

## GC-MS analysis of steroids and triterpenoids occurring in leaves and tubers of *Tamus edulis* Lowe

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### ABSTRACT

*Tamus edulis* Lowe is an endemic perennial plant belonging to Dioscoreaceae family. The plant has long climbing stems, ovate leaves, flowers in spikes, fleshy red berries and long tuberous roots. Young shoots and tuberous roots of *T. edulis* were used traditionally for nourishment and as a herbal medicine. Leaves and roots analyzed in the present study were collected in the northwest of Madeira island. The GC-MS analysis allowed to detect several steroids in free forms in diethyl ether extracts; and diosgenin with its isomer, yamogenin, in hydrolyzates from methanolic extracts. The obtained results reveal that *Tamus edulis* has some features common with other *Dioscorea* species, e.g., the presence of steroidal saponins with diosgenin and yamogenin as aglycones, or the phytosterol composition with predominating sitosterol. However, some other traits, like the relatively high content of free steroids (more than 1 mg/g d.w.) and their profile rich in cholesterol derivatives, can distinguish *Tamus edulis* from other *Dioscorea* species studied previously for their steroid and triterpenoid profile.

### 1. Introduction

The Dioscoreaceae is a large plant family, that includes more than 600 species. *Tamus edulis* Lowe, a species belonging to this family, was described for the first time by Lowe (1831) as a species occurring in Madeira. This perennial plant was sporadically cultivated in remote areas of this island and widely known by common name „norça” (Pinheiro de Carvalho et al., 2016). Tubers and young shoots of *T. edulis* were used traditionally for nourishment and as a herbal medicine. The plant has long climbing stems, ovate leaves (Fig. 1), flowers in spikes, fleshy reddish berries when mature, and long brown tuberous roots with white-yellowish flesh. This species is considered as an endemic plant occurring naturally in Canary Islands and Madeira (Press and Short, 1994). Its endemism is determined by its geographical and reproductive isolation. For a long time, *Tamus* has been considered as a monophyletic genus of flora of Madeira, holding *T. edulis* Lowe as a species with high genetic singularity (Press and Short, 1994; Borges et al., 2008). *Tamus* and *Dioscorea* genera were separated on the basis of fruit morphology: *T. edulis* forms a fleshy berry while *Dioscorea* forms dry capsule, however, recent biochemical and genetic data shows their

close relation (Wilkin et al., 2005). Based on the analysis of herbarium voucher material, but in the absence of other studies, *T. edulis* has been currently classified as one of numerous synonyms of *Dioscorea communis* (L.) Caddick and Wilkin (The Plant List, 2013). However, phylogenetic relationships between *Dioscoreales* used to be difficult to resolve (Chair et al., 2016). The confusion can be explained by high phenotypic variability of these species (Ferrer-Gallego and Boisset, 2016).

Plants belonging to Dioscoreaceae family are interesting from pharmaceutical point of view due to saponin content. Saponins possess cytotoxic, cytostatic, anti-tumoral and chemo-preventive activity (Lacaille-Dubois and Wagner, 2000). They also positively affect anti-oxidative enzymes (Son et al., 2007). González et al. (1970) isolated several steroidal saponin from *T. edulis* leaves and twigs collected in Canary Islands. Besides diosgenin, tamusgenin, eduligenin and lowegenin, some others, more rare aglycons, i.e. 7-ketotamusgenin, 7-ketodiosgenin, 25S-hydroxytamusgenin and afurigenin were also identified (González et al., 1970, 1971). However, the whole profile of steroids and triterpenoids, particularly occurring in this plant in a free form, has not been investigated yet. Thus, the main aim of the present study was the qualitative and quantitative GC-MS analysis of non-

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Fig. 1. *Tamus edulis* Lowe.

glycosylated steroids and triterpenoids occurring in leaves and tubers of *Tamus edulis* from Madeira island. Moreover, the determination of the main saponin occurring in the leaves and tubers were performed. Detailed biochemical investigation of *T. edulis* Lowe can shed more light on its taxonomy and help to solve some nomenclatural questions.

