of the plant was tested for cytotoxic activity against the following human tumor cell lines:

•U25l (brain tumor cell line) •MCF7 (breast carcinoma cell line)
•Hela (Cervix carcinoma cell line) •Hepg2 (Liver carcinoma cell line)

•H460 (Lung carcinoma cell line) •HCT116 (Colon carcinoma cell line)

Cells were plated in 96 - multi-well plate (10^4 cells / well) for 24 hours before treatment with the total ethanolic extract of the plant to allow attachment of the cell to the wall of the plate. Different concentrations of the extract under test (0, 1, 2.5, 5 and 10µg/mL DMSO) were added to the cell monolayer, triplicate wells being prepared for each individual dose. Monolayer cells were incubated with the total alcohol extract of the plant for 48 hours at 37°C and in atmosphere of 5% CO_2. After 48 hours cells were fixed, washed and stained with sulforhodamine B (SRB) stain (Sigma). Excess stain was washed with acetic acid and attached stain was recovered with tris-EDTA buffer (Sigma). Color intensity was measured in an ELISA reader. The relation between surviving fraction and the plant extract concentration was plotted to get the survival curve of each tumor cell line after treatment. The potency was compared with reference Cisplatin (Glaxo). The same method was repeated on the successive extracts using the cell line that showed lowest survival fraction.

RESULTS AND DISCUSSION

Identification of compounds

Compound **(I)** was isolated as light brown amorphous powder. It was found to possess chromatographic and color properties (dark spot on PC and UV light, intense blue $FeCl_3$ color reaction and a positive rose color with aqueous KIO_3 specific for galloyl esters (Barakat *et al*., 1999) and UV absorption maxima consistent with galloylated quercetin -3- glycoside (Markhams and Mohan Shari, 1982). On complete acid hydrolysis, (2N HCl at 100° C for 5 hours) yielded quercetin, gallic acid in the organic layer (CoPC, UV and ^{1}HNMR analysis) and glucose in the aqueous layer (CoPC). ^{1}HNMR spectrum in $(CD_3)_2CO$ of **(I)** exhibited the characteristic resonance pattern of quercetin, the anomeric glucose proton signal appeared as doublet (*J*= 7Hz) at δ 5.50 ppm and the galloyl protons appeared as doublet (*J*= 2 Hz) at δ 7.25 ppm. Attachment of the galloyl moiety to C- 6" of glucose has been deduced from the downfield shift of the two doublets of H- 6_a" and H- 6_b" at 5.27 and 5.20 respectively. The chemical shift values of **(I)** were in accordance with the structure of quercetin 3-*O*-*β*-(6"-galloyl glucopyranoside) (Sohretoglu *et al.*, 2009) (see Fig. 1). This represented the first report of this flavonoid galloylglucoside in *Ehretia wallichiana* Hook.f. & Thomson ex Gamble and even in the genus *Ehretia*.

Compound **(II)** was isolated as dark yellow amorphous powder, dark spot on PC, under UV light, turned orange yellow upon exposure to NH_3 vapour, and blue with $FeCl_3$ reagent. UV absorption maxima consistent with quercetin-3-*O*-glycoside (Mabry *et al.*, 1970). On complete acid hydrolysis, it yielded quercetin (CoPC, UV and ^{1}HNMR) and glucose (CoPC). ^{1}HNMR spectrum in DMSO- d_6 and UV with different shift reagents confirmed its structure as quercetin- 3-*O*-*β*-glucopyranoside (Ibrahim *et al.*, 2007) (see Fig. 2).

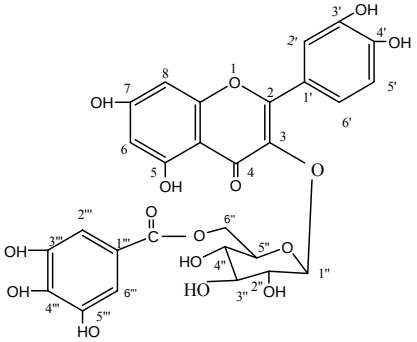

Fig. 1. quercetin 3-*O*-*β*-(6"-galloyl glucopyranoside).

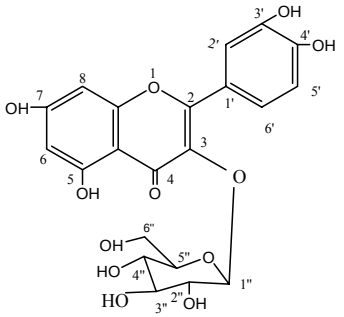

Fig. 2. quercetin- 3-*O*- *β*-glucopyranoside.

