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Phenolics from *Caesalpinia ferrea* Mart.: antioxidant, cytotoxic and hypolipidemic activity

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Received January 23, 2015, accepted February 27, 2015

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Pharmazie 70: 553–558 (2015) *doi: 10.1691/ph.2015.5526*

Nine phenolics were isolated from the aqueous ethanol extract of the leaves of *Caesalpinia ferrea*. The isolates were characterized for the first time from that plant. The structures of all isolates (**1**–**9**) were elucidated by conventional methods, spectroscopic analysis, including 1D and 2D NMR, and by HR-ESIMS as well. The antioxidant capacities using the ORAC method and the cytotoxic activity using the neutral red assay (NRU) for that extract and three major isolates have been evaluated. In addition, the hypolipidemic activity (*in vitro* and *in vivo*) of the extract has been assessed.

1. Introduction

The genus *Caesalpinia* (Caesalpinioideae, Fabaceae) which comprises tropical or subtropical trees and shrubs contains more than 500 species of worldwide distribution (Joly 1998). Previous studies reported remarkable biological activities for several *Caesalpinia* plants. Those activities include antimicrobial (Saaed and Sabir 2001), antidiabetic (*C. bonducella*, Sharma et al. 1997), antimalarial (*C. volkensii and C. pluviosa*, Kuria et al. 2001) and antiinflammatory activities (*C. sappan and C. ferrea*, Hikino et al. 1996). To date, less than 30 species of this genus have been studied for their phytoconstituents. The described metabolites include predominantly flavonoids and terpenoids (Bahia et al. 2005; Zanin et al. 2012).

Among the *Caesalpinia* plants, *Caesalpinia ferrea* Mart., known in English as leopard tree, is a leguminous tree of worldwide distribution (Lorenzi 2002). The tree grows up to 15 m, forming a broad flat-topped crown. The trunk is typically short, branching often close to the base. The leaves are bi-pinnately compound, 15-19 cm long. The individual leaflets range between 8-24 per pinnae. The flowers are bright yellow, typical of the Caesalpinioideae in form, and appear in terminal or axillary inflorescences. The seeds are dark brown, hard and contained in a flat pod (Lorenzi 2002).

In traditional medicine, the aqueous extract from fruits of this species is used in the treatment of diabetes and coughs. It exhibits antifungal, antiulcerogenic, anti-inflammatory and analgesic properties (Carvalho et al. 1996). Possible antitumour effects of the fruits of *C. ferrea* were tested *in vitro* by assessing the activation of the Epstein-Barr virus-early antigen (EBV-EA), which causes mononucleosis and other diseases, such as malignancy. The active constituents were identified as gallic acid and methyl gallate (Zanin et al. 2012). Recently, two chalcone dimers, pauferrol B and pauferrol C, were isolated from the stems of *C. ferrea*. These chalcone dimers exhibited potent inhibitory activities against human topoisomerase II and induced apoptosis in human leukemia HL 60 cells (Ohiraet al. 2013). Pauferrol A, a unique chalcone trimer, was isolated from the stems of the plant (Nozaki et al. 2007). In our own studies we isolated three formerly unknown di-*O*-glycosyl-*C*-glucosyl-flavones from the leaves of this species (Nawwar et al. 2014) and determined hypoglycemic activities of plant extract in hypoglycemic rats (Hassan et al. 2015).

During the current study we isolated and identified nine phenolics (**1** - **9**) from the aqueous ethanol extract of the leaves of *C. ferrea*. The isolates are characterized for the first time from that plant. Also, we evaluated the antioxidant capacities using the ORAC method and assessed the cytotoxicity against the keratinocyte cell line HaCaT using the neutral red assay (NRU) for that extract and three major isolates. In addition, the hypolipidemic activity (*in vitro* and *in vivo*) of the extract has been assessed.

2. Investigations, results and discussion

Specimens of the *Caesalpinia ferrea* leaves were exhaustively extracted with aqueous ethanol (75 %). The received extract was subjected to a series of column and preparative PC separations to isolate compounds **1** – **9**.