## 2. Results and discussion

### 2.1. Identification of steroids and triterpenoids occurring in leaves and tubers of *T. edulis*

GC–MS analysis of the fractions containing steroids and neutral triterpenoids obtained from diethyl ether extracts of leaves and tubers of analyzed plant revealed the presence of numerous steroids. Namely, several typical sterols (numbered according to their retention times), i.e. dominating sitosterol (6), accompanied by its saturated derivative, sitostanol (7), followed by cholesterol (1), campesterol (2), stigmasterol (5) and isofucosterol (8) were identified. Additionally, several steroids, i.e. cycloartanol (9), 24-methylenecycloartanol (14), cycloartenol (11), two derivatives of cholest-7-en-3-ol (4-methyl- and 2,2-dimethyl-cholest-7-en-3-ol, 3 and 4), and two ketones, tremulone (stigmasta-3,5-dien-7-one, 13) and sitostenone (15) were found in both leaf and tuber extracts. In turn, small peaks of pentacyclic triterpenoids, i.e.  $\alpha$ - and  $\beta$ -amyryns (10 and 12), were detected exclusively in tuber extract.

Table 1

Retention times and content of steroids and triterpenoids identified in *Tamus edulis* leaves and tubers.

Compound	Retention time [min]	LRI*	Tuber	Leaf
			( $\mu\text{g/g d.w.} \pm \text{S.D.}$ )	
Cholesterol (1)	31.52	3128	59.38 $\pm$ 7.22	46.25 $\pm$ 5.70
Campesterol (2)	34.22	3234	69.97 $\pm$ 8.01	41.93 $\pm$ 4.69
4-methyl-cholest-7-en-3-ol (3)	34.52	3245	11.06 $\pm$ 1.90	60.13 $\pm$ 5.81
2,2-dimethyl-cholest-7-en-3-ol (4)	34.86	3257	8.64 $\pm$ 1.16	17.15 $\pm$ 2.03
Stigmasterol (5)	35.12	3266	156.30 $\pm$ 22.05	91.50 $\pm$ 8.92
Sitosterol (6)	36.99	3325	453.79 $\pm$ 56.23	494.08 $\pm$ 61.38
Sitostanol (7)	37.23	3332	24.36 $\pm$ 2.88	25.72 $\pm$ 2.92
Isofucosterol (8)	37.48	3339	43.91 $\pm$ 4.75	70.01 $\pm$ 6.95
Cycloartanol (9)	37.87	3350	36.58 $\pm$ 4.02	50.16 $\pm$ 4.88
$\beta$ -Amyrin (in tubers) (10)	38.01	3354	16.42 $\pm$ 2.14	–
Cycloartenol (11)	39.34	3391	31.64 $\pm$ 3.02	30.54 $\pm$ 2.96
$\alpha$ -Amyrin (in tubers) (12)	39.62	3399	14.92 $\pm$ 1.66	–
Stigmasta-3,5-dien-7-one (13)	40.08	3410	56.81 $\pm$ 6.37	95.12 $\pm$ 8.04
24-methylenecycloartanol (14)	41.52	3442	27.19 $\pm$ 3.45	25.64 $\pm$ 2.80
Sitostenone (15)	42.40	3462	24.44 $\pm$ 2.90	9.02 $\pm$ 1.62
Sum of free triterpenoids:			1035.41	1057.25
Diosgenin (16)	35.80	3289	1600.56 $\pm$ 182.64	562.47 $\pm$ 60.15
Yamogenin (17)	37.30	3334	1264.28 $\pm$ 133.02	447.07 $\pm$ 51.11
Sum of aglycones:			2864.84	1009.54

\* Linear retention index.

As in the previous report on leaf extracts of other twelve *Dioscorea* spp. (Szakiel et al., 2017), no free form of diosgenin, the aglycone of the saponin dioscin typical for numerous *Dioscoreaceae* species, was detected in *T. edulis* samples analyzed in this study.

