Antioxidant and Immunomodulatory Constituents of Henna Leaves

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The immunomodulatory bioassay-guided fractionation of the methanolic extract of henna (*Lawsonia inermis* L.; syn. *Lawsonia alba* L.) leaves resulted in the isolation of seven compounds; three have been isolated for the first time from the genus, namely *p*-coumaric acid, 2-methoxy-3-methyl-1,4-naphthoquinone and apiin, along with the previously isolated compounds: lawsone, apigenin, luteolin, and cosmosiin. Structural elucidation of the isolated compounds was based upon their physical, chemical as well as spectroscopic characters. Their immuomodulatory profile was studied using an *in vitro* immunoassay, the lymphocyte transformation assay. The ABTS [2,2′-azino-bis (3-ethyl benzthiazoline-6-sulfonic acid)], free radical scavenging assay depicted that all isolated compounds exhibited antioxidant activity comparable to that of ascorbic acid.

Key words: Antioxidant Activity, *Lawsonia inermis* L., Henna

Introduction

For centuries, henna leaves were renowned as the most extensively used natural hair dying and tattooing agent in many civilizations and cultures. The word henna which means "to become queen" is indicative of something highly elegant. The plant has got several vernacular names *e.g. Ligusturum egypticum* which is the Latinized English synonym for henna. It arises from the common name for the same plant in England, namely of the Egyptian privet. The plant extract or its purified compounds exhibit a variety of biological activities such as anticomplementary activity (Handa *et al.*, 1997), dihydroorotate dehydrogenase inhibitory activity (Knecht *et al.*, 2000), macrophage-stimulating activity as a result of stimulation of secretion of GM-CSF (granulocyte macrophage colony stimulating factor) (Wagner *et al.*, 1988), antimicrobial activity (Malekzadeh, 1968; Abd-el-Malek *et al.*, 1973), anti-sickling activity (Chang and Suzuka, 1982; Clarke *et al.*,1986), hepatoprotective activity (Anaad *et al.*, 1992), cytotoxic activity (Ali and Grever, 1998), anti-inflammatory, antipyretic, and analgesic activities (Ali *et al.*, 1995). In the light of the previous activities, we investigated the compounds responsible for the immunomodulatory effects of henna. In addition, an antioxidant-screening assay was conducted to detect whether a redox mechanism may be involved in the immunomodulatory action of henna.

Results and Discussion

Seven compounds were isolated adopting the lymphocyte transformation assay (LTA)-guided fractionation of the total methanolic extract of henna (*Lawsonia inermis* L.) leaves. Immunoactive fractions were subjected to further phytochemical investigation. Seven compounds were isolated (Fig. 1), five from the chloroformic fraction [*p*-coumaric acid (**1**), lawsone (**2**), apigenin (**3**), luteolin (**4**), and 2-methoxy-3-methyl-1,4-naphthoquinone (**5**) as well as two compounds from the ethyl acetate fraction [cosmosiin (**6**) and apiin (**7**)] . The LTA assay was adapted as a test for cell-mediated immunity (Stites, 1987; Nores *et al.*, 1997). The cell-mediated immune response was determined in the peripheral blood lymphocytes (PBL) in response to mitogenic stimulation using either phytohaemagglutinin (PHA) or concanavalin A (Con A) as mitogens that stimulate human T- and B-cells but T-cells more vigorously. The Lymphocyte transformation (mitogenesis) assay studies a specific immune response *i.e.* it studies the mitogenic effect of the crude drug on T-lymphocyte proliferation. Initiation of T-lymphocyte proliferative responses means immunostimulation, whereas suppression of T-lymphocyte proliferative activities is indicative of immunosuppression (Sairam *et al.*, 1997). The results of the LTA expressed in terms of the lymphocyte transformation

