# Anticancer and Antioxidant Tannins from *Pimenta dioica* Leaves

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Two galloylglucosides, 6-hydroxy-eugenol 4-*O*-(6′-*O*-galloyl)-*β*-D-$^4C_1$-glucopyranoside (**4**) and 3-(4-hydroxy-3-methoxyphenyl)-propane-1,2-diol-2-*O*-(2′,6′-di-*O*-galloyl)-*β*-D-$^4C_1$-glucopyranoside (**7**), and two *C*-glycosidic tannins, vascalaginone (**10**) and grandininol (**14**), together with fourteen known metabolites, gallic acid (**1**), methyl gallate (**2**), nilocitin (**3**), 1-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-(*α*/*β*)-D-glucopyranose (**5**), 4,6-(*S*)-hexahydroxydiphenoyl-(*α*/*β*)-D-glucopyranose (**6**), 3,4,6-valoneoyl-(*α*/*β*)-D-glucopyranose (**8**), pedunculagin (**9**), casuariin (**11**), castalagin (**12**), vascalagin (**13**), casuarinin (**15**), grandinin (**16**), methylflavogallonate (**17**) and ellagic acid (**18**), were identified from the leaves of *Pimenta dioica* (Merr.) L. (Myrtaceae) on the basis of their chemical and physicochemical analysis (UV, HRESI-MS, 1D and 2D NMR). It was found that **9** is the most cytotoxic compound against solid tumour cancer cells, the most potent scavenger against the artificial radical DPPH and physiological radicals including $ROO^{\bullet}$, $OH^{\bullet}$, and $O_2^{-\bullet}$, and strongly inhibited the NO generation and induced the proliferation of T-lymphocytes and macrophages. On the other hand, **3** was the strongest NO inhibitor and **16** the highest stimulator for the proliferation of T-lymphocytes, while **10** was the most active inducer of macrophage proliferation.

*Key words: Pimenta dioica*, Galloylglucosides, Antioxidant and Anticancer

## Introduction

*Pimenta dioica* (Merr.) L., syn. *Pimenta officinalis* (Berg) L. (Myrtaceae) is widely distributed in West Indies, Mexico, and South America (Riffle, 1998) and traditionally known as allspice, pimenta, pimento, clove pepper and Jamaica pepper. The plant has been cultivated in Egypt, where it is known as "fulful afrangi". It is traditionally used as a spice and condiment, while being industrially used for tanning purposes and as flavouring and perfuming agent in soaps, tonics, as well as for appetizing medicines. Different plant parts have been used to relieve dental and muscle aches, as well as against rheumatic pains, colds, menstrual cramps, indigestion, flatulence, diabetes, viral infections, sinusitis, bronchitis, depression, nervous exhaustion, hysterical paroxysms, arthritis and fatigue (Christman, 2004; Nakatani, 1994). In Caribbean folk medicine, decoctions of *P. racemosa* leaves are used for their anti-inflammatory and analgesic properties (Fernandez *et al.*, 2001a; Garcia *et al.*, 2004). *P. officinalis* was reported to exhibit an antihyperlipidemic effect and to retrieve the inhibited glutathione, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) towards the normal levels (Shyamala *et al.*, 2005). The extract of its berries showed strong antioxidant activity (Kikuzaki *et al.*, 1999, 2000; Miyajima *et al.*, 2004; Ramos *et al.*, 2003), modulated the induced tetrabutyl hydroperoxidemutagenicity in *Escherichia coli* (Ramos *et al.*, 2003), and exhibited a cytotoxic effect (Logarto Parra *et al.*, 2001), while the extract of the whole plant exhibited analgesic, hypothermis, hypotensive and vasorelaxing effects (Suarez Urhan *et al.*, 1997a, b, 2000).

The high content as well as wide structural and biological diversity of the active constituents, *e.g.* phenolic acids, flavonoids, catechins, galloylglucosides (Kikuzaki *et al.*, 2000), phenylpropanoids

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(Kikuzaki *et al*., 1999), diterpenes (Fernandez *et al*., 2001a) and lupeol (Fernandez *et al*., 2001b), reported in different *Pimenta* species stimulated us to isolate and characterize polyphenols from *P. dioica* leaves and evaluate the antioxidant and anticancer activities of the major compounds against different tumour cell lines.

## Materials and Methods

### *Equipment*

The NMR spectra were recorded at 300, 500 ($^1$H) and 75, 125 ($^{13}$C) MHz on a Varian Mercury 300 and JEOL GX-500 NMR spectrometer and $\delta$ values are reported in ppm relative to TMS in the convenient solvent. HRESI-MS analyses were run on a LTQ-FT-MS spectrometer (Thermo Electron, Germany). UV analyses of pure samples were recorded, separately, in MeOH solutions and with different diagnostic UV shift reagents on a Shimadzu UV 240 spectrophotometer. Optical rotation values were measured on an ATAGO POLAX-D, No. 936216 (AEAGO Co., LTD., Japan) polarimeter with a 1 dm cell (ATAGO 901048). For column chromatography (CC), Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck, Germany) and polyamide S (Flucka) were used. For paper chromatography Whatman No. 1 sheets (Whatman Ltd., England) were used, while silica $F_{254}$ and cellulose plates (Merck) were used for TLC.

### *Plant material*

Leaves of *P. dioica* (Merr.) L. were collected in April 2003 from Zohria Botanical Garden, Cairo, Egypt. The identification of the plant was performed by Dr. Amal Abdel-Aziz, Lecturer of Taxonomy, Institute of Horticulture, Zohria Botanical Garden. A voucher sample (No.: P-1) is kept in the Herbarium, Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Egypt.