The prevailing compounds identified in the fractions of aglycones were diosgenin, (25R)-spirost-5-en-3-ol (16), and its isomer, yamogenin, (25S)-spirost-5-en-3-ol (17). They were found in both analyzed extracts, pointing to the occurrence of significant amounts of steroid saponins not only in the tubers, what is typical for *Dioscoreaceae* plants, but also in the leaves of *T. edulis*. The occurrence of several structurally related steroidal saponins in leaves and twigs of *T. edulis* was reported before (González et al., 1970, 1971), but only small amounts of unidentified saponin other than diosgenin and yamogenin were detected in the present study. From several steroids detected in free forms in diethyl ether extracts, in hydrolyzed methanolic extracts only small peaks of cholesterol and sitosterol were found, suggesting that these two sterols were present in plant also in the form of glycosides.

Free steroids can be unequivocally identified by their fairly consistent mass spectra, in which a molecular ion is usually easily observed, and it can have significant intensity (e.g., ion 386 of cholesterol or ion 414 in sitosterol). Another typical ion is  $[\text{M}-18]^+$ , which is produced after the loss of  $3\beta$ -hydroxy group as water. Further fragmentation by a retro-Diels-Alder reaction leads to other diagnostic ions. MS recognition of various types of triterpenoid isomers like  $\alpha$ -amyryn and  $\beta$ -amyryn, or diosgenin and yamogenin, can be made by comparison of intensity of some characteristic ion fragments. Fragmentation of both amyryns gives the base peak at  $m/z$  218 (due to retro-Diels-Alder rearrangement at C12) and accompanying ions at  $m/z$  189 and 203. However, the intensity of accompanying ions 189 and 203 is different, i.e. 189 is markedly higher than 203 in  $\alpha$ -amyryn, and inversely, 203 is higher than 189 in  $\beta$ -amyryn. Fragmentation of diosgenin and yamogenin gives one of the base ions at  $m/z$  282, and the characteristic accompanying ion at  $m/z$  139. The intensity of these two ions is significantly different in both isomers, i.e. 139 is notably higher than 282 in diosgenin, whereas in yamogenin the inverse ratio of these two peaks can be observed.

The calculated linear retention indices (Table 1) of analysed compounds were comparable to those reported in the literature (Isidorov and Szczepaniak, 2009).

The structures of identified compounds are presented in Figs. 2 and 3.

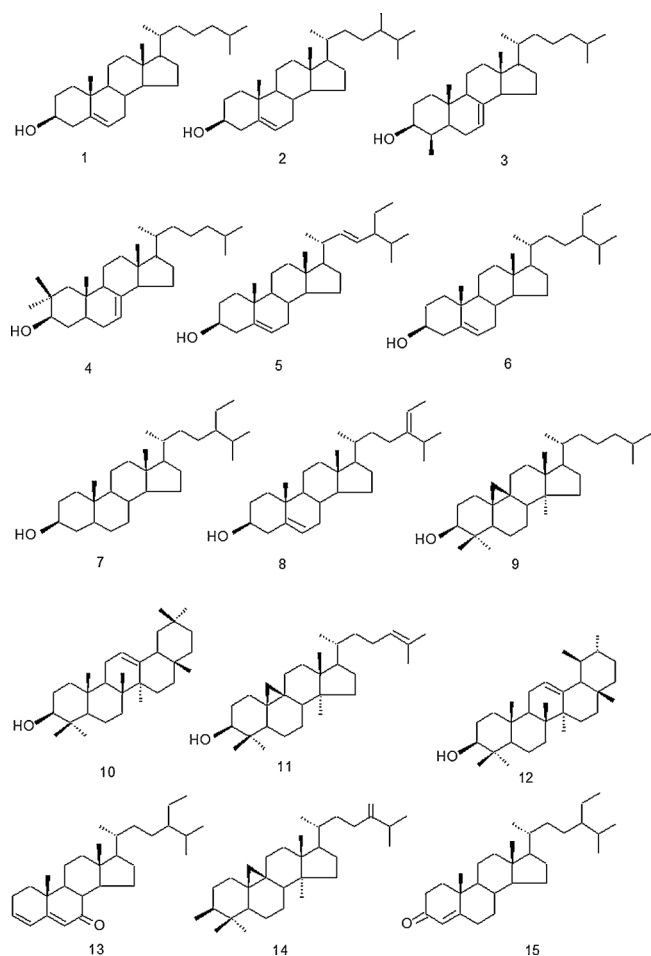


Fig. 2. Chemical structures of free steroids and triterpenoids identified in *T. edulis* leaves and tubers.