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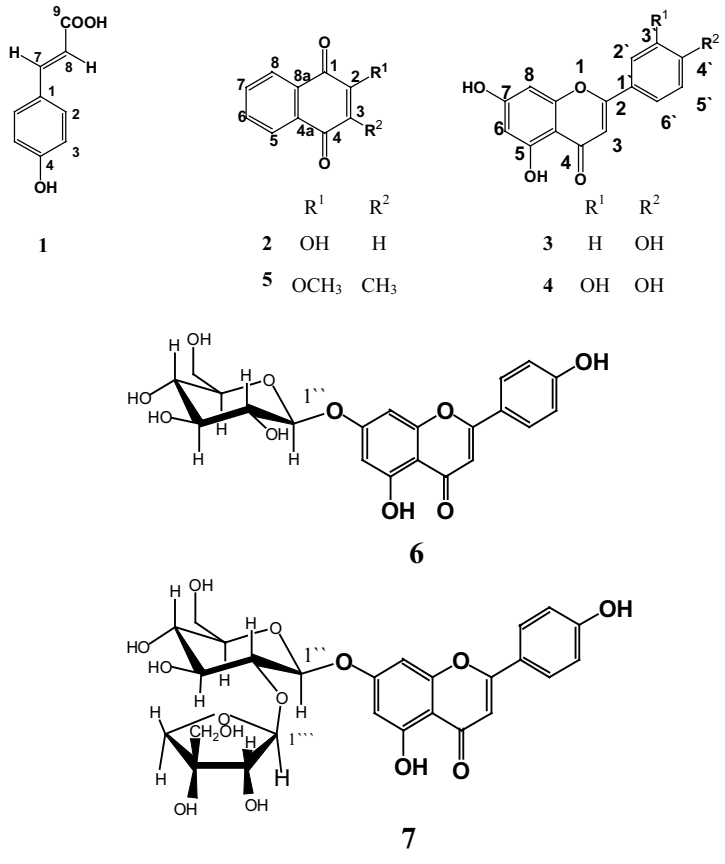

Fig. 1. Compounds isolated through bioassay-guided fractionation of henna (*Lawsonia inermis* L.) leaves; *p*-coumaric acid (**1**), lawsone (**2**), apigenin (**3**), luteolin (**4**), 2-methoxy-3-methyl-1,4-naphthoquinone (**5**), cosmosiin (**6**), apiin (**7**).

Table I. Results of lymphocyte transformation (proliferation) assay of total MeOH extract, different solvent fractions and pure compounds of henna (*Lawsonia inermis* L.) leaves.

Henna total methanolic extract 90% transformation)*, TC_{50}** = 0.55 mg/ml

Chloroform fraction*** (20% transformation), TC_{50} = 2.50 mg/ml				Ethyl acetate fraction (30% transformation), TC_{50} = 1.67 mg/ml			
Compound	*c* [μM]	Transformation (%)	TC_{50} [μM]	Compound	*c* [μM]	Transformation (%)	TC_{50} [μM]
1	6.09	10	30.49				
2	5.74	30	9.58	**6**	2.31	40	2.89
3	3.70	35	5.29				
4	3.50	50	3.50	**7**	1.77	25	3.55
5	4.95	20	12.37				

* Lymphocyte transformation in % at 1 mg/ml concentration is indicated in between brackets.
** Concentration showing 50% lymphocyte transformation.
*** Petroleum ether exhibited no immunomodulatory activity (0% transformation), while *n*-butanol extract elicited mild immunomodulatory activity (25% transformation, TC_{50} = 2.00 mg/ml), but unfortunately we were not able to isolate any compounds from it.

