Phytoconstituents of *Jatropha curcas* L. Leaves and their Immunomodulatory Activity on Humoral and Cell-Mediated Immune Response in Chicks

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A novel biflavone di-*C*-glucoside, 6,6″-di-*C*-*β*-D-glucopyranoside-methylene-(8,8″)-biapigenin (**1**), was isolated from the leaves of *Jatropha curcas* L. (Euphorbiaceae), together with six known compounds; apigenin 7-*O*-*β*-D-neohesperidoside (**2**), apigenin 7-*O*-*β*-D-galactoside (**3**), orientin (**4**), vitexin (**5**), vicenin II (**6**), and apigenin (**7**). Their structures were determined on the basis of extensive chemical and spectroscopic analyses (UV, NMR and HRESI-MS). The immunomodulatory effect of an 80% aqueous methanol extract (AME) and compounds **1**–**5** (0.25 mg/kg body wt) to one-day-old specific pathogen-free (SPF) chicks was determined. Stimulation of both humoral and cell-mediated seroresponse was observed, especially those of AME and compound **1**. Remarkable effective increases of the antibody titers, lymphocyte and macrophage cells, in blood were recorded. SPF chicks treated with the tested samples exhibited protection against Newcastle disease challenge virus after being vaccinated.

Key words: Jatropha curcas, Biflavone Di-*C*-glucoside, Immune Response

Introduction

The genus *Jatropha* is one of 300 genera belonging to the family Euphorbiaceae (Bailey, 1953). It comprises about 175 species, being distributed in tropical and subtropical regions but also in North America and Southern Africa. Several biological effects were reported for the plant like wound-healing (Shetty *et al.*, 2006), anti-inflammatory (Mujumdar and Misar, 2004), antimalaria (Ankrah *et al.*, 2003), antiparasitic (Fagbenro-Beyioku *et al.*, 1998), antimicrobial (Matsuse *et al.*, 1998), molluscicidal (Liu *et al.*, 1997), and antifertility activity (Makonnen *et al.*, 1997). The majority of research programs are directed towards the investigation of constitutive diterpenes and lignans in *Jatropha curcas* due to their antitumour activity (Lin *et al.*, 2003; Naengchomnong *et al.*, 1994; Haas *et al.*, 2002; Muangman *et al.*, 2005). The constitutive polyphenols, particularly flavonoids, were studied in several *Jatropha* species (Masaoud *et al.*, 1995; Okuyama *et al.*, 1996; Aiyelaagbe *et al.*, 1998), but nothing was reported about the constitutive polyphenols of *J. curcas*. The present communication describes the isolation and structural elucidation of a new biflavone di-*C*-glucoside, **1**. Also, the immunomodulatory role of an 80% aqueous methanol extract (AME) and compounds **1**–**5** on humoral and cell-mediated immune response was evaluated in specific pathogen-free (SPF) chickens vaccinated against Newcastle disease virus.

Material and Methods

General

The NMR spectra were recorded at 300 and 500 (^{1}H) and 75 and 125 (^{13}C) MHz on Varian Mercury 300 and JEOL GX-500 NMR spectrometers; δ values are reported as ppm relative to TMS in the convenient solvent. LC/ESI-MS and HRESI-MS analyses were recorded on LCQ (Finnigan MAT, Bremen, Germany) and LTQ-FT-MS mass spectrometers (Thermo Electron 400, Waltham, MA, USA), respectively. UV analyses of pure samples were recorded, separately, as MeOH so-

lutions and with different diagnostic UV shift reagents on a Shimadzu UV 240 (P/N 240–58000) instrument. For column chromatography, Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose (Merck, Darmstadt, Germany), and polyamide S (Fluka, Steinheim, Switzerland) were used. For paper chromatography, Whatman No. 1 paper sheets (Whatman Ltd., Maidstone, England) were used. The pure compounds were visualized by spraying with Naturstoff reagent [(a) 1% diphenyl boryloxyethanolamine in ethanol, (b) 5% polyethylene glycol 400 in methanol], heating the dry chromatogram at 120 °C for 10 min, and visualizing under UV light (365 nm) or with $FeCl_3$ (1% in ethanol). Solvent systems S_1 [*n*-BuOH/HOAc/H_2O (4:1:5, v/v/v, top layer)], S_2 (15% aqueous HOAc), and S_3 [*n*-BuOH/*iso*-propanol/H_2O (4:1:5, v/v/v, top layer)] were used.