### *Extraction and isolation*

Powdered air-dried leaves (3 kg) of *P. dioica* were exhaustively extracted with 80% MeOH ($5 \times 6$ L) under reflux (70 °C). After removal of methanol, the concentrated aqueous solution was desalted by precipitation with excess EtOH to give a dry residue (305 g). Defatting this residue by refluxing with petroleum ether (60–80 °C) yielded a dry extract (270 g) that was suspended in $H_2O$ and fractionated on a polyamide column ($12 \times 115$ cm, 400 g) eluted with $H_2O$ followed by a gradient of $H_2O$/MeOH mixtures up to pure MeOH. On the basis of TLC and PC with the use of UV light, 1% $FeCl_3$, 10% $H_2SO_4$ or Naturstoff spray reagents for detection, the individual 52 fractions (each 1 L) were pooled in 13 collective fractions (I–XIII). Fraction I ($H_2O$/10% MeOH, 14.5 g) was phenolic-free, while in fraction II (20%, 320 mg) gallic acid (**1**) and methyl gallate (**2**) were identified by PC in comparison with authentic samples. Fraction III (30%, 2.5 g) was fractionated on a cellulose column (20–50% EtOH) to give three subfractions, each being applied on a Sephadex LH-20 column eluted with *n*-BuOH/2-propanol/$H_2O$ (BIW, 4:1:5 v/v/v, organic layer) to afford pure **3** (67 mg), **4** (23 mg) and **5** (18 mg), respectively. Repeated fractionation of fraction IV (30–40%, 565 mg) on Sephadex using 20% EtOH, BIW, then MeOH, respectively, yielded pure samples of **6** (34 mg), crude **7** (precipitated with $Et_2O$ from its concentrated acetone solution, 41 mg) and **8** (14 mg). A crude saponin was spontaneously precipitated on concentrating the acetone solution of fraction V (40%, 3.4 g), while compounds **9** and **10** (42 and 18 mg) were obtained through fractionation of the mother liquor on Sephadex LH-20 with BIW as eluent. Fraction VI (40–60%, 8.2 g) was consecutively chromatographed on columns of cellulose (30–65% EtOH), Sephadex (BIW followed by EtOH) to give crude samples of **11**, **12** and **13** which were individually precipitated in a pure form from this concentrated EtOH solution (yield: 32, 148, 228 mg, respectively). Sephadex LH-20 CC of fraction VII (60%, 5.3 g) in three stages – elution in the 1$^{st}$ stage with EtOH, followed by EtOH with gradient additions of $H_2O$/$Me_2CO$, 1:1, the 2$^{nd}$ with BIW, and the 3$^{rd}$ with EtOH – afforded crude samples of **14** and **15**. Both compounds were purified by precipitation from MeOH solutions with excess EtOAc, giving **14** (37 mg) and **15** (44 mg). A pure sample of the major tannin **16** (280 mg) was precipitated from the concentrated MeOH solution of fraction VIII (60%, 3.3 g) with excess $Et_2O$ for twice. A pure sample of **17** (52 mg) was spontaneously precipitated in EtOH solution of fraction IX (70%, 6.4 g), and then washed with cold $Et_2O$. A part of fraction XI (95%, 3.1 g) was separated on PPC with $S_1$ followed by purification of each component on Sephadex with EtOH as an eluent to give pure **18** (11 mg) and an unknown phenolic acid. The crude

saponin from fraction V, mother liquor of IX and fractions X–XIII were saved for further investigations. All separation processes were followed up by Co-TLC with solvent systems: MeOH/$CHCl_3$ (2:8), EtOAc/$CHCl_3$ (7:3), MeOH/EtOAc/$CHCl_3$/$H_2O$ (35:32:28:7) and *n*-BuOH/MeOH/$H_2O$ (4:1:1) or by 2D-PC and Comp-PC with $S_1$ [*n*-BuOH/HOAc/$H_2O$ (4:1:5, top layer)] and $S_2$ (15% aqueous HOAc) solvents.

*Cell culture*

The human hepatoalluear and breast carcinoma cells Hep-G2 and MCF-7, human HCT-116 colon cancer cells and 1301 lymphoblastic leukemia cells (generous gifts from the German Cancer Research Center, Heidelberg, Germany and Training Centre of DakoCytomation, Elly, UK) and Raw murine macrophage RAW 264.7 cells (ATCC, VA, USA) were used in testing the anticancer activity. The cells were routinely cultured at 37 °C in humidified air containing 5% $CO_2$ in DMEM (Dulbeco's Modified Eagle's Medium), except for HCT-116 and RAW 264.7 cells, which were grown in McCoy's medium and RPMI-1640, respectively. Media were supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 U/mL penicillin G sodium, 100 U/mL streptomycin sulphate, and 250 ng/mL amphotericin B. The extract and the tested compounds were dissolved in DMSO (99.9%, HPLC grade) and diluted 1000-fold in the assays. In all the cellular experiments, results were compared with DMSO-treated cells. Monolayer cells were harvested by trypsin/EDTA treatment, except for RAW 264.7 cells, which were collected by gentle scraping. All experiments were repeated four times, unless mentioned and the data were represented as mean ± S.D. Unless mentioned, all culture material was obtained from Cambrex BioScience (Copenhagen, Denmark) and all chemicals were from Sigma (USA).

*Cytotoxicity assay*

Antiproliferative activity against various tumour cell lines was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay, based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells (Hansen *et al*., 1989). The relative cell viability was expressed as the mean percentage of viable cells relative to the respective DMSO-treated cells (control).

*DPPH assay*

The antioxidant capacity of the compounds was studied through their scavenging activity against DPPH radicals (Gerhäuser *et al.*, 2003). The percentage of DPPH bleaching utilized for $SC_{50}$ (half maximal scavenging concentration) was calculated with reference to the DPPH absorbance (0%) and the absorbance in the presence of ascorbic acid (100%).

*Oxygen radical absorbance capacity (ORAC)*

The peroxyl and hydroxyl radical absorbance capacity of the compounds was tested in a modified ORAC assay adapted to a 96-well plate format (Gamal-Eldeen *et al*., 2004). $\beta$-Phycoerythrin ($\beta$-PE) was used as a radical-sensitive fluorescent indicator, 2,2-azobis-(2-amidinopropane) dihydrochloride as a peroxyl radical initiator, and a mixture of $H_2O_2$/$CuSO_4$ as $OH^{\bullet}$ generator. Final concentration of the extract (1 $\mu$g/mL) and compounds (1 $\mu$M) was used. The decay of $\beta$-PE fluorescence was measured kinetically using a microplate fluorescence reader (FluoStarOptima, BMG). One ORAC unit equals the net protection of $\alpha$-PE produced by 1 $\mu$M Trolox.

*Scavenging of superoxide anion radicals assay*

Scavenging of superoxide anion radicals ($O_2^{\bullet-}$), generated by the oxidation of 50 $\mu$M hypoxanthine to uric acid by 12 mU xanthine oxidase, was quantified by the reduction rate of nitro-blue tetrazolium to dark-blue formazan measured at 550 nm (Gerhäuser *et al.*, 2002). The $SC_{50}$ was calculated from serial dilutions of gums and superoxide dismutase was used as a standard $O_2^{\bullet-}$ inhibitor.