(%) are shown in Table I. The total methanolic extract as well as the different solvent fractions of henna leaves at 1 mg/ml concentration have displayed immunostimulant action as indicated by promotion of T-lymphocyte proliferative responses. It was found that the immunostimulant activity of the total methanolic extract of henna leaves (90% lymphocyte transformation) is greater than that of individual solvent fractions at the same concentration (1 mg/ml), suggesting a synergism between the different components in each fraction. Adopting the bioassay-guided fractionation, the petroleum ether extract of henna leaves was not further investigated chemically due to lack of immunoactivity. Unfortunately, we were not able to isolate any compounds from the *n*-butanol extract, despite the extract exhibited mild immuomodulatory activity (25%). This activity may be due to the presence of mucilage (highly polar polysaccharide) (Youngken, 1950; Wagner, 1986). In order to detect whether a redox mechanism is involved in the immunomodulatory action of henna, we conducted an antioxidant screening assay. The antioxidant activity assay employed here is one of several assays (Masakis, 1995; Yen, 1995; Salah, 1995; and Unno, 1997) that depend on measuring the consumption of stable free radicals derived from the reaction of 2,2′-azino-bis(3-ethyl benzthiazoline-6-sulfonic acid) (ABTS) and manganese dioxide (MnO_2). The decrease in color intensity of the free radical solution due to scavenging of the free radicals by the antioxidant material is followed colorimetrically at a specific wavelength. The assay employs the radical cations as stable free radicals to assess the antioxidant potential of the isolated compounds. The advantage of ABTS-derived free radical scavenging method over other antioxidant screening methods is that, the produced color remains stable for more than 1 h and the reaction is stoichiometric. The results of the assay in terms of inhibition (%) of the test compounds are expressed in Table II. It is clear that a significant antioxidant activity was exhibited by the active constituents of *Lawsonia inermis* (henna) leaves, especially by the flavonoids and naphthoquinones. Nearly all compounds isolated from henna exhibited a free radical scavenging activity comparable with that of ascorbic acid (vitamin C) at 2 mM concentration. Compound **7** (apiin; apigenin-7-apiosyl-glucoside) has shown to be the most potent free radical scavenger. It has showed a greater antioxidant activity than the standard well-known antioxidant ascorbic acid. These results may, in part, explain the mechanism of the immunomodulatory actions of henna, which is based on its antioxidant potential. Thus, the constituents of henna act as free radical scavengers that prevent the phospholipid membrane peroxidation and protect immuno-compromised cells from free radical damage. This also may account for the previously reported hepatoprotective effect of henna against CCl_4-induced hepatotoxicity (Anaad *et al.*, 1992), as well as for the anti-sickling activity through protecting the membrane of normal and sickle erythrocytes by reaction with a transient oxidative species (Clarke *et al.*, 1986).

The ^{13}C NMR spectrum of compound **1** showed the presence of 9 carbon atom signals, their multiplicities were determined using the DEPT 135° experiment that revealed the presence of six methine signals. By subtracting DEPT 135° signals from the original ^{13}C NMR spectrum, three signals remain that can be assigned to three quaternary carbons. The EI/MS molecular ion peak at *m/z* 164 suggested a molecular formula of $C_9H_8O_3$ and thus giving a double bond equivalent (DBE) of six,

Table II. Results of ABTS antioxidant activity screening assay.

Test component (2 mM)	A_{Test}*	Inhibition (%)	DM**	IC_{50}*** [mM]
Blank	0.5	0	–	–
Ascorbic acid	0.3	40	2.1	2.5
Henna total MeOH extract	0.35	30	1.7	–
1	0.31	38	1.1	2.6
2	0.49	2	0.6	50.0
3	0.35	30	3.1	3.3
4	0.4	20	1.8	5.0
5	0.3	40	2.4	2.5
6	0.33	34	2.7	2.9
7	0.19	62	4.1	1.6

* Absorbance of test solution at λ_{max} 734 nm.
** Deviation from the mean.
*** Concentration showing 50% inhibition.

four of which were assigned to a *p*-disubstituted phenyl moiety as reflected by the strong IR band for aromatic C-H out of plane bending at 856 cm^{-1} and confirmed by the appearance of a two double intensity ^{13}C NMR signal for the aromatic methines C-2,6 and C-3,5 at δ 130.7 and 116.3, respectively. This was corroborated by the appearance of two ^{1}H NMR doublets at δ 6.78 (2H, d, J = 8.0 Hz) and 7.52 (2H, d, J = 8.0 Hz) for the *o*-coupled protons H-2,6 and H-3,5, respectively. The oxygenated quaternary carbon signal at δ 160.1 could be assigned to the hydroxylated carbon C-4 that was confirmed by the appearance of a broad IR band at 3392 cm^{-1}. The quaternary carbon signal at δ 125.8 was assigned to C-1. Another DBE was assigned to a C_7-C_8 double bond that is obvious from the appearance of the two methine ^{13}C NMR signals at δ 115.9 and 144.7 assigned to the olefinic carbons C-8 and C-7, respectively. This was assured by the appearance of two olefinic ^{1}H NMR signals at δ 6.29 (1H, d, J = 16.0 Hz) and 7.49 (1H, d, J = 16.0 Hz) for protons H-8 and H-7, respectively. This large coupling constant suggested that the double bond is in *trans* (*E*) configuration. The ultimate DBE could be assigned to the carboxylic carbonyl carbon C-9 appearing at δ 168.5. The EI/MS mass spectrum of compound **1** confirmed the above assignments; showing a molecular ion peak [M^+] and in the same time the base peak at *m/z* 164 corresponding to the molecular formula $C_9H_8O_3$. The peak at *m/z* 146 resulted from the dehydration of the parent ion [$M\text{-}H_2O^+$]. Decarboxylation of the parent ion resulted in the peak at *m/z* 119. Other fragments characteristic of the cinnamoyl moiety were those at *m/z* 91 that correspond to the 7-membered tropylium ion [$C_7H_7^+$]; subsequent elimination of a neutral acetylene molecule resulted in the fragment at *m/z* 65 which corresponds to the 5-membered ion [$C_5H_5^+$] (Silverstein *et al.*, 1991). The above mentioned assignments and comparison with the previously reported literature data (Pouchert and Behnke, 1993) made us deduce that compound **1** is *trans*-4-hydroxy-cinnamic acid, known as *p*-coumaric acid. To the best of our knowledge, this is the first report of an isolation of such an aromatic acid from *Lawsonia inermis*.