2.1. Phytochemical analysis

The following compounds have been found for the first time in *C. ferrea*: gallic acid (**1,** Nawwar et al. 1982), brevifolin carboxylic acid (**2**, Nawwar et al. 1994), brevifolin (**3**, Nawwar et al. 1994),

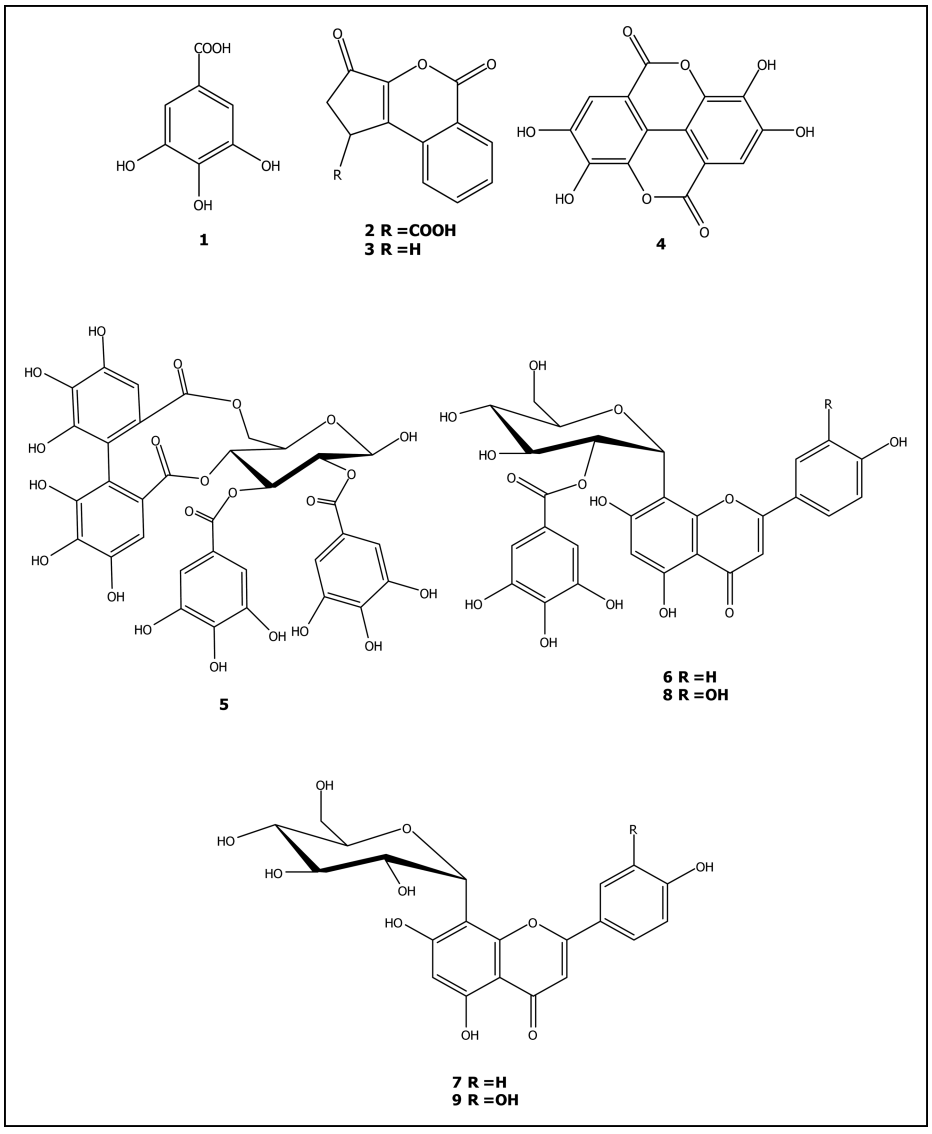

Fig. 1: Structures of isolated compounds.

ellagic acid (**4**, Nawwar et al. 1994), tellimagrandin-I (**5**, Hatano et al. 1988), 2"-*O*-galloylvitexin (**6**, Latté et al. 2002), vitexin (**7**, Latté et al. 2002), 2"-*O*-galloylorientin (**8**, Latté et al. 2002) and orientin (**9**, Latté et al. 2002), (Fig. 1).