Plant material

The leaves of *J. curcas* L. were collected from plants growing in Benha region, Egypt, during July 2003. The identification of the plants was performed by Dr. Wafaa M. Amer, Professor of Botany, Botany Department, Faculty of Science, Cairo University Cairo, Egypt. A voucher specimen (No. J.2) is deposited in the herbarium of Pharmacognosy Department, Faculty of Pharmacy, Helwan University, Helwan, Egypt.

Specific pathogen-free (SPF) chicks

Fertile SPF eggs were taken from Nile SPF eggs, Koom Oshiem, Fayoum, Egypt. They were hatched in Central Laboratory for Evaluation of Veterinary Biologics (CLVB), Cairo, Egypt from which we took 360 SPF chicks.

Vaccines

Live Newcastle disease (ND) vaccine, La Sota strain with a titer of $10^{6.5}$ EID_{50}/mL (embryo infective dose), was used for vaccination of the experimental birds via drinking water. The vaccine was titered by measuring the hemagglutinating activity (HA) using a microtiter plate test (Anon, 1971).

ND-virulent strain

A very virulent Newcastle disease (VVND) virus strain (10^7 EID_{50}/mL) obtained from the bank of strains of CLVB, Abbassia, Egypt as used as challenging virus.

Extraction and isolation

The air-dried powdered leaves (1.0 kg) were extracted with 80% aqueous methanol (4 L, then 3 × 3 L). The combined methanolic extract was concentrated under reduced pressure and low temperature. The residue (93 g) was defatted with petroleum ether (60–80 °C, 2 × 3 L). The defatted residue obtained (85 g) was preliminary fractionated on a polyamide column (250 g, 110 × 7 cm) using a step gradient of H_2O/MeOH (100:0–0:100, v/v) for elution to give 26 fractions, which were collected, monitored by Comp-PC (solvent systems S_1 and S_2), and divided by UV light into seven major collective fractions (I–VII). A major compound, **2** (145 mg), was obtained from fraction II and purified by Sephadex LH-20 column chromatography eluted with MeOH. Fraction III (150 mg) was subjected to repeated column chromatography on microcrystalline cellulose using *n*-BuOH/*iso*-propanol/H_2O (BIW, 4:1:5, v/v/v, top layer) as an eluent, followed by repeated cellulose column chromatography for each major subfraction using MeOH/BIW (50%) to give pure **3** (25 mg) and **4** (23 mg). Fraction IV (60 mg) was chromatographed on a Sephadex column and eluted with a gradient of MeOH/H_2O (100:0–0:100, v/v) to give pure **5** (28 mg). Fraction V (40 mg) was subjected to Sephadex column chromatography (10% aqueous MeOH as an eluent) which led to pure **6** (12 mg). Fraction VI (25 mg) was applied to a Sephadex column with BIW as an eluent to afford pure **1** (11 mg). Fraction VII (20 mg) was separated on a Sephadex column eluted with MeOH to give pure **7** (9 mg). The homogeneity of the fractions was tested by 2D-PC and Comp-PC using Whatman No. 1 paper sheets, and solvent systems S_1 and S_2 for elution.