*Nitrite assay*

The accumulation of nitrite is an indicator of NO synthesis, which was measured in the culture medium by the Griess reaction (Gerhäuser *et al*., 2002). RAW 264.7 cells were grown in phenol red-free RPMI medium containing 10% FBS. Cells were incubated for 2 h with bacterial lipopolysaccharide (LPS, 1 $\mu$g/mL) before treated with the extract (10 $\mu$g/mL), compounds (10 $\mu$M), or DMSO. A standard curve was blotted using serial concentrations of sodium nitrite. Data was normalized to the cellular protein content measured by the bicinchoninic acid (BCA) assay (Smith *et al*., 1985).

*6-Hydroxy-eugenol 4-O-(6′-O-galloyl)-β-D-$^4C_1$-glucopyranoside (**4**):* White amorphous powder. – $R_f$-values: 0.51 ($S_2$), 0.68 ($S_1$). – Under short UV-light, it appeared as dark purple spot, turned to deep blue colour with $FeCl_3$ spray reagent on PC. – UV (MeOH): $\lambda_{max}$ = 225, 275 nm. – $^1$H NMR (500 MHz, DMSO-$d_6$): $\delta_H$ = 6.94 (2H, s, H-2″/6″), 6.43 (1H, s, H-2), 6.41 (1H, s, H-5), 5.75 (1H, m, H-8), 4.88 (1H, d, $J$ = 7.2 Hz, H-1′), 4.85 (2H, s, H-7), 4.63 (1H, br s, H-9$_a$), 4.42 (1H, br d, $J$ = 11.5 Hz, H-6′$_a$), 4.26 (1H, dd, $J$ = 12 and 6 Hz, H-6′$_b$ overlapped with H-9$_b$), 3.67 (3H, s, OC<u>H</u>$_3$), 3.60–3.10 (4H, remaining sugar protons). – $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta_C$ = 166.20 (C-7″), 148.31 (C-4), 145.70 (C-3″/5″ and C-3), 138.80 (C-4″), 138.40 (C-6), 135.00 (C-8), 125.40 (C-1), 119.80 (C-1″), 115.50 (C-2), 109.50 (C-9), 108.90 (C-2″/6″), 108.20 (C-5), 102.80 (C-1′), 76.00 (C-3′), 74.50 (C-5′), 73.50 (C-2′), 70.20 (C-4′), 63.60 (C-6′), 56.21 (C-O<u>Me</u>), 38.20 (C-7). – Negative HRESI-MS: $m/z$ = 987.28033 [2M-H]$^-$, 493.13515 (calcd. for $C_{23}H_{25}O_{12}$ [M-H]$^-$ 493.13528), 341.11131 [M-H-gallyl]$^-$, 331.32980 [M-H-hydroxyeugenol]$^-$ = [monogalloylglucose-H]$^-$, 313.10094 [monogalloylglucose-H-$H_2O$]$^-$, 179.04651 [M-galloylglucose]$^-$ = [hydroxyeugenol-H]$^-$, 168.96399 [gallate]$^-$, 125.04117 [gallate-H-$CO_2$]$^-$.

*3-(4-Hydroxy-3-methoxyphenyl)-propane-1,2-diol-2-O-(2′,6′-di-O-galloyl)-β-D-$^4C_1$-glucopyranoside (**7**):* Brown amorphous powder. – $R_f$-values: 0.71 ($S_2$), 0.58 ($S_1$). – Under short UV-light, it appeared as dark purple spot, turned to deep blue colour with $FeCl_3$ spray reagent on PC. – UV (MeOH): $\lambda_{max}$ = 222, 274 nm. – $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta_H$ = 6.97, 7.00 (each 2H, s, H-2″/6″ and H-2‴/6‴), 6.99 (1H, br d, $J$ = 7.8 Hz, H-6 hidden by the two previous singlets), 6.86 (1H, d, $J$ = 8.4 Hz, H-5), 6.75 (1H, br s, H-2), 4.73 (1H, dd, $J$ = 9 and 8 Hz, H-2′), 4.59 (1H, d, $J$ = 7 Hz, H-1′), 4.43 (1H, br d, $J$ = 10.2 Hz, H-6′$_a$), 4.32 (1H, dd, $J$ = 12 and 5.1 Hz, H-6′$_b$), 3.75 (1H, m, H-8), 3.62 (3H, s, O-C<u>H</u>$_3$), 3.60–3.10 (5H, remaining sugar protons and C<u>H</u>$_2$-9), 2.69 (1H, dd, $J$ = 14.1 and 6 Hz, H-7$_a$), 2.57 (1H, dd, $J$ = 14.1 and 5.1 Hz, H-7$_b$). – $^{13}$C NMR (75 MHz, DMSO-$d_6$): $\delta_C$ = 165.90, 165.06 (C-7″/7‴), 147.03 (C-4), 145.59, 145.46 (C-3″/5″, 3‴/5‴), 144.51 (C-3), 138.58, 138.47 (C-4″/4‴), 128.71 (C-1), 121.75 (C-6), 119.91, 119.47 (C-1″/1‴), 115.02 (C-2), 113.68 (C-5), 108.87, 108.72 (C-2″/6″, 2‴/6‴), 100.45 (C-1′), 80.26 (C-8), 74.26 (C-2′/5′), 73.99 (C-3′), 70.16 (C-4′), 62.83 (C-6′/9), 55.39 (C-O<u>Me</u>), 37.80 (C-7). – Negative HRESI-MS: $m/z$ = 1327.09787 [2M-H]$^-$, 663.15686 (calcd. for $C_{30}H_{31}O_{17}$ [M-H]$^-$ 663.15667), 511.32980 [M-H-gallyl]$^-$, 331.32980 [M-H-gallyl-aglycone]$^-$, 313.11800 [M-H-gallyl-aglycone-$H_2O$]$^-$, 169.02848 [gallate]$^-$.