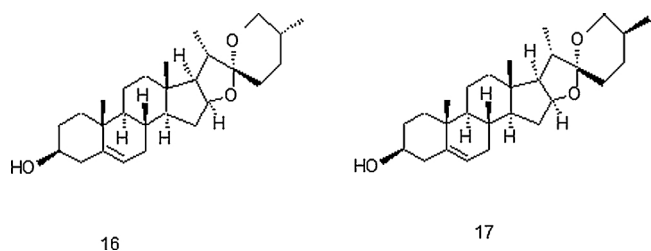


Fig. 3. Chemical structures of steroidal saponogenins identified in *T. edulis* leaves and tubers.

## 2.2. Quantification of steroids and triterpenoids occurring in leaves and tubers of *T. edulis*

The results of quantitative determination of identified compounds are presented in Table 1. The total contents of free steroids and triterpenoids in leaf and tuber extracts were similar and added up to approx. 1 mg/g of dry weight. This amount is relatively high as compared to the contents previously determined in other *Dioscorea* species, in which the amount of free steroids and triterpenoids ranged from 0.5 to 0.8 mg/g (with one exception of *Dioscorea caucasica* leaves containing very high level of pentacyclic triterpenoids). Sitosterol was the most abundant compound, constituting 44 and 47% of total contents in tubers and leaves, respectively. The second abundant phytosterol was stigmasterol. Tuber extracts additionally contained very small amount (approx. 31 µg/g) of pentacyclic triterpenoids.

The report on sterol profile in *Tamus communis* (current synonym:

*Dioscorea communis*) collected in Italy (Capasso et al., 1983) revealed that sitosterol, stigmasterol and campesterol were the most abundant in roots and leaves of this plant, with the relative ratio of approx. 2:1:1, similar in both organs (although different from the ratio of these compounds in berries). In the present study on *T. edulis* the ratios of these phytosterols were approx. 7:2:1 in tubers, and 12:2:1 in leaves, thus significantly different than in *D. communis*.

Analyzed hydrolyzates of methanolic extracts of *T. edulis* tubers and leaves contained high amounts of saponogens, i.e. diosgenin and yamogenin, and their total content accounted for almost 3 mg/g d.w. in tubers and 1 mg/g d.w. in leaves. Diosgenin was the prevailing isomer, constituting 56% of the sum of saponogens both in tubers and leaves. Although the amount of aglycones in *T. edulis* leaves was found to be almost 3-times lower than in tubers, the leaves can still be considered as an important source of steroidal saponins.

Diosgenin is the dominant aglycone of the majority of saponins found in plants of various *Dioscorea* species, including *D. communis*, in which several glycosides having diosgenin as aglycone (i.e. dioscin and gracillin, as well as methylprotodioscin, methylprotogracillin and methylprotoneodioscin) were identified (Aquino et al., 1986, 1991). However, the saponin profile of *T. edulis* reported previously by González et al. (1970, 1971) seems to support a supposition of a distinction of these two species.

The obtained results reveal that *Tamus edulis* has some features typical for all *Dioscorea* species, e.g., the presence of steroidal saponins with diosgenin and yamogenin as aglycones, the absence of free saponogens, the phytosterol composition with predominating sitosterol. However, some other traits, like the relatively high content of free steroids (more than 1 mg/g d.w.) and their profile rich in cholesterol derivatives, can distinguish *Tamus edulis* from other *Dioscorea* species studied to date for their steroid and triterpenoid profile (Szakiel et al., 2017).