2.2. Biological activities

2.2.1. Oxygen radical absorbance capacity and cytotoxic activities

In order to get information about biological activities of the extract of *C. ferrea* and and some of the isolated substances, samples of the extract and of three isolates were tested for oxygen radical absorbance capacity by ORAC assay (Lucas-Abellán et al. 2008) and for cytotoxicity on HaCaT keratinocytes by NRU neutral red assay (Mothana et al. 2011). Table 1 summarizes the EC_{50} values for radical scavenging activity and the IC_{50} values for cytotoxicity. Among the test samples 2"-*O*-galloylorientin (**8**) has the highest radical scavenging activity with an EC_{50} value of 1.9 μg/ml. This was much lower than the IC_{50} value for cytotoxicity (67.5 μg/ml). Brevifolin carboxylic acid (**2**), 2"-*O*-galloylvitexin (**6**) and the whole extract exhibit radical scavenging activity in much lower concentrations than cytotoxic effects too (Table 1). The vehicle in which the test samples were dissolved had no influence on the viability of HaCaT cells.

2.2.2. In vitro and in vivo hypolipidemic effects

Because of the traditional use of the plant against diabetes and close connections between diabetes and disorders in lipid metabolism, hypolipidemic effects of the extract of *C. ferrea* has been investigated. Table 2 shows the *in vitro* effect of the extract on the activity of HMG CoA-reductase; the key limiting enzyme of cholesterol biosynthesis. The extract inhibited enzyme activity by about 86 % (control = 100 %). The reference drug lipanthyl caused about 90 % inhibition of enzyme activity.

Table 1: Cytotoxicity (IC50) and radical scavenging activity (ED50) of isolated compounds in comparison to the extract of *Caesalpinia ferrea*

Test sample	Radical Scavenging Activity [ED_{50}(μg/ml)]	Cytotoxicity [IC_{50} (μg/ml)]
Extract of *Caesalpinia ferrea*	12.5 ± 2.9	114.4 ± 18.9
Brevifolin carboxylic acid (2)	5.0 ± 1.0	124.9 ± 22.5
2"-*O*-Galloylvitexin (6)	3.8 ± 0.4	59.7 ± 3.8
2"-*O*-Galloylorientin (8)	1.9	67.9 ± 5.9
Trolox	28.0 ± 14.3	n.d.
Etoposide	n.d.	1.47

Results are given in mean ± SD of 3 independent experiments

Table 2: ***In vitro*** **hypolipidemic effect of extract of** ***Caesalpinia ferrea***

Test sample	Activity of HMG CoA reductase (μmol/mg)	Inhibition (%)
Control	17.00 ± 0.50	–
Extract	2.27 ± 0.14	86.64
Lipanthyl	1.60 ± 0.34	90.58

Enzyme activity is represented by μmole/mg dried extract. Data are mean ± SD of three readings.

In comparison to the control group, hypercholesterolemic rats (cholesterol group) revealed a significant increase in content of total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C) and total lipid contents (153.91, 59.45, 104.33 and 66.38 % respectively), while high density lipoprotein cholesterol was decreased (Table 3). These observations were in accordance with the finding of Hamed (2011) and Awad et al. (2012) in hypercholesterolemic rats fed with high fat diet.

Treatment with plant extract (extract group) resulted in a significant decrease in TC (53.08 %), LDL-C (25.03 %), TG (48.84 %) and total lipids (23.28 %), while HDL-C showed significant increase by 158.71 % as compared to untreated hypercholesterolemic group. Lipanthyl (lipanthyl group) caused significant decrease in TC, HDL-C, LDL-C, TG and total lipids by 54.33, 32.06, 50.49 and 26.66 %, respectively, while HDL-C showed a significant increase after drug treatment by 127.05 % (Table 3).