*Vascalaginone (**10**):* White amorphous powder. – $R_f$-values: 0.41 ($S_2$), 0.76 ($S_1$). – Under short UV-light, it appeared as dark purple spot and gave a deep blue colour with $FeCl_3$ and indigo-red with $HNO_2$ reagent. – UV (MeOH): $\lambda_{max}$ = a strong hump between 240 and 400 nm with 285sh. – $[\alpha]^D$ = –23.7° ($c$ = 1.4, MeOH). – $^1$H NMR (500 MHz, DMSO-$d_6$): furan: $\delta_H$ = 8.24 (1H, d, $J$ = 1.8 Hz, H-5), 7.80 (1H, d, $J$ = 3.9 Hz, H-3), 6.84 (1H, dd, $J$ = 3.9 and 1.8 Hz, H-4); phenoyl moieties: $\delta_H$ = 6.65, 6.49 (1H each, s, 2 X H-6), 6.31 (1H, s, H-6); glucose: $\delta_H$ = 5.37 (1H, dd, $J$ = 6.9 and 1.5 Hz, H-5), 5.28 (1H, t-like, $J$ = 1.5 Hz, H-2), 5.06 (1H, t-like, $J$ = 6.9 Hz, H-4), 5.02 (1H, d, $J$ = 1.5 Hz, H-1), 4.77 (1H, dd, $J$ = 12.5 and 6 Hz, H-6$_a$), 4.72 (1H, dd, $J$ = 6.9 and 1.5 Hz, H-3), 3.88 (1H, br d, $J$ = 12 Hz, H-6$_b$). – $^{13}$C NMR (125 MHz, DMSO-$d_6$): $\delta_C$ = 184.94 (C=O); furan: $\delta_C$ = 151.23 (C-2), 148.55 (C-5), 121.02 (C-3), 112.23 (C-4); phenoyl moieties: 168.52 (C-7⁗), 166.29 (C-7‴), 166.22 (C-7⁗), 165.63 (C-7″), 163.20 (C-7′), 146.95 (C-5‴), 144.50 (C-3⁗), 144.15 (C-3⁗/5⁗), 144.08 (C-5⁗/5″), 143.71 (C-5′/3′), 142.00 (C-3‴/3″), 137.11 (C-4‴), 136.40 (C-4⁗), 135.60 (C-4⁗), 135.24 (C-4″), 133.21 (C-4′), 125.80 (C-1⁗), 125.53 (C-1″), 123.60 (C-1⁗), 123.52 (C-1‴), 121.12 (C-1′), 116.53 (C-2′), 114.95 (C-2′’/2‴), 114.84, 114.37 (C-2⁗/2⁗), 113.25 (C-6′), 113.11 (C-6″), 107.91 (C-6‴), 107.12 (C-6⁗), 105.11 (C-6⁗); glucose: $\delta_C$ = 73.15 (C-2), 72.62 (C-5), 70.02 (C-3), 68.77 (C-4), 64.50 (C-6), 47.90 (C-1). – Negative HRESI-MS: $m/z$ = 1011.07397 (calcd. for $C_{46}H_{27}O_{27}$ [M-H]$^-$ 1011.07531), 966.93302 [M-H-$CO_2$]$^-$, 948.98453 [M-H-$CO_2$-$H_2O$]$^-$, 709.04623 [M-H-deoxyHHDP]$^-$, 665.06397 [M-H-$CO_2$-deoxyHHDP]$^-$, 505.18254 [M-2H]$^{2-}$, 300.99947 [ellagic acid-H]$^-$.

*Grandininol (**14**):* White amorphous powder. – $R_f$-values: 0.35 ($S_2$), 0.56 ($S_1$). – Under short UV-light, it appeared as dark purple spot and gave deep blue colour with $FeCl_3$ and indigo-red with $HNO_2$ reagent. – UV (MeOH): $\lambda_{max}$ = a strong hump between 240 and 400 nm with 280sh. $[\alpha]^D$ = –63.1° ($c$ = 1.4, MeOH). – $^1$H NMR (500 MHz, DMSO-$d_6$): phenoyl moieties: $\delta_H$ = 7.22, 6.72, 6.67, 6.65 (1H in total, each s, H-6‴), 6.52, 6.50, 6.37, 6.35, 6.30 (2H in total, each s, H-6⁗/

6‴″); sugar: $\delta_H$ = 5.20–5.57 (2H in total, m, H-2/5), 4.91 (1H, t-like, $J$ = 7 Hz, H-4), 4.84 (2H in total, m, H-2/3$_{pentose}$), 4.75 (1H, dd, $J$ = 12.5 and 1.5 Hz, H-6$_a$), 4.32 (1H, br d, $J$ = 6.9 Hz, H-3), 4.02–3.20 (4H, m, H-6$_b$, 4$_{pentose}$, 2 X H-5$_{pentose}$). – $^{13}$C NMR (125 MHz, DMSO-d$_6$): phenoyl: $\delta_C$ = 168.7, 168.4 (C-7‴″), 166.6, 166.3 (C-7‴), 166.0 (C-7″″), 165.5 (C-7″), 164.0, 163.8 (C-7′), 147.2, 147.0 (C-5‴), 145.0, 144.9 (C-3‴″/3″″), 144.8, 144.7 (C-5‴″/5″″), 144.5, 144.3, 143.9 (C-5″/5′), 143.5, 143.4, 142.8, 142.5, 142.3 (C-3′/3′′/3‴), 137.5, 137.0 (C-4‴), 136.3, 136.0, 135.9 (C-4‴″/4″″), 135.5, 134.3 (C-4″), 133.9, 133.7 (C-4′), 126.0, 125.9 (C-1″), 124.5, 124.3, 124.1 (C-1‴″/1″″), 123.5, 123.3 (C-1‴), 123.0, 122.9 (C-1′), 116.2, 115.2, 115.1, 114.4, 114.0 (C-2′/2′′/2‴/2‴″/2″″), 114.6 (C-6′), 112.0 (C-6″), 108.9, 108.8 (C-6‴), 107.0, 106.8 (C-6‴″), 105.8, 105.6 (C-6‴″); sugar: $\delta_C$ = 101.9, 100.0, 97.5 (C-1$_{pentose}$), 92.3 (C-1), 81.5, 78.1, 73.0, 68.9 (C-4$_{pentose}$), 74.0, 73.9 (C-2$_{pentose}$), 73.5 (C-3$_{pentose}$), 70.1, 70.0 (C-2), 69.9 (C-5), 68.7 (C-4), 68.4 (C-3), 66.9, 66.0 (C-5$_{pentose}$), 64.3, 64.0, 63.5 (C-6). – Negative HRESI-MS: $m/z$ = 1081.10113 (calcd. for $C_{46}H_{33}O_{31}$ [M-H]$^-$ 1081.10171), 540.14084 [M-2H]$^{2-}$, 933.06859 [M-H-dehydropentoside]$^-$, 779.03138 [M-deoxyHHDP-H]$^-$, 631.08195 [M-H-dehydropentoside-deoxyHHDP]$^-$, 300.99968 [ellagic acid-H]$^-$

## Results

### *General*

A total of 18 polyphenols was isolated from the desalted and defatted 80% methanol extract of *P. dioica* leaves through consecutive column chromatographic separations. On the basis of chemical and physicochemical analyses as well as comparison with published data (Kikuzaki *et al*., 2000; Moharram *et al*., 2003; Hervé du Penhoat *et al*., 1991; Nonaka *et al*., 1989), their structures were identified as four phenolic acid derivatives (**1**, **2**, **17**, **18**), two galloylglucosides (**4**, **7**), five hydrolysable tannins (**3**, **5**, **6**, **8**, **9**) and seven *C*-glycosidic tannins (**10**–**16**) (Fig. 1).