The spot of compound **2** on TLC plates acquired an orange color that was intensified by alkalis such as ammonia, NaOH, and Na_2CO_3 and after heating with vanillin sulphuric acid spray reagent using precoated silica gel plates G60 F_{254} suggesting a naphthoquinone structure. It exhibited UV absorption maxima at 269 and 453 nm (MeOH solution) suggesting a highly conjugated system. Its IR spectrum showed the presence of a hydroxyl group (3410 cm^{-1}), two α,β-unsaturated carbonyl bands at 1676 and 1644 cm^{-1} (C=O chelated with an α-hydroxyl), suggesting also a 1,4-naphthoquinone structure (Harborne, 1984). The ^{13}C NMR spectrum of compound **2** showed the presence of ten carbon signals, the multiplicities of which were determined using an APT experiment that revealed the presence of five methine signals and five quaternary carbon signals confirming the naphthoquinone 10 carbons skeleton. Four ^{13}C NMR methine signals were assigned to four aromatic methines C-5, C-8, C-6, and C-7 at δ 126.5, 126.7, 133.1, and 135.3, respectively. The remaining olefinic methine signal at δ 110.7 could be assigned to C-3. The two quaternary carbon signals at δ 129.4 and 132.9 were assigned to C-8a, and C-4a, respectively. An oxygenated quaternary carbon signal at δ 156.3 was assigned to the hydroxylated carbon C-2. The remaining two quaternary carbon signals at δ 181.9 and 184.9 were assigned to two upfield-shifted α,β-unsaturated carbonyls C-1 and C-4, respectively, corroborating the 1,4-naphthoquinone structure. The ^{1}H NMR spectrum was consistent with the above ^{13}C NMR assignments, displaying an olefinic proton signal at δ 6.36 (1H, s) for H-3, a set of four double doublets at 7.7–8.2 ppm that were assigned to four *m*-, *o*-coupled aromatic protons at δ 7.71 (1H, dd, J = 2, 10.2 Hz), 7.73 (1H, dd, J = 2 , 10 Hz), 7.76 (1H, dd, J = 2, 11.2 Hz), and 8.12 (1H, dd, J = 2.6, 9.4 Hz) for protons H-6, H-5, H-7, and H-8, respectively. The above assignments as well as co-chromatography with the authentic sample made us to deduce that compound **2** is 2-hydroxy-1,4-naphthoquinone (2-hydroxy-1,4-naphthalenedione) known as lawsone, isojuglone, or Natural Orange 6.