6,6″-Di-C-β-D-$^4C_1$-glucopyranoside-methylene-(8,8″)-biapigenin (**1**): Yellow amorphous powder ($1.1 \cdot 10^{-4}$ μg/mg dry plant wt); R_f 0.32 (S_1), 0.37 (S_2) on PC; deep green colour and greenish yellow fluorescence with $FeCl_3$ and Naturstoff spray reagents, respectively. – UV (MeOH): λ_{max} = 277, 332 nm; (+NaOMe) 280, 320(sh), 396 nm; (+NaOAc) 278, 310(sh), 350 nm; (+NaOAc/H_3BO_3) 279, 310(sh), 350 nm; (+$AlCl_3$) 282, 306(sh), 335, 390 nm; (+$AlCl_3$/HCl) 282, 305(sh), 332, 388(sh) nm. – ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 7.59

(d, J = 8.4 Hz, H-2′/6′, H-2‴/6‴), 6.58 (d, J = 8.4 Hz, H-3′/5′, 3‴/5‴), 6.54 (s, H-3/3″), 4.69 (d, J = 9.2 Hz, 2 × H-$1_{Glucose}$), 4.28 (t-like, J = 9.3 Hz, 2 × H-$2_{Glucose}$), 3.7–3.1 (m, remaining sugar protons and CH_2-bridge), OH-5 (H-bound). – ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 181.25 (C-4), 163.85 (C-2), 162.71 (C-7), 160.20 (C-5), 159.33 (C-4′), 154.28 (C-9), 127.71 (C-2′, C-6′), 122.10 (C-1′), 115.63 (C-3′, C-5′), 109.61 (C-6), 105.00 (C-8), 103.60 (C-10), 100.85 (C-3), 81.36 (C-5), 79.48 (C-3), 73.91 (C-1), 71.01 (C-2), 70.07 (C-4), 61.75 (C-5); the resonance of the CH_2-bridge was expected to be masked by the DMSO-d_6 signal.

Immunomodulatory activity

A pilot experiment was carried out to choose the suitable dose which neither caused degeneration nor necrosis in livers and kidneys. 0.25, 0.5, and 1 mg/kg body wt of AME and compounds **1**–**5** were given orally to chicks. It was found that the selected dose for studying the immunomodulatory activity should be 0.25 mg/kg body wt.

360 one-day-old SPF chicks were reared and kept in isolators under complete hygienic measure and divided into 8 groups of 45 chicks each. The first non-treated, non-vaccinated group was considered as control. The second group was vaccinated at the 7th day with living attenuated ND vaccine. The remaining six groups received 0.25 mg/kg body wt of AME and compounds **1**–**5** orally daily from the 2nd to the 6th day of life. Then they were vaccinated at the 7th day with living attenuated ND vaccines *via* drinking water. Twenty random blood samples were collected from each group 3, 7, 10, 14, 21, 28, and 35 days post vaccination (DPV).

Humoral immune response

Ten of the previously mentioned 20 random blood samples were used for estimation of the humoral immune response after serum separation using the hemagglutination inhibition (HI) test (Majiyabe and Hitchner, 1977).

Cell-mediated immune response

The other 10 samples were used for evaluation of the cell-mediated immune response *via* the following tests:

Assay of lymphocyte blastogenesis

The lymphocyte blastogenesis assay (Charles *et al.*, 1978) was carried out and evaluated using the MTT test (Mosmann, 1983). The results were expressed as changes in the optical density recorded at λ_{max} = 490 nm by the aid of an automatic Titertek multiscan reader model ELX 800 UV (New York, USA) for reading ELISA plated.

Macrophage activity test

Macrophage activity was proceeded (El-Enbawy, 1990) and the phagocytic index (Richardson and Smith, 1981) was determined according to the following formula: phagocytic percentage = total no. of phagocytes which ingest more than 2 *Candida*/total no. of phagocytes which ingest *Candida*.