Regarding to liver function enzymes, hypercholesterolemic rats showed a significant increase in AST, ALT and ALP enzymes activity by 45.26, 48.30 and 78.32 %, respectively as compared to the control group. Hamed (2011) attributed the increase in enzyme activity to the elevation of free radicals and lipid peroxidation process that affected the permeability of hepatocyte membranes and led to enzyme leakage into the circulation. Treatment with plant extract caused significant decrease in AST, ALT and ALP enzyme activities by 28.77, 22.83 and 22.15 %, respectively as compared to the hypercholesterolemicrats. The positive control lipanthyl caused significant decrease by 27.03, 24.30 and 18.83 %, respectively (Table 4).

The histopathological picture of the control liver revealed thenormal appearance of hepatocytes surrounding the central vein (Fig. 2A). In hypercholesterolemic rats degenerative changes in the hepatocytes including hydropic degeneration and fatty changes could be seen (Fig. 2B). Congestion of the central veins and leucocytes infiltrations were observed. Numbers of circumscribed masses (granuloma) were seen within the hepatic parenchyma (Fig. 2C). These masses included different types of cells as epithelioid cells, macrophages and lymphocytes with the presence of a few giant cells in some masses. Fibrous connective tissue encapsulation was seen around these structures. Separating these structures from the healthy tissue, leucocytic infiltration was found. In some areas, early fibrous connective tissue proliferation was seen within the hepatic lobules *via* the subendothelial areas of hepatic sinusoids (Fig. 2C). The same histopathological pattern in hypercholesterolemic rats has been described previously (Hamed 2011; Awad et al. 2012). In the group treated with plant extract, the liver appeared more or less normal with the presence of very mild congestion and very mild degenerative changes (Fig. 2D). Minimal numbers of granulomas could be seen (Fig. 2E). Group treated with positive control lipanthylshowed mild degeneration and congestion in the livers with minimal granulomas (Fig. 2F and G).

3. Experimental

3.1. General

NMR spectra were acquired in DMSO-d_6 on a Bruker 400-MHz. Standard pulse sequence and parameters were used to obtain one-dimensional ^{1}H and^{13}C- NMR, two-dimensional COSY, HSQC, and HMBC spectra, respectively. Chemical shifts (δ) were measured in ppm, ^{1}H NMR chemical shifts relative to tetramethylsilane (TMS) and^{13}C- NMR chemical shifts to acetone-d_6 and were converted to the TMS scale by adding 29.8. High-resolution ESI mass spectra were measured using a Finnigan LTQ FT Ultra mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a Nanomate ESI interface (Advion, Berlin, Germany). An electrospray voltage of 1.7 kV (+/-) and a transfer capillary temperature of 200 °C were applied. Collision-induced dissociation (CID) was performed in the iron trap using normalized collision energy of 35 Kv, activation time of 30 ms, 0.25 activation Q, and a precursor ion isolation width of 2 amu. High-resolution product ions were detected in the Fourier transform ion cyclotron resonance (FTICR) cell of the mass spectrometer. UV spectra were recorded on a Shimadzu UV-Visible-1601 spectrophotometer (Kyoto, Japan). Flame atomic absorption analysis was performed on a Varian Spectra-AA220 instrument (Cairo, Egypt) (lamp current, 5 mA; fuel, acetylene; oxidant, air). Paper chromatography (PC) was carried out on Whatmanpaper No. 1 using the following solvent system: H_2O; 15 vol.-% HOAc; 6 vol.-% HOAc; BAW [n-BuOH/HOAc/H_2O (4: 1: 5, v/v/v), upper layer)].

3.2. Plant material

Leaves of *Caesalpinia ferrea* were collected from a tree cultivated in the Zoological Garden, Cairo, Egypt, in May 2012. The plant was identified by Prof. Salwa Quashti, National Research Center (NRC), Cairo, Egypt. A voucher specimen (C 253) has been deposited at the herbarium of the NRC.

