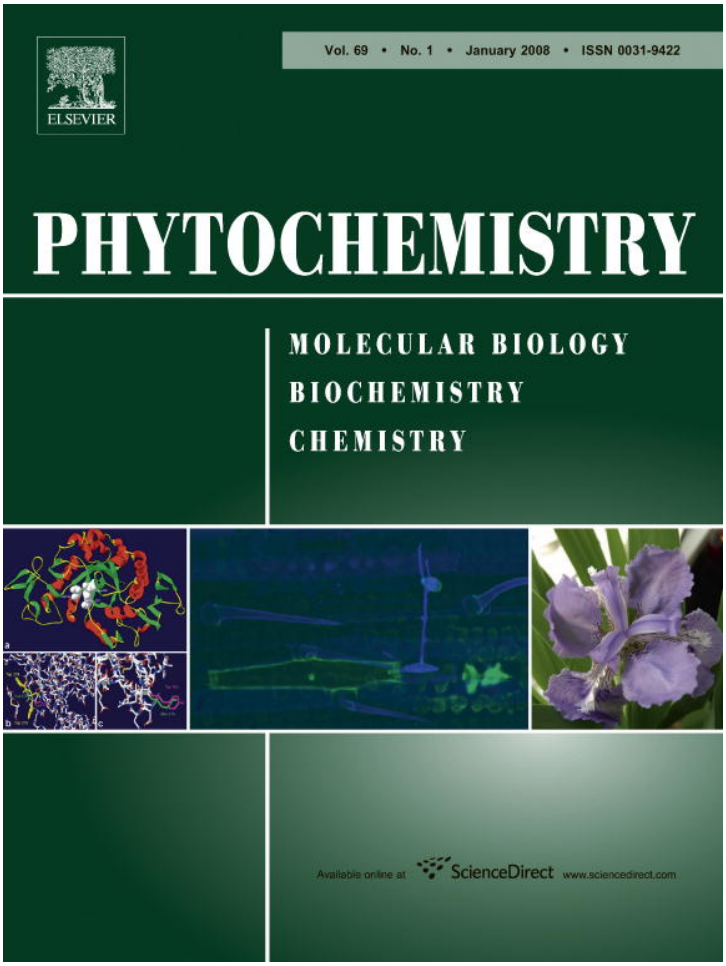

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Phytochemistry 69 (2008) 176–186
PHYTOCHEMISTRY
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Distribution of steroidal saponins in *Tribulus terrestris* from different geographical regions

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Received 4 May 2007; received in revised form 29 June 2007
Available online 23 August 2007

Abstract

The steroidal saponins of *Tribulus terrestris* L. (Zygophyllaceae) are considered to be the factor responsible for biological activity of products derived from this plant. The activity depends on the concentration and the composition of active saponins, which in turn is influenced by the geographical origin of plant material. Samples of *T. terrestris* collected in Bulgaria, Greece, Serbia, Macedonia, Turkey, Georgia, Iran, Vietnam and India were analyzed by LC-ESI/MS/MS for the presence and the concentration of protodioscin (**1**), prototribestin (**2**), pseudoprotodioscin (**3**), dioscin (**4**), tribestin (**5**) and tribulosin (**6**). The flavonoid rutin (**7**) was also included in the comparison. The results revealed distinct differences in the content of these compounds depending on region of sample collection, plant part studied and stage of plant development. The samples from Bulgaria, Turkey, Greece, Serbia, Macedonia, Georgia and Iran exhibited similar chemical profile and only some quantitative difference in the content of **1–7** with protodioscin (**1**) and prototribestin (**2**) as main components. The Vietnamese and Indian samples exhibit totally different chemical profile. They lack **2** and **5**, while tribulosin (**6**) is present in high amounts. Compounds different from **1** to **7** are dominating in these 3 samples. The presented results suggested the existence of one chemotype common to the East South European and West Asian regions. Most probably, the Vietnamese and Indian samples belong to other chemotypes which are still to be studied and characterized. No clear correlation between the burrs morphology and the chemical composition of the samples has been found.