Compound (**III**): It was isolated as white powder, with violet color under UV turned to dark violet by I_2 / KI spray reagent and exhibited an UV spectrum characteristic of 5-hydroxy-6-methoxybenzofuran-3-carbaldehyde, with maxima at 274 and 330 nm^{-1}. The EIMS of the compound exhibited a molecular ion peak at m/z 192 (M^+), and its molecular formula $C_{10}H_8O_4$. The downfield signal was observed in the ^{1}HNMR spectrum at δ 7.65 ppm and was allocated to H-7 and the singlet signal at δ 7.45 ppm is for proton H-4, while H-2 appeared as singlet at δ 5.30 ppm and the methoxy group was confirmed at C-6 a singlet at δ 3.6 ppm signal. The structure of compound **(III)** has been established on the basis of its chemical, chromatographic and spectral analysis and found to be identical with 5-hydroxy-6-methoxybenzofuran-3-carbaldehyde (Fig. 3). It is a precursor of coumarin.

Compound **(IV):** The structure of compound **(IV)** was identified on the basis of its spectral data (UV, ^{1}HNMR and EI-MS) which were identical with *p*- methoxybenzoic acid (Shabana *et al.*, 2013). ^{1}HNMR proved 1, 4-disubstituted benzene structure as could be concluded from the recognized mode of splitting of the recorded proton resonances. These resonances appeared as doublet (*J*=8.5 Hz) at δ7.74 and 6.78 ppm attributed to H-2, 6 and H-3, 5, respectively. The methoxy group at position 4

Table 1. Cytotoxic activity of total 90% ethanol extract of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble.

Cell line	Conc μg/mL	90% Ethanol extract		Cisplatin	
		SF	MSE	SF	MSE
Brain	10.000	0.866	0.006	0.494	0.051
Breast	10.000	0.538	0.008	No effect	-----
Cervix	10.000	0.692	0.005	0.059	0.024
Colon	10.000	0.737	0.002	No effect	-----
Liver	10.000	0.780	0.004	0.518	0.054
Lung	10.000	0.896	0.005	0.401	0.007

*SF: Survival fraction. *MSE: Mean standard error

Table 2. Cytotoxic activity of successive extracts (10 μg/mL) of Ehretia *wallichiana* Hook.f. & Thomson ex Gamble on breast cell line.

Extract	Petroleum Ether	Chloroform	Ethyl Acetate	Ethanol
Survival Fraction	0.630	0.634	0.602	0.670

appeared as singlet at δ 3.75 ppm. The positive EI-MS confirmed the structure and gave signal at m/z 153 (M^+ +H) corresponding to molecular weight (M^+) 152 and molecular formula ($C_8H_8O_3$) of 4-methoxy benzoic acid (Fig.4). Compound **(IV)** was found to be 4-methoxy benzoic acid (*p*-anisic acid).

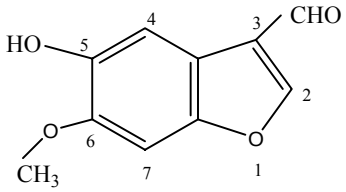

Fig. 3. 5-hydroxy-6-methoxybenzofuran-3-carbaldehyde.

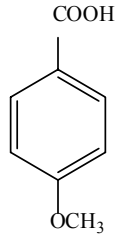

Fig. 4. *p*-methoxybenzoic acid.

Cytotoxic activity of *Ehretia wallichiana* Hook.f. & Thomson ex Gambleby SRB assay

The total ethanol extract showed strong cytotoxic activity against breast cancer cell line (IC_{50}=10 μg/mL) when compared to cisplastin as reference drug (Table 1), which was not sensitive to this cancer cell line. As the preliminary cytotoxic screening indicates that the total ethanol extract inhibited breast cancer cell line, potential cytotoxicity of the successive extracts of *Ehretia wallichiana* leaves was carried out against breast cancer cell line by the same SRB assay (Table 2). The four successive extracts (petroleum ether, chloroform, ethyl acetate and ethanol) showed nearly the same cytotoxic activity against breast cancer cell line (surviving fraction 0.630, 0.634, 0.602 and 0.670/10μg mL^{-1}), respectively. The strong *in vitro* cytotoxic activity of petroleum ether and chloroform extracts on breast carcinoma cell line may be attributed to its hydrocarbon, steroidal and terpenoidal contents. The significant cytotoxic activity of polar extracts (ethyl acetate and ethanol) could possibly attribute to their flavonoidal and phenolic contents.

CONCLUSION

Cytotoxic activity of *Ehretia wallichiana* was investigated by SRB assay against 6 cell lines. Total ethanol extract showed strong cytotoxic activity against breast cancer cell line followed by ethyl acetate extract. Phytochemical investigation of ethanolic extract resulted in isolation of four phenolic compounds which may be responsible for this cytotoxic activity. *Ehretia wallichiana* could be considered a good candidate for protection of breast cancer.

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