3.3. Extraction and isolation

Leaves (2.5 kg), dried in the shadow in the air, were crushed and exhaustively extracted with 70 % (v/v) aqueous EtOH under reflux (three times, each extraction for 8 h with 21). Two hundred g of the dried extract were dissolved in 150 ml aqueous EtOH and applied to a polyamide column (2 kg;

Table 3: Effect of ***Caesalpinia ferrea*** **extract on lipid profile of hyperlipidemic rats**

Parameters	Control group	Cholesterol group	Extract group	Lipanthyl group
TC	$115.00^{d} \pm 8.09$	292.00 ± 17.86^{a}	137.00 ± 7.58^{c}	133.33 ± 4.21^{c}
	—	(+153.91)	[−53.08]	[−54.33]
HDL-C	64.48 ± 2.85^{a}	15.60 ± 1.70^{d}	40.36 ± 3.37^{b}	35.42 ± 4.75^{c}
	—	(−75.80)	[+158.71]	[+127.05]
LDL-C	71.73 ± 12.43^{b}	114.38 ± 4.76^{a}	85.75 ± 13.08^{b}	77.70 ± 13.21^{b}
	—	(+59.45)	[−25.03]	[−32.06]
TG	132.75 ± 4.11^{c}	271.26 ± 23.34^{a}	138.76 ± 4.18^{c}	134.28 ± 10.54^{c}
	—	(+104.33)	[−48.84]	[−50.49]
Total lipids	5.86 ± 0.86^{d}	9.75 ± 0.41^{a}	7.48 ± 0.21^{c}	7.15 ± 0.29^{b}
	—	(+66.38)	[−23.28]	[−26.66]

Data are means ± SD of eight rats in each group. Data are expressed as mg/dl.

Total lipids are expressed as g/dl. Unshared superscript letters between groups are the significance values at $p < 0.0001$. Values between brackets are percentage change over control group = [(mean treated- mean control)/ mean control] x 100. Values between parentheses are percentage change over cholesterolemic group = [(mean treated- mean hypercholesterolemia)/ mean hypercholesterolemia] x 100.

Statistical analysis is carried out by one way analysis of variance (ANOVA) – Costat Software Computer Program.

Table 4: Effect of *Caesalpinia ferrea* extract on liver enzymes of hypercholesterolemic rats

Parameters	Control group	Cholesterol group	Extract group	Lipanthyl group
AST	38.04 ± 3.18^{b}	55.26 ± 3.51^{a}	39.36 ± 2.34^{b}	40.32 ± 2.19^{b}
	—	(+45.26)	[−28.77]	[−27.03]
ALT	41.16 ± 3.57^{c}	65.16 ± 4.11^{a}	50.28 ± 4.15^{b}	49.32 ± 3.68^{b}
	—	(+48.30)	[−22.83]	[−24.30]
ALP	44.30 ± 1.78^{d}	79.00 ± 2.32^{a}	61.50 ± 2.34^{b}	64.12 ± 1.70^{b}
	—	(+78.32)	[−22.15]	[−18.83]

Data are means ± SD of eight rats in each group. Data are expressed as Unit/L.
Unshared superscript letters between groups are the significance values at $p < 0.01$.
Values between brackets are percentage change over control group = [(mean treated- mean control)/ mean control] x 100. Values between parentheses are percentage change over cholesterolemic group = [(mean treated- mean hypercholesterolemia)/ mean hypercholesterolemia] x 100.
Statistical analysis is carried out by one way analysis of variance (ANOVA) – Costat Software Computer Program.