Keywords: *Tribulus terrestris*; Zygophyllaceae; LC-MS analysis; Steroidal saponins; Protodioscin; Prototribestin; Flavonoid; Rutin; Chemotypes; Morphology-chemical composition correlation

1. Introduction

Tribulus terrestris L. (Zygophyllaceae) is an annual plant native of Mediterranean region, but now widely distributed in the warm regions all over the world (Frohe, 1999). It is used in the folk medicine of India, China, Bulgaria and South Africa against sexual impotence, oedemas, abdominal distention and cardiovascular diseases (Kostova et al., 2002). Many pharmaceutical preparations and food supplements based on the saponin fraction from this plant are on sale worldwide (Tomova et al., 1981; Xu et al., 2000; Cai et al., 2001; Adimoelja and Adaikan, 1997; Mulinacci et al., 2003).

Most of the phytochemical investigations described in the literature refer to *T. terrestris* of Chinese, Indian and Bulgarian origin. There are limited studies on the saponins in the same plant species from Turkey, Moldova, South Africa, Australia, Azerbeidjan and Romania. A careful examination of the available literature data (Kostova and Dinchev, 2005) revealed that:

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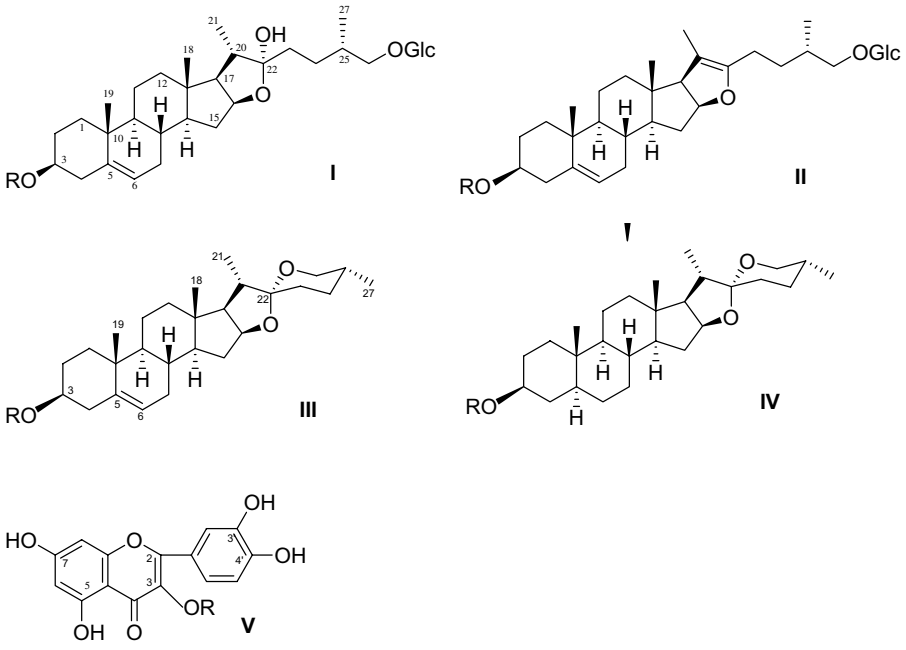
doi:10.1016/j.phytochem.2007.07.003

(a) Saponins with a *cis* A/B – rings juncture are found only in *T. terrestris* from China.
(b) Saponins of gitogenin type are not present in *T. terrestris* of Indian and Bulgarian origin, while saponins of tigogenin, gitogenin and hecogenin type predominate in *T. terrestris* from China.
(c) Sulphated spirostanol and furostanol saponins are isolated only from *T. terrestris* of Bulgarian origin.
(d) Protodioscin (**1**) has been considered as the most dominant component of *T. terrestris*.