### *Antioxidant activity*

The extract of the leaves proved to be a strong scavenger of DPPH radicals as indicated by the low $SC_{50}$ value (Table I). Moreover, 1 $\mu$g/mL of the extract possessed multiple-folded antioxidant capacity, against the physiological radicals ROO$^\bullet$ and OH$^\bullet$ higher than that of 1 $\mu$M of Trolox, a known antioxidant (Table I). However, the extract exhibited a moderate scavenging activity against $O_2^{-\bullet}$. All the pure examined isolates were extremely efficient in bleaching DPPH radicals as obviously concluded from their low $SC_{50}$ values compared to ascorbic acid ($SC_{50}$ 6.8 $\mu$M). On the other hand, compounds **3**, **8**, **9** and **12** exhibited the lowest effective $SC_{50}$ values. The ORAC assay results indicated that the most effective antioxidant compounds are against ROO$^\bullet$ **9** > **16** > **3** > **12** > **6** and against OH$^\bullet$ **9** > **5** > **16** > **8** > **12** > **3** that exhibited more than double-fold of the antioxidant capacity of 1 $\mu$M Trolox against those radicals. The tested compounds revealed a moderate antioxidant activity against $O_2^{-\bullet}$, except for **9**, **12** and **16**, which were potent scavengers of $O_2^{-\bullet}$ with a low effective $SC_{50}$ value.

Measuring the nitrite levels (as NO index) in macrophage culture supernatants (Fig. 2) indicated that the extract inhibited the generated NO by 87% ($P < 0.001$), while inhibition by the examined isolates ranged from 80 to 95% ($P < 0.001$), the less effective compounds being **6**, **9** and **16** exhibiting an inhibition of 65, 72 and 68%, ($P < 0.01$), respectively. In addition to the normalization of the nitrite concentration to the cellular protein content, the effect on the macrophage pro-

| Sample | DPPH $SC_{50}$ [$\mu$M][a] | $ORAC_{ROO^\bullet}$ [units][b] | $ORAC_{OH^\bullet}$ [units][b] | X/XO$_{(O_2^{-\bullet})}$ $SC_{50}$ [$\mu$M][b] |
|---|---|---|---|---|
| Extract[c] | 1.42 ± 0.24 | 3.83 ± 0.35 | 4.26 ± 0.44 | 38.16 ± 4.41 |
| **3** | 0.25 ± 0.03 | 2.98 ± 0.21 | 2.62 ± 0.19 | 42.21 ± 3.80 |
| **5** | 1.12 ± 0.11 | 1.52 ± 0.05 | 3.91 ± 0.03 | 24.85 ± 2.16 |
| **6** | 1.33 ± 1.60 | 2.56 ± 0.06 | 1.94 ± 0.02 | 36.61 ± 4.23 |
| **8** | 0.12 ± 0.08 | 1.95 ± 0.22 | 3.63 ± 0.29 | 22.73 ± 3.77 |
| **9** | 0.11 ± 0.04 | 4.11 ± 0.42 | 4.38 ± 0.31 | 3.28 ± 0.84 |
| **10** | 1.20 ± 0.03 | 1.59 ± 0.038 | 1.63 ± 0.66 | 19.40 ± 2.91 |
| **12** | 0.32 ± 0.06 | 2.93 ± 0.36 | 3.19 ± 0.23 | 2.72 ± 0.45 |
| **16** | 1.94 ± 0.13 | 3.27 ± 0.35 | 3.71 ± 0.42 | 7.14 ± 1.17 |

Table I. Radical scavenging activity and antioxidant capacity of the extract of *P. dioica* leaves and selected polyphenolic constituents.

[a] $SC_{50}$, halfmaximal scavenging concentration.
[b] 1.0 ORAC unit equals the net protection of $\alpha$-PE produced by 1.0 $\mu$M Trolox.
[c] $SC_{50}$ and ORAC units are expressed as $\mu$g/mL for the extract.

