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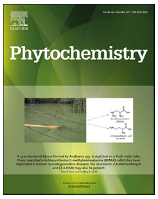

Phytochemical study of *Juglans regia* L. leaves

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Dedicated to Prof. Dr. Gerhard Franz on the occasion of his 80th birthday and decades of outstanding scientific work in the field of pharmaceutical biology.

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ABSTRACT

A methanolic extract of *Juglans regia* L. leaves was fractioned by various chromatographic techniques yielding a total of 40 metabolites belonging to megastigmane, tetralone, phenylpropanoid, neolignane and juglone glycosides. Ten unknown megastigmane glucoside derivatives (juglanionosides A-K, **1**–**10**) and six unknown tetralone glucoside derivatives (juglanosides J-O, **11**–**16**) together with 24 known compounds - among them 16 described for the first time in *Juglans* - were isolated. As characteristic structural feature, the previously undescribed compounds showed acylation of the sugar units with sinapic, ferulic, coumaric, benzoic or salicylic acid. Their chemical structures were elucidated on the basis of 1D and 2D NMR techniques, HRESIMS as well as CD spectroscopy. Absolute stereochemistry was revealed by mild alkaline hydrolysis and comparison of CD and polarimetric data to literature values.

1. Introduction

Juglans regia L. is a deciduous tree and one of approximately 64 species belonging to the Juglandaceae. The species has aromatic odd-pinnate leaves and unisexual, monoecious, wind-pollinated flowers. Male flowers appear at one year old shoots as drooping catkins while the female flowers appear at this year's shoots. The fruit is a nutritious nut which is utilized since prehistoric times by humans. It is thought that *J. regia* is native to Central Asia, Anatolia, northern parts of Iran and Himalaya. Today it is cultivated almost around the world for nut production (Schaarschmidt, 2006). Leaves of *J. regia* are used since a long time in folk medicine of different cultures to cure various diseases, preferably by use of a decoction. Among others, claimed indications are malaria (Fujita et al., 1995), rheumatic pain (Wichtl and Czygan, 2002; Yeşilada, 2002), venous insufficiency, inflammations of mouth and pharynx, dandruff (Bruneton, 1993), diarrhea (Wichtl and Czygan, 2002), hypoglycemia (Subramoniam, 2016), skin tuberculosis (Eisenman et al., 2013) and marginal blepharitis (Geßner et al., 1974). Further effects of the decoct are described as antibacterial (Pereira et al., 2007), antiviral (Mouhajir et al., 2008), astringent (Wichtl and Czygan, 2002), anthelmintic (Bruni et al., 1997; Eisenman et al., 2013; Geßner et al., 1974), sweat regulating (Schilcher et al., 2016), keratolytic (Subramoniam, 2016) and sedative (Gîrzu et al., 2008).

The metabolites identified for *J. regia* leaves are mainly polyphenols i.e. naphthoquinones like juglone or tetralones, hydrolysable tannins, flavonoids, phenol carboxylic acids like ferulic and caffeic acid as well as diarylheptanoids (Amaral et al., 2004; Pereira et al., 2007; Wagner and Bauer, 1999). Terpenes were also identified especially monoterpenes, sesquiterpenes and megastigmanes (Bou Abdallah et al., 2016; Forino et al., 2016). More recent studies tested the efficacy of *J. regia* leaf extracts against indications like hypoglycemia though the biological active compounds are still unclear (Hosseini et al., 2014; Jelodar et al., 2007). Nevertheless, TLC analyses of a methanolic extract suggested that a number of metabolites of *J. regia* leaves remain unreported (TLC analyses available in

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Supplementary Material). In order to comprehensively isolate and characterize the metabolites of *J. regia* leaves, the compounds present in a 70% (v/v) methanolic extract were separated by various chromatographic techniques. This study deals with the isolation and structure elucidation of forty metabolites of which sixteen were previously undescribed and another sixteen have been isolated in the genus *Juglans* for the first time.

The comprehensive description and isolation of different classes of specialized metabolites in *J. regia* offers interesting aspects with regard to *in vitro* pharmacological testing and described pharmacological effects of *Juglans* extracts. Whereas comprehensive pharmacological investigations have been done on the cytotoxic (Zhang et al., 2015) and anti-inflammatory (Peng et al., 2015) effects of the naphthoquinones data on megastigmanes are scarce. Some megastigmane glucosides have been reported to inhibit NO production in mouse macrophages (Qiu et al., 2008; Thao et al., 2015) and thus these metabolites may have also anti-inflammatory effects. Nevertheless, neither molecular targets nor structure activity relationships have been evaluated. As studies on the occurrence of acylated megastigmane glucosides are rare (Matsunami et al., 2006; Nomoto et al., 2013) and the characterization of phytochemical profiles in *Juglans* species concentrated more on the aromatic compounds (Huo et al., 2017; Wang et al., 2017) it should be also clarified whether these structural variation of megastigmane glycosides is specifically realized in *J. regia* or can be also found in other species of the genus *Juglans* (Juglandaceae).