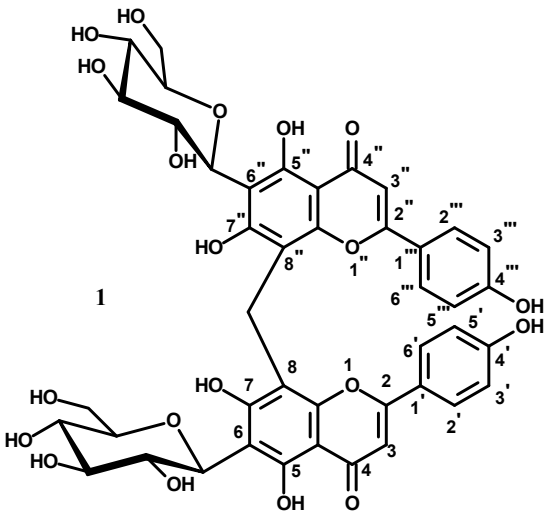
Newcastle disease virus (NDV) challenge test

The humoral and cellular immune response were confirmed by the NDV challenge test and proceeded by choosing 15 chickens randomly from each group at 3, 14, 35 DPV and subjecting to the challenge test with 0.5 mL of VVNDV strain (10^7 EID_{50}/mL). The chickens were observed for 15 DPV. Chickens dying within this period were collected and subjected to detailed *post mortem* examination.

Results and Discussion

Seven flavonoids were isolated from the AME. All isolates were identified by comparative PC with authentic samples, and their ^{1}H and ^{13}C NMR resonances were assigned by comparison with those of the corresponding published data of structurally related compounds (Mabry *et al.*, 1970; Agrawal and Bansal, 1989).

Compound **1** showed chromatographic behaviour, acid cleavage products and UV spectral data of an apigenin *C*-glycoside-like structure with free OH-7, OH-5 and OH-4′ (Mabry *et al.*, 1970). The negative HRESI-MS/MS spectrum showed a molecular ion peak at *m/z* 875.22043 $[M-H]^-$ (ca. 875.22920) corresponding to the molecular weight 876.23143 and molecular formula $C_{43}H_{40}O_{20}$ of a biapigenin di-*C*-hexoside with additional 14.0149 mu, the exact mass of an extra CH_2 group. The ^{1}H NMR spectrum showed, in the aromatic region, only an A_2X_2 spin coupling system of the two *ortho* doublets each of 4H at 7.59 (H-2′/6′, 2‴/6‴) and 6.58 (H-3′/5′, 3‴/5‴) together with a



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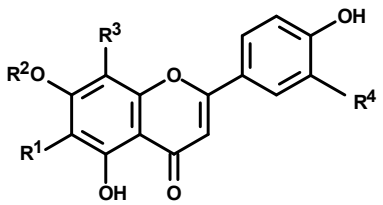

2: $R^1 = R^3 = R^4 = H$, R^2 = β-D-neohesperidoside
3: $R^1 = R^3 = R^4 = H$, R^2 = β-D-galactopyranoside
4: $R^1 = R^2 = H$, R^3 = β-D-glucopyranoside, R^3 = OH
5: $R^1 = R^2 = R^4 = H$, R^3 = β-D-glucopyranoside
6: $R^1 = R^3$ = β-D-glucopyranoside, $R^2 = R^4 = H$
7: $R^1 = R^2 = R^3 = R^4 = H$

Fig. 1. Chemical structures of compounds **1**–**7** isolated from *J. curcas* leaves.

Table I. The average log 2 of hemagglutination inhibition (HI) titer to Newcastle disease virus (NDV) in chickens.

Group	HI			
	7 d	14 d	21 d	35 d
Control	0.0	0.0	0.0	0.0
Vaccinated control	$2^{3.0}$	$2^{6.0}$	$2^{9.1}$	$2^{4.0}$
AME[a]	$2^{3.8}$	$2^{6.6}$	$2^{9.7}$	$2^{4.6}$
1	$2^{3.6}$	$2^{6.4}$	$2^{9.5}$	$2^{4.4}$
2	$2^{3.4}$	$2^{6.2}$	$2^{9.3}$	$2^{4.2}$
3	$2^{3.2}$	$2^{6.3}$	$2^{9.2}$	$2^{4.1}$
4	$2^{3.1}$	$2^{6.1}$	$2^{9.1}$	$2^{4.3}$
5	$2^{3.0}$	$2^{6.1}$	$2^{9.2}$	$2^{4.0}$

[a] AME, 80% aqueous methanol extract.