The close similarity in the UV spectrum between compound **5** and compound **2** (lawsone) indicated that they possess one and the same chromophore (1,4-naphthoquinone moiety). The IR spectrum of compound **5** revealed two strong and sharp absorption bands at 1679 and 1652 cm^{-1} for C=O stretching indicating the presence of two carbonyl groups conjugated with a double bond (α,β-unsaturated carbonyls) and thus endorsing the possibility of having a 1,4-naphthoquinone nucleus. A major difference from compound **2** is the lack of the IR broad absorption band around

3500 cm^{-1} excluding the presence of a free hydroxyl group. This was corroborated through the 4–5 ppm upfield shifting of the ^{13}C NMR signal for C-2. Methoxylation of C-2 was deduced from the appearance of the ^{13}C NMR signal at 54.9 ppm for the methoxyl carbon, as well as the ^{1}H NMR signal for methoxyl protons at 3.75 ppm. Another difference from compound **2** was the disappearance of the olefinic ^{1}H NMR signal for H-3 indicating that it was substituted. This was confirmed by the appearance of a ^{13}C NMR signal at 18.5 ppm for the methyl substituent at C-3 in addition to the ^{1}H NMR signal at 1.98 ppm for methyl protons. The mass spectral data of compound **5** were very useful in elucidating its structure. The EI/MS mass spectrum assured the above assignments showing a molecular ion peak [M^+] at *m/z* 202 indicating the molecular formula $C_{12}H_{10}O_3$. The fragment ion at *m/z* 187.9 indicated demethylation, thus suggesting the presence of a methyl group. The loss of a 31 mass unit fragment, giving the peak at *m/z* 157, indicated losing a methoxyl (-OCH_3) group. Subsequent loss of carbon monoxide moieties (28 mass units) resulted in the peak at *m/z* 129. Another pathway for fragmentation could be proposed starting from the parent ion at *m/z* 202 which loses a CO moiety leading to the fragment at *m/z* 174 followed by demethylation and deprotonation yielding the fragment at *m/z* 158. The fragment at *m/z* 174 can also lose a CO moiety resulting in the peak at *m/z* 146. Subsequent demethoxylation gives rise to the fragment at *m/z* 115. The base peak at *m/z* 105 [$C_8H_9^+$] resulted from the fragmentation of the naphthalene cation.

The above assignments made us to conclude that compound **5** is 2-methoxy-3-methyl-1,4-naphthoquinone. To the best of our knowledge, this compound is reported in henna for the first time.

The TLC spot of compound **3** attained a yellow color if visualized with NaOH or ammonia, and after heating with vanillin sulphuric acid spray reagent using precoated silica gel plates G60 F_{254} indicating its flavonoidal nature. Its UV spectrum in MeOH displayed absorption bands I and II at λ_{max} 338 and 268 nm, respectively, indicating that it has a flavone nucleus. A bathochromic shift in band I of + 54 nm with increasing intensity on addition of sodium methoxide indicated the presence of a free hydroxyl group at C-4′. A bathochromic shift in band I of + 44 nm with aluminium chloride shift reagent, that persisted after addition of HCl, reflected the presence of a free hydroxyl group at C-5, and the absence of 3′,4′-*ortho*-dihydroxy groups in ring B. The bathochromic shift (+ 7 nm) in band II, on addition of sodium acetate, indicated the presence of a free hydroxyl group at C-7. Little (+ 2 nm) or no bathochromic shift in band II with sodium acetate/boric acid indicated the absence of *ortho*-dihydroxy groups at ring A. The above data showed that compound **3** is a flavone with free 5,7,4′ hydroxyls (Mabry *et al.*, 1970). The flavone nucleus was confirmed from the ^{13}C NMR signal at 181.5 ppm that was assigned to C-4 (Agrawal, 1989). This was emphasized by ^{1}H NMR signals at δ 6.76 (1H, s) for H-3. The two ^{13}C NMR signals at δ 93.9 and 98.7 were assigned to C-8 and C-6, respectively, indicating that these positions are unsubstituted. The double intensive ^{13}C NMR signals at δ 115.8 and 128.3 for carbons C-3′, 5′ and C-2′, 6′, respectively, proved that ring B is mono-substituted at position 4′. This was confirmed by the ^{1}H NMR spectrum that displayed two doublets at δ 6.9 (d, J = 8.7 Hz) and 7.9 (d, J = 8.7 Hz), each integrated for 2 protons that are corresponding to the *o*-coupled protons H-3′, H-5′, and H-2′, H-6′, respectively. The oxygenation pattern of ring A was established from the ^{1}H NMR signals at δ 6.18 (1H, d, J = 1.5 Hz) and 6.47 (1H, d, J = 1.8 Hz) for the *m*-coupled protons H-6 and H-8, respectively, indicating that these positions are unsubstituted. Further confirmation of the *m*-oxygenation pattern of ring A came from the ^{13}C NMR signals at δ 160.9 and 163.9 for the two oxygenated carbons C-5 and C-7, respectively. EI/MS fragments corroborated the aforementioned assignments showing a molecular ion peak which is also the base peak [M^+] at *m/z* 270 corresponding to the molecular formula $C_{15}H_{10}O_5$ indicating that compound **3** is apigenin.