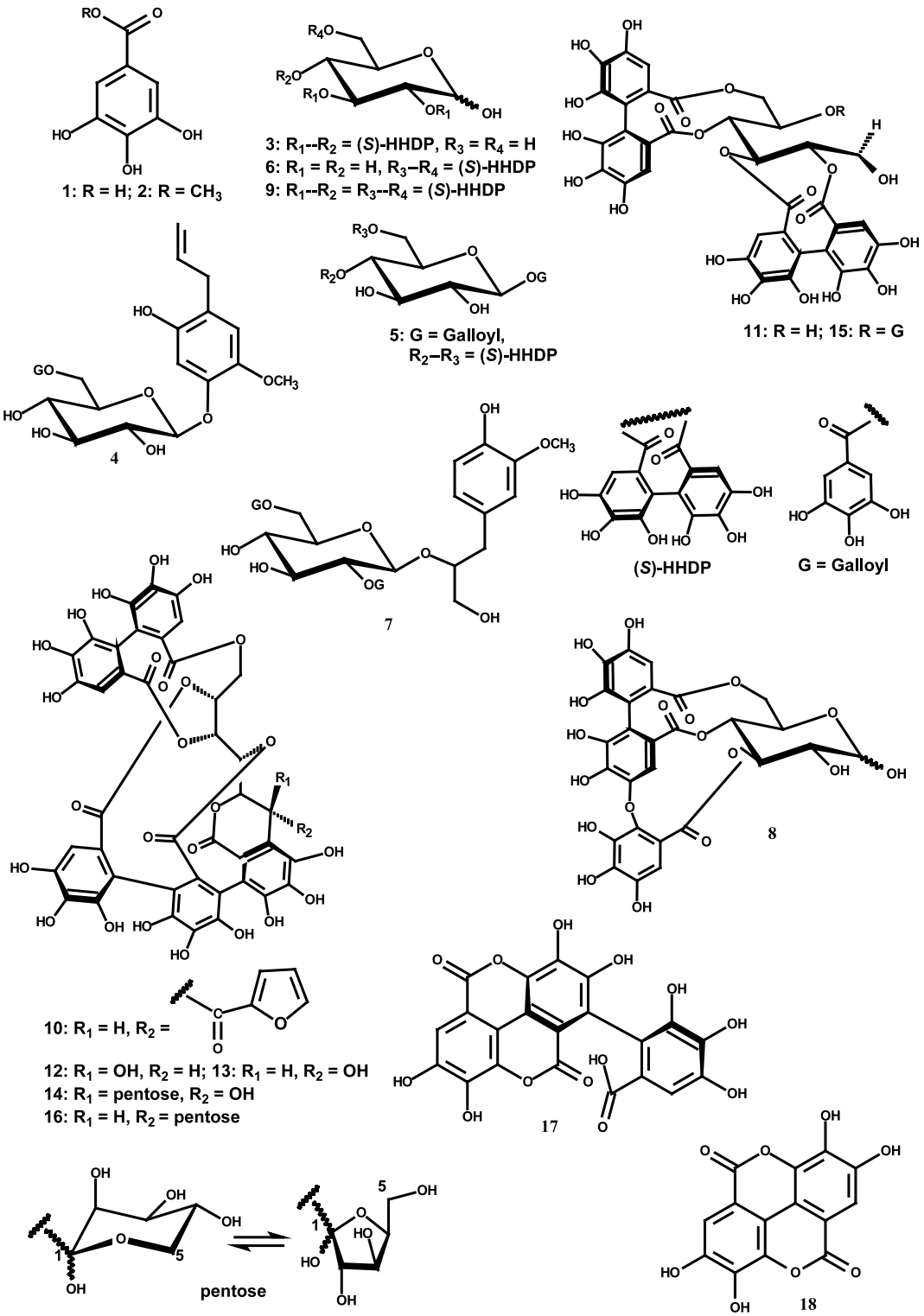


Fig. 1. Chemical structures of the isolates **1**–**18** from *P. dioica* leaves.

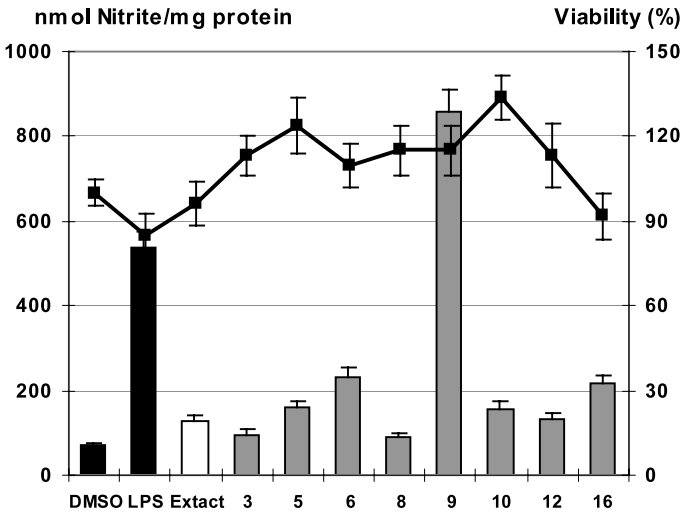


Fig. 2. Effect of the treatment with *P. dioica* leaves extract (10 $\mu$g/mL), isolated compounds (10 $\mu$M) and DMSO on nitrite accumulation as an index for NO generation from LPS-stimulated RAW 264.7 investigated by the Griess assay in comparison with the nitrite level of control cells (untreated with LPS). Black bars represent control (DMSO-treated cells) and positive control (DMSO + LPS-treated cells). The squared line represents the macrophage viability percentage at the examined fixed concentration.

liferation was explored. Using the MTT assay after 24 h of treatment, RAW 264.7 cell growth was stimulated by **3**, **5**, **6**, **8**, **9**, **10** and **12** (Fig. 2) up to 113, 123, 109, 115, 115, 133.8 and 113% of the control cells, respectively.

*Antiproliferative activity*

Although treatment of different human cancer cell lines with the extract depressed remarkably the cell growth of Hep-G2 and HCT-116 cells (Table II), it exhibited a lower cytotoxic effect against MCF-7 cells. However, the extract showed a growth stimulating effect on 1301 cells of 48.81 $\mu$g/mL twice that of the control growth. Treatment with **5**, **6**, **8**, **12** and **16** resulted in a strong cytotoxic effect on both Hep-G2 and HCT-116 cells. In contrast, they exhibited a proliferative activity of the immune cells as indicated by the growth rate of 1301 and RAW 264.7 cells. In addition, it was found that **9** was the strongest cytotoxic compound against different solid tumour cell lines and the strongest inducer of RAW 264.7 cell proliferation almost 133.8% of the control. Moreover, treatment with 28.87 $\mu$M of **9** resulted in a double-fold induction of the proliferation of 1301 cells, while **16** was the most powerful compound in the induction of 1301 cells proliferation at low concentration of 10.39 $\mu$M.

## Discussion

Compounds **4** and **7** showed the chromatographic behaviour and UV absorption (~275 nm)

| Sample | Hep-G2 cells $IC_{50}$ [$\mu$M][a] | MCF-7 cells $IC_{50}$ [$\mu$M][a] | HCT-116 cells $IC_{50}$ [$\mu$M][a] | 1301 cells [$\mu$M][b] |
|---|---|---|---|---|
| Extract[c] | 22.82 ± 3.31 | 40.81 ± 2.7 | 19.42 ± 2.62 | 48.81 ± 2.9 |
| **3** | 29.85 ± 3.08 | 71.49 ± 6.3 | 34.61 ± 4.61 | 34.31 ± 2.8 |
| **5** | 11.42 ± 0.89 | 53.71 ± 4.2 | 13.31 ± 2.14 | 36.91 ± 3.1 |
| **6** | 16.24 ± 1.99 | 41.76 ± 2.8 | 18.35 ± 2.87 | 29.70 ± 2.2 |
| **8** | 18.22 ± 1.88 | 46.76 ± 5.1 | 12.74 ± 2.06 | 67.29 ± 5.4 |
| **9** | 6.42 ± 0.98 | 18.39 ± 1.9 | 4.37 ± 0.84 | 28.87 ± 2.2 |
| **10** | 24.62 ± 3.21 | 82.15 ± 6.2 | 19.23 ± 3.11 | 36.87 ± 3.8 |
| **12** | 9.82 ± 1.40 | 26.16 ± 2.2 | 7.35 ± 0.94 | 41.70 ± 3.1 |
| **16** | 18.41 ± 1.91 | 22.06 ± 1.9 | 13.76 ± 1.83 | 10.39 ± 1.2 |

Table II. Effect of the extract of *P. dioica* leaves and selected polyphenolic constituents on the proliferation of different cancer cell lines.