2. Results and discussion

A 70% (w/w) methanolic extract of *J. regia* leaves was first fractionated by subsequent liquid-liquid partition against solvents with increasing polarity. The EtOAc soluble fraction was further separated by several chromatographic techniques like Sephadex® LH-20, MCI-Gel® CHP20P, RP-18 flash chromatography as well as Centrifugal Partition Chromatography (CPC). Using normal and reversed phase HPLC in a semi-preparative or analytical mode the isolation of ten unknown megastigmane glucosides (**1**–**10**), six unknown tetralone glucosides (**11**–**16**) and 24 already known compounds (**17**–**40**) was achieved (Fig. 1). Among the known compounds eight belong to the megastigmane glucosides (**17**–**24**), four are tetralone glucosides (**25**–**27**), two naphthalenyl glucosides (**29**–**30**), seven phenylpropanoid glucosides (**31**–**37**) and three neolignan glucosides (**38**–**40**). In accordance to literature data they were identified as 3-hydroxy-5,6-epoxy-*β*-ionol-9-*O*-*β*-D-glucopyranoside (**17**) (Sudo et al., 2000), citroside A (**18**) (Umehara et al., 1988; Zhang et al., 2010), roseoside (**19**) (Otsuka et al., 1992), (6*R*,9*R*)-3-oxo-*α*-ionol 9-*O*-*β*-D-glucopyranoside (**20**) (Pabst et al., 1992), sedumoside F1 (**21**) (Morikawa et al., 2007), linarionoside A (**22**) (Otsuka, 1994), linarionoside B (**23**) (Otsuka, 1994), byzantionoside B (**24**) (Matsunami et al., 2010), juglanoside A (**25**), B (**26**), D (**28**), and E (**27**) (Liu et al., 2004), 4-hydroxy-1-naphthalenyl *β*-D-glucopyranoside (**29**), and 4,8-dihydroxy-1-naphthalenyl *β*-D-glucopyranoside (**30**) (Müller and Leistner, 1978), eugenyl-*O*-*β*-apiofuranosyl-(1″-6′)-*O*-*β*-D-glucopyranoside (**31**) (Takeda et al., 1998), eugenyl-*O*-*β*-apiofuranosyl-(1″-2′)-*O*-*β*-D-glucopyranoside (**32**) (Bin, 2015), eugenol rutinoside (**33**) (Orihara et al., 1992), citrusin C (**34**) (Kim et al., 2004), phoeniceoside (**35**) (Ina et al., 1987), 6-*O*-*E*-caffeoyl-*β*-D-glucopyranose (**36**), and 6-*O*-*E*-caffeoyl-*α*-D-glucopyranose (**37**) (Shimomura et al., 1986), icariside E4 (**38**) (Miyase et al., 1989; Nakanishi et al., 2004), (−)-dihydrodehydrodiconiferyl alcohol 9-*O*-*β*-D-glucopyranoside (**39**), (+)-dihydrodehydrodiconiferyl alcohol 9-*O*-*β*-D-glucopyranoside (**40**) (Abe and Yamauchi, 1986; Changzeng and Zhongjian, 1997) by comprehensive comparison of the spectroscopic (1D and 2D NMR, UV and CD) and spectrometric (HRESIMS) data. Known compounds **17**, **18**, **20**–**23** and **31**–**40** were found in the genus *Juglans* for the first time.