## 3. Experimental

### 3.1. Plant material

Leaves and tuberous roots of *T. edulis* used in this study were collected in September 2017, in the northwest part of Madeira Island (32°51.3043' N, 17°10.4078' W). After collection, samples were dried and homogenized.

### 3.2. Extraction and fractionation

Dried tubers and leaves were homogenized to a fine powder in a grinding mortar, then extracted first with diethyl ether, and afterwards with methanol (each extraction lasting 8 h) in a Soxhlet apparatus. The obtained extracts were evaporated to dryness at 40 °C under reduced pressure. The diethyl ether extracts were fractionated by preparative TLC in the solvent system CHCl<sub>3</sub>/MeOH (97:3, v/v). The methanolic extracts were subjected to acid hydrolysis by boiling under reflux with 10% hydrochloric acid in methanol for 120 min and the aglycones liberated from glycosides were extracted from hydrolyzates with diethyl ether. The fractions of diethyl ether extracts containing free triterpenoids, and the aglycones from hydrolyzed saponins obtained from methanolic extracts, were analyzed directly by GC–MS.

### 3.3. Identification and quantification

The analyses were performed using an Agilent Technologies 7890 A gas chromatograph (Perlan Technologies, Warsaw, Poland) coupled to 5975C mass spectrometric detector and equipped with a flame ionization detector, FID. All of the samples were dissolved in diethyl ether/methanol (5:1, v/v) and a volume of 1–4 µl was applied by split injection 1:10. The GC separation was made on a HP-5MS UI column of 30 m x 0.25 mm i.d., film thickness 0.25 µm (Agilent Technologies) in

the temperature programme: initial temperature of 160 °C held for 2 min, then increased to 280 °C at 5 °C/1 min and the final temperature of 280 °C held for further 44 min with constant helium flow rate of 1 ml min<sup>-1</sup>. For GC–MS/FID detection the following parameters were employed: inlet and a flame ionization detector (FID) temp. 290 °C, MS transfer line temp. 275 °C, quadrupole temp. 150 °C, ion source temp. 230 °C, EI 70 eV, *m/z* range 33–500; FID–H<sub>2</sub> flow 30 ml min<sup>-1</sup> (hydrogen generator), air flow 400 ml min<sup>-1</sup>. The individual compounds were identified by comparing their mass spectra with library data from Wiley 9th ED. & NIST 2008 Lib. SW (Version 2010) or data from the literature, and by comparison of their retention times and corresponding mass spectra with those of authentic standards: α-amyrin (Roth, Karlsruhe, Germany); β-amyrin, campesterol, cholesterol, diosgenin, sitosterol, and stigmaterol (Sigma-Aldrich, Steinheim, Germany). The standards were of GC grade > 98.5%. Retention indices were calculated with the use of equation  $LRI = 100(n + t_n/t_{n+1} - t_n)$ , where *t* = retention time of component, *n* = carbon number of preceding n-alkane, *n* + 1 = carbon number of subsequent n-alkane from homologous series of n-alkanes. The identified compounds were quantified using external standard method based on calibration curves determined for typical representatives of each triterpenoid group analyzed in this study: α-amyrin for monohydroxyalcohols, and stigmaterol for steroids. Calibration curves were drawn between the peaks areas versus the concentration of α-amyrin (linear regression equation  $y = 45800x - 300$ ) and stigmaterol ( $y = 35900x - 550$ ) in the range of 0.002–2.0 mg/ml. The correlation coefficient (*r*<sup>2</sup>) values of α-amyrin was 0.999 and for stigmaterol 0.998. RSD values of peak areas were less than 4%. The LOD (Limit of Detection) was determined by analysis of samples with decreasing level of concentration of analyte, value for α-amyrin was 0.8 μg/ml, for stigmaterol 1.0 μg/ml. A 2.5 μg/ml and 3.2 μg/ml limit of quantification (LOQ) was attained for α-amyrin and stigmaterol, respectively.

### 3.4. Statistical analysis of data

All data are presented as the means ± standard deviation of three independent samples analyzed in triplicate.

### Declaration of interest

None.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.phytol.2019.01.012>.

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