Table II. Evaluation of cell-mediated immune response for chickens by lymphocyte transformation suppressed by optical density (λ_{max} = 490 nm).

Group	7 d		14 d		21 d		35 d	
	PHA[a]	NDV[b]	PHA	NDV	PHA	NDV	PHA	NDV
Control	0.012	0.015	0.010	0.013	0.012	0.014	0.013	0.014
Vaccinated control	0.098	0.118	0.129	0.163	0.111	0.145	0.053	0.077
AME	0.106	0.124	0.137	0.169	0.118	0.149	0.068	0.088
1	0.101	0.122	0.136	0.168	0.116	0.147	0.065	0.086
2	0.099	0.121	0.133	0.166	0.114	0.145	0.063	0.081
3	0.098	0.119	0.132	0.164	0.114	0.146	0.064	0.071
4	0.102	0.120	0.130	0.164	0.112	0.145	0.061	0.079
5	0.103	0.103	0.131	0.163	0.112	0.145	0.057	0.078

[a] PHA, phytohemagglutination.
[b] NDV, Newcastle disease virus.

singlet at 6.54 for H-3/3′′. These evidences proved a symmetric structure of a biapigenin aglycone expected to be connected through a methylene bridge. The δ and J values of the two equivalent anomeric proton doublets ($2 \times$ H-$1_{Glucose}$) at 4.69 (J = 9.2 Hz) and the triplet-like ($2 \times$ H-$2_{Glucose}$) at 4.28 (J = 9.3 Hz) were diagnostic for the di-*C*-glycoside on the two aglycone moieties at similar carbon atoms. The positions of the connection between the two aglycone monomers and glycosidation of the two *C*-glucosides were determined to be C_8-CH_2-C_8 and 6-*C*-glycoside, based on the absence of the two H-8 and the two H-6 of aglycones, in a symmetric manner. The ^{13}C NMR spectrum showed 19 signals; 13 were assigned to the aglycone and 6 to the sugar moiety in a total symmetric structure of two equivalent apigenin and glycoside moieties. On the other hand, the downfield shift ($\Delta \sim 10$ ppm) of C-8 and C-6 to 105.00 and 109.61, respectively, reflected the di-6-*C*-glycoside and the C_8-CH_2-C_8 linkage between the two apigenin moieties. The characteristic upfield shift ($\cong$ 0.4 ppm) of the two A_2X_2 doublets was explained, due to the anisotropic effect of stereo phenyl-phenyl interaction, as another confirmative evidence for the symmetric connection of the aglycones. Based on ^{1}H and ^{13}C NMR spectral data, the two sugar moieties matched with a $^4C_1$-pyranose structure. The other ^{13}C NMR resonances were assigned by application of α/β down/up ^{13}C substituent additive rules and comparison with reported data of similar structure (Agrawal and Bansal, 1989). Therefore, **1** was confirmed as 6,6′′-di-*C*-β-D-$^4C_1$-glucopyranoside-methylene-(8,8′′)-biapigenin (Fig. 1).

The biological study was planned to assess the immunomodulatory role of the AME and compounds **1**–**5** in chickens vaccinated against the NDV. There was a remarkable increase of the hemagglutinating antibodies titer in groups treated with the AME and **1**. Separately they were vaccinated with living attenuated NDV vaccine at the 7th day. These groups recorded high antibody titers of $2^{9.7}$ and $2^{9.5}$, respectively, at 21 DPV (peak titer), while groups of compounds **2**–**5** gave a slight increase in the antibody titers ($2^{9.3}$, $2^{9.2}$, $2^{9.1}$, and $2^{9.2}$, respectively) in comparison with the group vaccinated only at the 7th day with living attenuated NDV vaccine which gave 2^6 14 DPV reaching peak ($10^{9.1}$) at 21 DPV (Table I). A progressive increase in the cellular and macrophage activity was produced. The test of lym-

Table III. Evaluation of cell-mediated immune response for chickens by macrophage activity using *Candida albicans*-expressed phagocytic percentage and phagocytic index.