Compound **1** was isolated as a pale beige amorphous powder and its molecular formula was determined to be $C_{28}H_{40}O_{12}$ by negative-ion HRESIMS showing a deprotonated ion $[M\text{-}H]^-$ at *m/z* 367.2450. The ^{13}C NMR spectrum indicated the presence of a megastigmane glucoside due to the characteristic shifts of four methyl (δ_C 21.7, 26.2, 27.5, 27.5), two methylene (δ_C 46.3, 45.7), two oxymethine (δ_C 65.3, 78.6), two olefinic (δ_C 133.0, 134.2) and three quaternary carbons (δ_C 40.7, 77.9, 79.1) beside the typical signal pattern of a *β*-glucose (Table 1). Additionally, nine further carbons resonated downfield shifted, resembling the signals of a phenylpropanoid moiety. Accordingly, the ^1H NMR spectrum (Table 2) showed additional signals of an aromatic ABX-spin system, in particular two doublets (δ_H 7.05, $J_{2'', 6''} = 1.9$ Hz, H-2″ and δ_H 6.76, $J_{5'', 6''} = 8.2$ Hz, H-5″) and a doublet of doublets (δ_H 6.94, $J_{6'', 2'', 5''} = 1.9, 8.2$ Hz, H-6″) as well as the presence of two additional *trans*-olefinic protons (δ_H 7.56, $J_{7'', 8''} = 15.9$ Hz, H-7″ and δ_H 6.33, $J_{8'', 7''} = 15.9$ Hz, H-8″) indicating the substitution of the megastigmane glucoside by a disubstituted *trans* configured phenylpropanoid. This was confirmed by $^1H,^{13}C$ HSQC and $^1H,^{13}C$ HMBC spectra the latter showing cross peaks of a quaternary carboxyl carbon (δ_C 169.3, C-9″) to the aromatic protons H-5″ (δ_H 6.76), H-6″ (δ_H 6.94) and H-2″ (δ_H 7.05) as well as to H-7″ and H-8″. An 3,4-dihydroxy-substitution of the aromatic ring and thus the presence of a caffeic acid were deduced from two low field shifted aromatic carbons (δ_C 147.0, C-3″ and δ_C 150.2, C-4″) coupling in HMBC with the aromatic protons. HMBC correlations from C-9″ to methylene signals (δ_H 4.26 and 4.46, H-6′a, b) of a *β*-glucopyranosyl moiety revealed the acylation at C-6′of the glucose. Mild alkaline hydrolysis of **1** with 0.1 M NaOH resulted after ester hydrolysis in **1a** (Table 2), which showed the same spectroscopic properties (polarimetry, NMR) as published for actinidioionoside. Therefore, the absolute stereochemistry of **1** could be deduced as 3*S*, 5*R*, 6*R*, 9*R*. Determination of absolute configuration of the glucose moiety of **1** revealed the presence of *β*-D-glucose. This was performed by hydrolyzation and derivatization with (*S*)-(+)-2-methylbutyric anhydride and comparison of the anomeric proton's ^1H shift with those of derivatized D- and L-sugars. As this signal of **1** (δ_H 5.948, $J_{1'', 2''} = 8.3$ Hz) was identical to that of derivatized D-(+)-glucose (δ_H 5.948, $J_{1'', 2''} = 8.3$ Hz) and different of that of derivatized L-(−)-glucose (δ_H 5.938, $J_{1'', 2''} = 8.4$ Hz) compound **1** was identified due to its spectroscopic data as actinidioionoside 6′-*O*-*E*-caffeate and named juglanionoside A.

Due to almost identical ^{13}C and ^1H shifts of the glucose moieties in compounds **1**–**10** the same *β*-D-glucose configurations could be presumed (York et al., 1997). Compounds **2**, **3**, **5** and **7**–**9** also showed identical ^1H and ^{13}C NMR shifts compared to **1** and referred to the aglycone. Thus the same 3*S*, 5*R*, 6*R*, 9*R*-actinidioionoside structure is postulated. ^1H, ^{13}C HMBC correlations of H-1′ and C-9 and *vice versa* confirmed the connection of the *β*-glucose to C-9 of the aglycone. These compounds only differed in their phenylpropanoid moiety which was substituted to C-6′ of the glucose in every case due to respective cross peaks in HMBC. In contrast, compounds **4** and **6** showed a substitution of an acylated *β*-glucose at C-3 of the aglycone resulting in shifted proton and carbon signals of the aglycone (Tables 1–3).

Compound **2** was isolated as a white amorphous powder and its molecular formula was assigned to be $C_{28}H_{40}O_{11}$ by positive-ion HRESIMS showing a $[M + NH_4]^+$ at *m/z* 570.2910. ^1H NMR spectrum showed signals corresponding to four aromatic and two *trans*-olefinic (δ_H 7.62, $J_{7'', 8''} = 15.9$ Hz, H-7″ and δ_H 6.38, $J_{8'', 7''} = 15.9$ Hz, H-8″) protons (Table 2) besides the typical megastigmane pattern. ^{13}C NMR spectrum (Table 1) gave again evidence for one carboxyl