The spectral data of compound **4** are more or less similar to that of compound **3** yet having an additional hydroxyl group in ring B at position 3′. This was corroborated by the significant bathochromic shift (+ 62 nm) in band I of the UV spectrum (MeOH) with aluminium chloride as shift reagent that declined upon addition of HCl as well as the bathochromic shift of band I (+ 21 nm) with sodium acetate/boric acid shift reagent indicating the presence of 3′,4′-*ortho*-dihydroxy groups in ring B. Additional confirmation for the 3′-hydroxyl function was provided by the ^{13}C NMR signals at δ 145.6 and 149.9 for the hydroxylated carbons C-3′ and C-4′. This was em-

phasized by the ^{1}H NMR signals at δ 7.43 (1H, d, J = 2.1 Hz), 6.89 (1H, d, J = 8.1 Hz), and 7.41 (1H, dd, J = 2.1, 8.1 Hz) that could be assigned for the protons H-6′, H-5′ and H-2′, respectively. EI/MS fragments of compound **4** corroborated the previous assignments showing a molecular ion peak which is also the base peak [M^+] at *m/z* 286 corresponding to the molecular formula $C_{15}H_{10}O_6$. The above assignments as well as co-chromatography with an authentic sample made us to conclude that compound **4** is luteolin.

Compound **6** is also flavonoid based on a similar basis like for compound **3**. It gave a positive Molisch's test suggesting that it is glycosidic in nature. It displayed UV and NMR spectral patterns resembling that of compound **3** suggesting that it has an apigenin aglycone. Nevertheless, the absence of a significant bathochromic shift of band II in the UV spectrum (MeOH) upon addition of sodium acetate as shift reagent reflected that the hydroxyl group at C-7 may be absent or blocked by glycosidation. Glycosidation of the 7-hydroxyl group was corroborated by the downfield shifts of about 0.5–1 ppm in the *ortho* related C-6 and C-8 signals and 1.5–2 ppm in the *para* related C-10 signal relative to those in the aglycone (compound **3**) *i.e.* apigenin. Strong acid hydrolysis of compound **6** and TLC of the hydrolysate afforded glucose. This was confirmed by the ^{13}C NMR signal at δ 99.9 that was assigned for the anomeric carbon C-1″ of glucose, and the ^{1}H NMR signal at δ 5.05 (1H, d, J = 6.6 H) for the anomeric proton of the β-configured glucopyranose moiety as deduced from the axial-axial coupling constant (Harborne, 1994). The above findings were confirmed through positive-ion FAB/MS-MS (TANDEM MS) that showed a fragment ion peak at *m/z* 433 for [M+H^+] corresponding to the molecular formula $C_{21}H_{21}O_{10}$, and at *m/z* 271 for [apigenin (aglycone)+H^+], as well as through negative-ion FAB/MS-MS (TANDEM MS) that showed fragment ion peaks at *m/z* 431 for [M-H^-] corresponding to the molecular formula $C_{21}H_{19}O_{10}$, and at *m/z* 269 for [apigenin (aglycone)-H^-]. The aforementioned data suggested that compound **6** is apigenin-7-*O*-β-D-glucopyranoside known as cosmosiin which has been previously isolated from the leaf extracts of *Lawsonia inermis* L. by Muhammad *et al.* (1980).