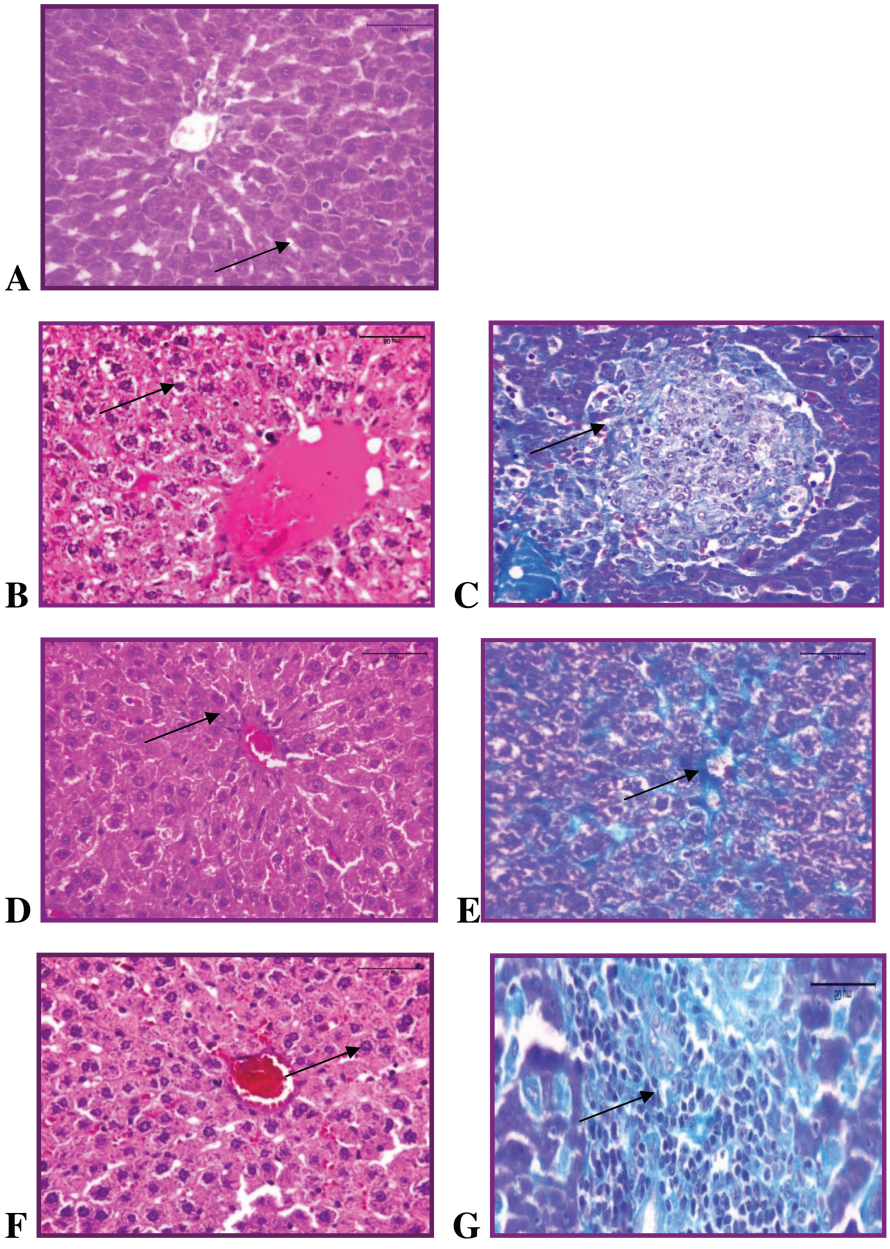

Fig. 2: Histological pictures of liver slices. Control liver showed normal hepatocytes appearance (A). Hypercholesterolemic liver (B and C) showed marked degenerative changes in the hepatocytes, numbers of circumscribed masses and steatosis. Extract treated group showed more or less normal hepatocytes with minimal granuolomas (D and E).Lipanthyl treated group showed mild degeneration in the livers with minimal granulomas (F and G). A,B, D and F were stained with Haematoxylin and Eosin. C, E and G were stained with Masson Trichrom (100X).