Group	3 d		7 d		10 d		14 d		21 d		28 d		35 d	
	PH-%[a]	PH-I[b]	PH-%	PH-I	PH-%	PH-I	PH-%	PH-%	PH-%	PH-I	PH-%	PH-I	PH-%	PH-I
Control	19.10	0.06	12.90	0.09	13.10	0.08	16.30	0.10	19.40	0.09	15.20	0.09	16.60	0.08
Vaccinated control	46.70	0.31	56.30	0.36	69.90	0.45	72.40	0.46	64.60	0.37	48.30	0.32	43.10	0.22
AME	49.30	0.34	58.40	0.38	74.40	0.48	78.80	0.49	66.80	0.40	52.70	0.36	44.20	0.28
1	49.00	0.33	58.20	0.37	74.20	0.47	77.80	0.48	66.20	0.39	52.10	0.35	44.00	0.27
2	48.60	0.33	57.80	0.36	73.10	0.46	77.20	0.47	65.60	0.38	51.80	0.33	43.80	0.24
3	49.10	0.32	57.40	0.37	70.80	0.46	74.30	0.46	64.80	0.38	49.80	0.32	43.60	0.26
4	47.40	0.31	56.90	0.36	69.60	0.45	73.60	0.47	65.10	0.37	48.60	0.33	43.60	0.23
5	47.20	0.31	56.60	0.36	69.40	0.45	73.20	0.46	64.80	0.37	48.40	0.31	43.30	0.22

[a] PH-%, phagocytic percentage.
[b] PH-I, phagocytic index.

Table IV. Number of dead and protected (protection percentage in parentheses) of chickens 3, 14 and 35 days post vaccination.

Group	Number of dead chickens			Number of protected chickens		
	3 d	14 d	35 d	3 d	14 d	35 d
Control	15	15	15	0 (0)	0 (0)	0 (0)
Vaccinated control	8	2	4	7 (46.6)	13 (90.0)	11 (73.3)
AME	7	1	3	8 (53.3)	14 (93.3)	12 (80.0)
1	9	2	3	6 (40.0)	13 (86.6)	12 (80.0)
2	9	2	3	6 (40.0)	13 (86.6)	12 (80.0)
3	10	2	4	5 (33.3)	13 (86.6)	11 (73.3)
4	10	2	5	5 (33.3)	13 (86.6)	10 (66.6)
5	9	3	5	6 (40.0)	12 (80.0)	10 (66.6)

phocyte transformation expressed as changes in the optical density (Table II) revealed maximum values at the 14th DPV in all groups. However, the magnitude of the value was noticed by applying the NDV as antigen compared with the use of phytohemagglutination (PHA) as a mitogen. The PHA value was 0.129 for the group vaccinated only (not treated) 14 DPV. There was a noticeable increase in the PHA values in vaccinated and treated groups, especially in the group treated with AME (0.137) and compound **1** (0.136) (Table II). On the other hand, more assertion for the former results was obtained after evaluation of the macrophage activity (Table III). The maximum phagocytic activity was 72.4 in the 14 DPV group, while a remarkable increase was in vaccinated and groups treated with AME (78.8), compound **1** (77.8) and compound **2** (77.2). The protection percentage against the VVNDV reflected the immunological status of the experimental birds (Table IV) as a maximum protection (87.0%) for the 14 DPV groups, whereas the percentage for the AME-treated and vaccinated groups at 3, 14, 35 DPV were 53%, 93%, and 80%, respectively.

The obtained results summarized the possible role played by *J. curcas* leaf extract and 6,6″-di-*C*-β-D-$^4C_1$-glucopyranoside-methylene-(8,8″)-biapigenin (**1**) in the immunity (humoral and cellular) against Newcastle disease which is severely endangering the poultry industry by causing economic losses (Aldous and Alexander, 2008).

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