Another apigenin-7-glycoside is compound **7** that was concluded by the same grounds as compound **6**, yet the glycosyl moiety is a little bit different being formed of a disaccharide. This was deduced from the strong acid hydrolysis and TLC of the hydrolysate that afforded glucose and apiose. This was corroborated by the ^{13}C NMR signals at δ 99.3 and 108.6 that were assigned for the anomeric carbons C-1″ of glucose and C-1‴ of apiose and the ^{1}H NMR signals at δ 5.14 (1H, d, J = 6.9 Hz), and 5.45 (1H, s) for the anomeric protons of the β-configured D-glucopyranosyl moiety and the β-configured D-apiofuranosyl moiety, respectively, as deduced from the axial-axial coupling constants (Harborne, 1994). The β-D-apiofuranosyl moiety is attached to C-2″ of glucose as reflected by the sizeable downfield shift of C-2″ of glucose from 73 to 76.8 ppm (Harborne and Mabry, 1982).

Experimental

Materials for chromatographic study

Silica gel G60 F_{254} for TLC (Merck, Germany), silica gel for column chromatography (70–230 mesh) (Merck), precoated silica gel GF_{254} aluminium and plastic plates for TLC (Merck and Machery-Nagel, Germany) were used.

Reagents for lymphocyte transformation assay

Heparinized peripheral venous blood was obtained from healthy volunteers from the blood bank of Mansoura University Hospital; Ficoll/Hypaque was obtained from Amersham Pharmacia, Uppsala, Sweden; phytohaemagglutinin (PHA) was obtained from Difco, Detroit, MI, USA; concanvalin A (ConA) was obtained from Merck, Germany; Hank's balanced salt solution (HBSS), foetal calf serum (FCS), glutamine, HEPES (*N*-2-hydroxyethylpiperazine-*N*′–2-ethanesulfonic acid)-buffer and RPMI-1640 medium were obtained from Gibco BRL, Life Technologies, Pailsey, Scotland; crystalline penicillin G and streptomycin were obtained from El-Nile Pharmaceutical Co., Cairo, Egypt; *Echinacea purpurea* extract (Immulone®) was obtained from Sekem Pharmaceutial Co., Cairo, Egypt; levamisole (Ketrax®) was obtained from Elkahira Pharmaceutial Co., Cairo, Egypt (manufactured under license from AstraZeneca, Wilmington, Delaware, USA); cyclophosphamide (Endoxan®) was obtained from ASTA Medica AG, Frankfurt, Germany; cyclosporin (Sandimmune Neoral®) was obtained from Novartis Pharma, Switzerland.

General instrumentation

UV spectra were recorded in MeOH using a Shimadzu 1601-PC UV/Visible spectrophotometer; IR spectra were recorded on a Buck model 500 Infrared spectrophotometer; NMR spectra were recorded using a Bruker AM-300 spectrometer, Drx-400, and a Varian Mercury-300 spectrometer with $CDCl_3$ and DMSO-d_6 as solvents and TMS as internal standard. Chemical shifts (δ) are expressed in ppm. DEPT and APT experiments were conducted under standard conditions. EI/MS were performed using a Finningan Mat SSQ 7000 mass spectrometer with a Digital DEC 3000 workstation.

Plant material

The air-dried powdered leaves of *Lawsonia inermis* L. were purchased from the local herbal stores in Mansoura, Egypt on January 1999. It was identified through microscopical examination and identification of diagnostic fragments previously described by Youngken (1950).

Extraction

The air-dried powdered leaves of *Lawsonia inermis* L. (2.0 kg) were extracted with about 15 l of methanol (by maceration) at room temperature several times until complete exhaustion. The methanolic extract was concentrated to a syrup consistency (300 g) under reduced pressure at a temperature not exceeding 45 °C, diluted in about 1 l of distilled water and then successively extracted with petroleum ether, chloroform, and ethyl acetate. The aqueous mother liquor was further subjected to partition with *n*-butanol saturated with water. The extracts, in each case, were treated with anhydrous Na_2SO_4 and evaporated almost to dryness under reduced pressure and kept for further investigation. The obtained fractions were weighed to obtain the petroleum ether fraction (109.9 g), chloroformic fraction (34.8 g), ethyl acetate fraction (37.2 g), and finally *n*-butanol fraction (56.8 g).