[a] $IC_{50}$, half maximal growth inhibitory concentration.
[b] The concentration needed to double-fold growth induction of 1301 cells.
[c] $IC_{50}$ and double-fold growth induction are expressed as $\mu$g/mL for the extract.

intrinsic of galloyl esters, which gave gallic acid and glucose (CoPC with authentic samples) on acid hydrolysis. Negative HRESI-MS of **4** exhibited a dimeric adduct ion (987.28033 [2M-H]$^-$) and a molecular ion (493.13515 [M-H]$^-$, calcd. 493.13528), corresponding to $C_{23}H_{26}O_{12}$ with 16 mu (OH) more than that of eugenol 4-*O*-(6′-*O*-galloyl)-glucoside reported from the same plant (Kikuzaki *et al*., 2000; Tanaka *et al*., 1993). The other assigned six fragments at 341.11131, 331.32980, 313.10094, 179.04651, 168.96399 and 125.04117 were in complete accordance with the proposed structure of **4** as hydroxyleugenol galloylglucoside (see MS data, for description). The presence of a galloyl moiety was indicated by a singlet at $\delta$ 6.94 (2H) and five intrinsic $^{13}$C signals at 166.20, 145.70, 138.80, 119.80 and 108.90 in the $^1$H and $^{13}$C NMR spectra (Kikuzaki *et al*., 2000). The galloylation of OH-6′-glucose was evidenced from the deshielding of $CH_2$-6′ diastereomeric protons at 4.42 (br d, $J$ = 11.5 Hz, H-6′$_a$) and 4.26 (dd, $J$ = 12 and 6 Hz, H-6′$_b$) and C-6′ at 63.60 ($\Delta$ + ~ 2.5 ppm). The glucoside moiety was assigned as $\beta$-$^4$C$_1$-pyranose due to the anomeric doublet at 4.88 (7.2 Hz) and six $^{13}$C signals (C-1′–C-6′, see $^{13}$C data), assigned for a $\beta$-$^4$C$_1$-glucopyranoside. The relatively deshielded H-1′ (4.74 or 4.93) led us to deduce the glucosidation of a phenolic OH group (Tanaka *et al*., 1993; Oya *et al*., 1997), not an aliphatic one (4.39 or 4.56) (Suarez Urhan *et al*., 1997a). Depending on the two singlets at 6.43 (H-2) and 6.41 (H-5) in the aromatic region instead of the eugenol three resonances (H-6, 5, 2), a hydroxy group at C-6 was deduced that was further evidenced from the downfield shift of C-6 at 138.40 and upfield of both C-5 and C-1 at 108.20 and 125.40, in comparison with those of the eugenol analogue (Tanaka *et al*., 1993; Oya *et al*., 1997). All other $^1$H and $^{13}$C resonances of 6-hydroxy-eugenol were assigned by the comparison with the corresponding published data for structure-related compounds (Kikuzaki *et al*., 2000; Tanaka *et al*., 1993; Oya *et al*., 1997). Thus, **4** was identified as 6-hydroxy-eugenol 4-*O*-(6′-*O*-galloyl)-$\beta$-D-$^4$C$_1$-glucopyranoside (Fig. 1).

The negative HRESI-MS of **7** showed a dimeric adduct ion at *m/z* 1327.09787 [2M-H]$^-$ and a molecular one at 663.15686 [M-H]$^-$ (calcd. 663.15667) for $C_{30}H_{32}O_{17}$ with 152 mu (one a galloyl group) more than the analogue 3-(4-hydroxy-3-methoxyphenyl)-propane-1,2-diol-2-*O*-(6′-*O*-galloyl)-$\beta$-D-glucoside (Kikuzaki *et al*., 2000) reported before from the same plant. The other recorded fragments at 511.32980, 331.32980, 313.11800 and 169.02848 (see MS data for description) were further evidences for the second galloyl and 3-(4-hydroxy-3-methoxyphenyl)-propane-1,2-diol aglycone that had previously been isolated from allspice (Kikuzaki *et al*., 1999). $^1$H and $^{13}$C data of **7** were more or less the same as those of its 6′-galloyl analogue (Kikuzaki *et al*., 2000) except for the duplication of the signals of the galloyl group, the strong downfield shift of H-2′ to 4.73 (dd, $J$ = 9 and 8 Hz, $\Delta$ + ~ 1 ppm) and the slight downfield shift of H-1′ to 4.59 (d, $J$ = 7 Hz, $\Delta$ + ~ 0.2), proving the position of the second galloyl group on OH-2′. Further documents for a 2′,6′-diester have been deduced from downfield shifts of C-6′ and C-2′ and upfield shifts of C-5′, C-3′ and C-1′ with respect to the free or monoacylated glucoside (Kikuzaki *et al*., 2000; Tanaka *et al*., 1993; Oya *et al*., 1997) to interpret **7** as 3-(4-hydroxy-3-methoxyphenyl)-propane-1,2-diol-2-*O*-(2′,6′-di-*O*-galloyl)-$\beta$-D-$^4$C$_1$-glucopyranoside (Fig. 1).

The tannins **10** and **14** showed more or less the same chromatographic properties ($R_f$, UV and indigo-red with $HNO_2$-response on PC) and UV spectral data of a *C*-glycosidic ellagitannin (**10**, **12**, **13**, **16**). Unlike the previous two galloyl esters **4** and **7**, **10** gave one mol equivalent of ellagic acid on treatment with methanolic hydrochloric acid, which indicates the presence of one HHDP as in case of **12**, **13**, **16** (Moharram *et al*., 2003; Hervé du Penhoat *et al*., 1991; Nonaka *et al*., 1989). The negative HRESI-MS showed a molecular ion at 1011.07397 [M-H]$^-$ (calcd. 1011.07531 for $C_{46}H_{27}O_{27}$), which is higher than that of **12** or **13** (933.06395, $C_{41}H_{25}O_{26}$) by the same increment (78 mu, $C_5H_2O$, furan) and lower than **16** (1065.10626, $C_{46}H_{33}O_{30}$) by 54 mu, corresponding to the loss of 3 $H_2O$ to reflect the fact that **10** is most propably a furfural derivative of **16** (Moharram *et al*., 2003; Hervé du Penhoat *et al*., 1991; Nonaka *et al*., 1989). This was further supported by the assignment of its double charged molecular ion at 505.18254 [M-2H]$^{2-}$, together with the other five diagnostic fragments at 966.93302, 948.98453, 709.04623, 665.06397 and 300.99947 (see MS data for description). The glucosyl moiety was established as an open *C*-glucose, neither $^4$C$_1$ nor $^1$C$_4$ conformers (Nonaka *et al*., 1989), depending on the splitting pattern of its $^1$H signals ($\delta$ and $J_{vic}$-values, see $^1$H NMR data), particularly that of $J_{12}$ (1.5 Hz), the same configuration in **13** and **16** and differs from