Macherey Nagel, Düren, Germany). Separation was initiated with H_2O followed by H_2O/MeOH (9:1, v/v), and the MeOH content was gradually increased in 10 % steps. The flavonoid-containing fraction II was eluted with H_2O/MeOH (2:8) to yield faintly yellow powder (16 g). Further fractionation of fraction II was performed on a MCI gel column (CHP-20P, 75 - 150 μm; Mitsubishi Chemical Co., Düsseldorf, Germany) and gradient elution with H_2O/MeOH mixtures afforded three individual compounds (**1**-**3**). The flavonoid-containing fraction III was resolved by application of preparative PC using Whatman paper No. 3 MM and BAW as solvent. This led to the separation of pure sample of **4** and **5**. Separation of the phenolics of fraction VI was performed on polyamide column and elution with MeOH/toluene/H_2O (60:38:2) (v/v/v) to afford six sub-fractions. Further polyamide column separation was used for the fifth sub-fraction using the same solvent, thus producing three individual compounds (**6**, **7** and **8**). Acetone extract of fraction VII was fractionated on a MCI gel column (CHP-20P, 75-150 μm; Mitsubishi Chemical Co., Düsseldorf, Germany) using gradient elution with H_2O/MeOH mixtures which led to the desorption of two individual compounds **1** and **9**).

3.4. Isolated phenolics

Gallic acid (**1**, Nawwar et al. 1982), brevifolin carboxylic acid (**2**, Nawwar et al. 1994, brevifolin (**3**, Nawwar et al. 1994), ellagic acid (**4**, Nawwar et al. 1994), tellimagrandin-I (**5**, Hatano et al. 1988), 2”-*O*-galloylvitexin (**6**, Latte et al. 2002), vitexin (**7**, Latte et al. 2002), 2”-*O*-galloylorientin (**8**, Latte et al. 2002) and orientin (**9**, Latte et al. 2002), (Fig. 1).

3.5. Biological assays

3.5.1. Determination of oxygen radical absorbance capacity by ORAC assay

Reactive oxygen species (ROS) are generated by the thermal decomposition of [2,2’-azobis(2-amidino-propane)]dihydrochloride (AAPH) and over time quench the signal of the fluorescent probe fluorescein. The subsequent addition of antioxidants reduces the quenching by preventing the oxidation of the fluorochrome. Briefly, the positive control 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and test compounds were dissolved/diluted in phosphate buffered saline (10 mM, pH 7.4). In each well of a black 96 well Plate 150 Plate 150 μL 10 nM fluorescein (final concentration: 2.5 nM), 25 μLTrolox (final concentrations: 0.2-3.13 μM) or 25 μL test compound were pipetted in quadruplicate. The plate was allowed to equilibrate at 37 °C for 30 min. After incubation, fluorescence measurements (Ex. 485 nm, Em. 520 nm) were taken every 90 s to determine the background signal. After 3 cycles, 25 μL (240 mM) of AAPH was injected into each well. Measurements were continued for 90 min. The percent inhibition of fluorescein decay at different concentrations of the test samples was determined using MS Excel software; from which the IC_{50} (concentration of substance that causes inhibition of half the fluorescein decay) was calculated. The ORAC values for all the tested substances at 12.45 mg/L were calculated using a Trolox standard curve.

3.5.2. Cytotoxicity assay

HaCaT cells were obtained from CLS Cell Lines Service (Eppelheim, Germany) and cultured in RPMI 1640 medium (BioWhittaker, Lonza, Verviers, Belgium) supplemented with 8 % fetal bovine serum (Sigma Aldrich, Taufkirchen, Germany) and antibiotics (100 U/ml penicillin/100 μg/ml streptomycin; Sigma Aldrich, Taufkirchen, Germany) at 95 % humidity, 5 % CO2 and 37 °C. The cells were sub-cultured twice a week and regularly tested for mycoplasma contamination. Cytotoxicity of test samples was investigated using the neutral red uptake (NRU) assay. After 24 h cultivation in 96 well plates (8 x 10^3 cells/well) medium was removed and cells were exposed for 72 h to various concentrations (max. 500 μg/ml) of test samples. After removal of the medium wells were washed with HBSS (Hanks Balanced Salt Solution, PAA). Cells were than incubated for 3 h with 100 μl 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red, Merck, Darmstadt, Germany, stock solution 3.3 μg/ml; working solution 33 ng/ml). Medium was removed and wells were washed twice with HBSS. Afterwards cells were lysed with 100 μl of 1 % acetic acid in 50 % EtOH. Finally, after 45 min optical density was measured at 450 nm in a plate reader (Fluostar Omega, BMG Labtech, Offenburg, Germany). The IC_{50} values were defined from obtained dose-response curves and expressed in mean ± SD. All samples were tested in duplicate and etoposide (Alexis Biochemicals, ≥ 98 % purity) served as positive control.