Isolation and identification

Only the chloroformic and ethyl acetate fractions were investigated as explained before. The chloroformic extract (20 g) was applied onto the top of a glass column (120 cm × 5 cm) previously packed with silica gel (300 gm; 70–230 mesh; Merck) in petroleum ether. The extract was gradiently eluted with petroleum ether containing an increasing proportion of ethyl acetate. The effluent was collected in 50 ml fractions. Each fraction was concentrated to a small volume and monitored by TLC. Fractions 1–13 revealed the presence of three spots. They were not further investigated due to poor yield. Fractions 14–15 were further purified by silica gel column chromatography (15 g, 60 cm × 2.5 cm) using chloroform containing increasing proportions of ethyl acetate. Sub-fractions 8–12 upon crystallization yielded compound **1** (27.6 mg). Fractions 16–19 were purified by preparative TLC on silica gel using petroleum ether/EtOAc (6:4) to afford compound **2** (500 mg). Fractions 20–23 were separated by normal phase silica gel column chromatography (30 gm, 50 cm × 2 cm column) using petroleum ether/ethyl acetate gradient elution, collecting 25 ml fractions. Sub-fractions 1–9, upon evaporation of solvent, deposited 112 mg of compound **3**. Fractions 24–30 were further purified by normal phase silica gel column chromatography (30 gm, 50 cm × 2 cm column) using petroleum ether/ethyl acetate gradient elution, collecting 20 ml fractions. Sub-fractions 8–11 upon crystallization afforded compound **4** (32 mg), whereas sub-fractions 21–27 upon evaporation of solvent and re-crystallization yielded compound **5** (30 mg).

About 20 g of the ethyl acetate extract were applied onto the top of a glass column (60 cm × 5 cm) previously packed with silica gel (300 gm) in ethyl acetate. The extract was gradiently eluted with ethyl acetate containing an increasing proportion of a methanol/water mixture. The effluent was collected in 50 ml fractions. Each fraction was concentrated to a small volume and monitored by TLC. Fractions 1–14 revealed the presence of 3 spots in TLC, yet they were not further investigated due to poor yield. Fractions 15–17 upon evaporation of solvent and re-crystallization afforded compound **6** (264 mg) as canary yellow crystals. Fractions 18–42 upon crystallization deposited light yellow crystals of compound **7** (90 mg).

The UV, IR, MS and NMR spectral data of the isolated compounds are available upon request from the corresponding author.

Assessment of the immunomodulatory activity: lymphocyte blast transformation (mitogensis) assay

A) Separation of peripheral blood lymphocytes (PBL)

Lymphocytes were separated from peripheral human venous blood by the Ficoll/Hypaque gradient technique (Boyum, 1976). For each sample, 5 ml of heparinized blood was diluted with an equal volume of Hank's balanced salt solution (HBSS) in a sterile plastic centrifuge tube. 6 ml of diluted blood were carefully overlaid on 4 ml Ficoll/Hypaque solution gradient without allowing the solution to become mixed by keeping the pipette against the tube wall 5–10 mm above the fluid meniscus. The tube was centrifuged at 1200 rpm ($200 \times g$) at room temperature. The lymphocytes were localized as a whitish layer on the upper meniscus of the gradient solution. Using a fine pasteur pipette, the zone containing lymphocytes was taken and washed twice in HBSS, 10 min at 1200 rpm ($200 \times g$). The residue is a buffy coat of polymorphnuclear leucocytes (PMNLs).

B) Lymphocyte transformation assay

The viable lymphocytes were adjusted to a concentration of 2×10^6 cells/ml in RPMI-1640 medium supplemented with 600 μl penicillin, 0.1 ml streptomycin, 1% glutamine, 25% HEPES-buffer, and 20% foetal calf serum (FCS). The lymphocytes were plated into 96-well tissue culture plates (or Eppendorf tubes). The test solution (100 μl) in DMF (100 μl/ml) and 20 μg of the mitogen (PHA) were added to each well. Cell cultures were incubated at 37 °C in 5% CO_2 atmosphere for 72 h, during which the mitogen produces its maximal effect on DNA synthesis. After culture, cell films were stained by Giemsa stain and the average count of percentage of transformed (proliferated) blasts was determined. Aqueous *Echinaceae purpurea* extract (Immulone®) and levamisole (Ketrax®) were used as positive control (standard immunostimulant) while cyclophosphamide (Endoxan®) and cyclosporin (Sandimmune Neoral®) were used as negative control (standard immunosuppressant) at 100 μg/ml of each drug in DMSO.

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