that of the epimer **12** ($J_{12} \geq 4$ Hz) (Moharram *et al*., 2003; Hervé du Penhoat *et al*., 1991; Nonaka *et al*., 1989). By comparison of $^{1}$H NMR spectral data of **10** with those of **12** and **13**, an extra AMX-spin coupling system of three types, 1H each, was assigned at 8.24 (d, $J$ = 1.8 Hz, H-5), 7.80 (d, $J$ = 3.9 Hz, H-3) and 6.84 (dd, $J$ = 3.9 and 1.8 Hz, H-4) for a 2-monosubstituted furan ring together with the normal three singlets, 1H each, at 6.65, 6.49 (4,6-*O*-*S*-hexahydroxydiphenoyl, HHDP) and 6.31 (1,2,3,5-*O*-flavogallonoyl, FL), in the aromatic region. The typical four $^{13}$C resonances of a furan ring were assigned at 151.23 (C-2), 148.55 (C-5), 121.02 (C-3) and 112.23 (C-4), which were in complete accordance with those analogues to furfural. In addition, an adjacent carbonyl carbon atom to the furan ring was deduced from its signal at 184.94 ppm. The complete assignment of $^{13}$C resonances of the HHDP and FL groups and their substitution positions on the glucose moiety were confirmed by the comparison of its NMR data with those of **12**, **13** and **16** and other structural related compounds (Moharram *et al*., 2003; Hervé du Penhoat *et al*., 1991; Nonaka *et al*., 1989). Therefore, **10** was identified herein as a degradative product of **16**, for the first time from nature, and named as vascalaginone (Fig. 1).

The HRESI-MS spectrum of **14** showed a molecular ion at 1081.10113 $[M\text{-}H]^-$ (calcd. 1081.10171 for $C_{46}H_{33}O_{31}$), with 16 mu (OH) more than that of **16**, 148 mu (dehydropentose) more than that of **12** or **13**, and a double charged ion at 540.14084 along with four diagnostic fragments at 933.06859, 779.03138, 631.08195 and 300.99968 for a hydroxy-grandinin identity. Accordingly, the identity of **14** was expected to be a lyxosyl, unlike in grandinin molecule, attached to C-1 of the open chain glucose core through an oxidation coupling to form a C-C-linkage. Like grandinin, it gave extremely complicated $^{1}$H and $^{13}$C NMR spectra (dublication, triplication) owing to the expected pyranose-furanose and anomeric equilibria of sugars (Moharram *et al*., 2003; Hervé du Penhoat *et al*., 1991; Nonaka *et al*., 1989). According to a comparison study of the NMR data of **14** with that of **16** and other related ellagitannins in the previous literature (Moharram *et al*., 2003; Hervé du Penhoat *et al*., 1991; Nonaka *et al*., 1989), its $^{1}$H and $^{13}$C resonances were completely assigned as mentioned above (see Materials and Methods). Taking into account the increment of the MWt by 16 mu than that of **16**, the similarity of $^{1}$H and $^{13}$C data ($\delta$ and $J$-values) of **16** and **14** except for the absence of the H-1 signal and the downfield shift of C-1 at 92.3 in case of **14**, its stereostructure was unequivocally established as an oxidative coupling product of a lyxose-type pentose with vascalagin (**13**) and named as grandininol (Fig. 1).

The extract of *P. dioica* leaves and the major pure tannins demonstrated an overall potent radical scavenging activity with variable action against different physiological and non-physiological radicals (Tables I and II, Fig. 2). The extract was an inactive cytotoxic agent against different human cancer cell lines, but it showed a lymphoproliferative effect towards 1301 cells (T-lymphocytes). To our knowledge, this is the first report investigating the cytotoxic effect of a *P. dioica* extract against five different solid and hematopoietic tumour cell lines.

In previous reports, **9** showed a protective effect towards footshock-induced chronic stress in rat brain, where it normalized the induced SOD, CAT, and GPX activities (Bhattacharya *et al*., 2000), and **9** and **12** were reported as strong lipid peroxidation inhibitors (Khennouf *et al*., 2003), which may be explained and supported by our results. In our findings, the strong antioxidant activity of such tannins, in pure or extract form, could be explained mainly due to the presence of a large number of hydroxyl groups in a huge extended $\pi$-electron conjugation system in galloyl, HHDP, valoneoyl (VL) or FL (**3**, **5**, **6**, **8**, **12**, **16**) groups that is the responsible factor for the stabilization of phenoxide radicals and hence increases its scavenging affinity in the oxidation reaction.

Particularly, **9** was reported as a mild inhibitor of the survival of human lung cancer cells (PC14) and gastric cancer cells (MKN45) (Lee and Yang, 1994). In our findings, **9** showed strong cytotoxic effects against human hepatocellular carcinoma cells (Hep-G2), human colon cancer cells (HCT-116), mild cytotoxic activity against human breast cancer cells (MCF-7) and pronounced immunoproliferative effects towards T-lymphocytes and macrophages. Tannins **9**, **12** and **16** were found to possess selective cytotoxic effects against PRMI-7951 melanoma cells but week cytotoxicity ($> 10\,\mu$g/mL) against lung carcinoma (A-549), ileocecal adenocarcinoma HCT-8), epidermoid carcinoma of nasopharnyx (KB) and medulloblastoma (TE-671) tumour cells (Kashiwada *et al*., 1992). The similar pattern of antioxidant, cytotoxic effect and immunoproliferative activity of our iso-

lates was explained due to the presence of the same phenoyl esters in their structures. It is also found that the glucose unit with HHDP (**5**, **6**, **9**, **12** and **16**) and VL (**8**) groups showed cooperatively inhibitory effects on poly(ADP-ribose) glycohydrolase purified from human placenta (Aoki *et al*., 1993). These tannins were found to act as potent inhibitors of induced histamine release from rat peritoneal mast cells (Kanoh *et al*., 2000) and as stimulators of the iodination of human peripheral blood monocytes (Sakagami *et al*., 1992).

Taken together, among all tested tannins, **9** was the most cytotoxic compound against solid tumour cancer cells, the most potent scavenger against the artificial radical DPPH and the physiological radicals $ROO^{\bullet}$, $OH^{\bullet}$ and $O_2^{-\bullet}$, and it inhibited strongly the NO generation and induced the proliferation of T-lymphocytes and macrophages. Moreover, **3** was the strongest NO inhibitor, while **16** was the best stimulator for the proliferation of T-lymphocytes and **10**, that contains a furan ring as an intrinsic structural difference among all tested tannins, was the most active inducer of macrophage proliferation.

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