3.5.3. In vitro hypolipidemic study

The activity of the key limiting enzyme of cholesterol biosynthesis; β-hydroxy- β- methyl glutaryl CoA reductase (HMG CoA reductase) was assayed by the method of Zhang et al. (1998). The decrease in absorbance due to the oxidation of NADPH to NADP was measured colorimetrically at 340 nm.

3.5.4. In vivo hypolipidemic study

Male Wistar albino rats (100: 120 g) were selected for this study. They were obtained from the Animal House, National Research Center, Egypt. All animals were kept in controlled environment of air and temp with access of water and diet. The control group was fed along the experimental period (thirteen weeks) with standard diet (El- Kahira Co. for Oil and Soap, Cairo, Egypt). The cholesterolemic group was fed with standard diet containing 150 g lard (fat abdomen of sheep)/kg diet (Auger et al. 2002) for nine weeks. By starting treatment with the extract or reference drug, they were fed with normal diet for 4 weeks.

Anesthetic procedures and handling with animals complied with the ethical guidelines of the Medical Ethical Committee of the National Research Centre in Egypt.

Thirty two male rats were divided into four groups (eight rats each) as follows: Group 1: normal healthy control rats. Group 2: cholesterol treated rats. Cholesterol was orally given at a dose of 30 mg/0.3 ml (Tween 0.7 %), five times/ week for nine consecutive weeks (Adaramoye et al., 2008). Group 3: rats forced with cholesterol for 9 weeks and treated with plant extract for 4 weeks. Extract was administrated orally at a dose 300 mg/kg b.w. (Kannur et al. 2006) daily for 4 weeks after cholesterol induction. Group 4: rats forced with cholesterol for 9 weeks and treated orally with the positive control lipanthyl (=fenofibrate, Mina Pharm, Egypt) at a dose of 50 mg (Petit et al. 1988) daily for 4 weeks.

Blood was collected from each animal by puncture the sub-lingual vein in clean and dry test tube, left 10 min to clot and centrifuged at 3000 r.p.m for serum separation. The separated serum was stored at –80 °C for further determination of lipid profile and liver function tests.

Cholesterol was determined by the method of Meiattini et al. (1978), high density lipoprotein-cholesterol (HDL-C) by the method of Bustein et al. (1980), low density lipoprotein-cholesterol (LDL-C) by the method of Assmann et al. (1984), triglycerides by the method of Fossati and Prencipe (1982), and total lipids by the method of Zollner and Kirsch (1962). Aspartate and alanine amintransferases were measured by the method of Gella et al. (1985) and alkaline phosphatase by those of Rosalki et al. (1993).

Representative slices of liver tissues were fixed in 10 % formalin. Paraffin-embedded sections (4 μm thick) were stained by haematoxylin and eosin (H&E) and Masson’s Trichrom. Slides were seen under light microscope according to Bancroft and Gamble (2008).

All data are expressed as mean ± SD of eight rats in each group. Statistical analysis was carried out by one-way analysis of variance (ANOVA), Costat Software Computer Program accompanied with least significance differences (LSD) between groups at $p < 0.05$.

Acknowledgements: This research was supported and financed by Alexander von Humboldt foundation through the group linkage program (joint project: “Bioactive phenolics from Egyptian folk medicinal plants”, 3.4-fokoop-DEU /1093980) awarded to U. Lindequist and M. Nawwar.

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