However, the studies showed that **1** is present in different amounts in samples from China, India and Bulgaria (Ganzera et al., 2001). Moreover, the Indian sample showed totally different saponin profile. These authors observed also significant differences in the saponin composition and the saponin content depending on the geographical region of sample collection and suggested the presence of chemotypes.

Till now the publications on the isolation and structure elucidation of the chemical components (including saponins and flavonoids) of *T. terrestris* do not offer any morphological description of the studied plant samples. This makes the proper explanation of the observed differences impossible. Any correlation between the chemical composition and the morphology that could lead to some taxonomic conclusions was not provided.

This prompted us to undertake a comparative investigation on the saponins in *T. terrestris* from different geo-



Compound	Genin	Name	R
1	I	protodioscin	−β-D-Glc^4-α-L-Rha ^{2} α-L-Rha
2	I	prototribestin	−β-Glc^2-α-Rha ^{4} OSO_3Na
3	II	pseudoprotodioscin	−β-D-Glc^4-α-L-Rha ^{2} α-L-Rha
4	III	dioscin	−β-D-Glc^4-α-L-Rha ^{2} α-L-Rha
5	III	tribestin	−β-Glc^2-α-Rha ^{4} OSO_3Na
6	IV	tribulosin	−β-D-Gal^4-β-D-Glc^3-β-D-Xyl ^{2} ^{2} α-L-Rha β-D-Xyl
7	V	rutin	-β-D-Glc^6-α-L-Rha

Fig. 1. Chemical structures of the standards for LC-MS/MS investigations.

graphical regions and to look for a reasonable explanation of the above mentioned differences. For the purposes of the comparison we have developed and validated a LC-ESI/MS method with selected ion monitoring to quantify some components. Compounds **1–5** and **7** are typical of Bulgarian *T. terrestrsis* (Kostova et al., 2002; Conrad et al., 2004; Panova and Tomova, 1970; Gyulemetova et al., 1982), while tribulosin (**6**) is the main component of Indian *T. terrestris* (Mahato et al., 1981; Deepak et al., 2002). The biostimulating activity of **1** and **2**, the cytotoxic effects of **1**, **3** and **4**, and the anthelmintic properties of **6** are reported in the literature (Tomova et al., 1981; Kostova et al., 2002; Hu and Yao, 2002; Chiang et al., 1991; Kostova and Dinchev, 2005 and the references therein; Deepak et al., 2002;

Table 1
Collected plant material

Origin	Voucher	Plant part	Stage	Collection date
Bulgaria				
Haskovo	SOM Co-1162	Aerial parts	Flowering–seeding	07 August 2003
		Fruits		07 August 2003
		Leaves		07 August 2003
		Stems		07 August 2003
Pomorie	SOM Co-1163	Aerial parts	Flowering–seeding	18 August 2004
Petrich	SOM Co-1164	Aerial parts	Flowering–seeding	22 July 2004
Svistov	SOM Co-1165	Aerial parts	Flowering–seeding	August 2004
Ropotamo	SOM Co-1166	Fruits		27 July 2004
Smokinia	SOM Co-1167	Fruits	Flowering–seeding	27 July 2004
Plovdiv	SOM Co-1168	Aerial parts	All stages of development	May–September 2005
Turkey				
Marmaris	SOM Co-1169	Aerial parts-*hirsutum* fruits	Flowering–seeding	08 July 2004
		Fruits		08 July 2004
	SOM Co-1179	Aerial parts-*glabrous fruits*		07 July 2004
		Fruits		07 July 2004
Ayvalak	SOM Co-1170	Aerial parts	Flowering–seeding	03 July 2004
Ankara[a]	SOM Co-1171	Aerial parts	Seeding	September 2004
		Fruits		September 2004
Yatagan	SOM Co-1172	Aerial parts	Flowering–seeding	04 July 2004
Greece				
Kalambaka, Thesalia	SOM Co1173	Aerial parts	Seeding	30 October 2004
Rhodes Island	SOM Co-1174	Aerial parts	Flowering–seeding	06 July 2004
Macedonia				
Bogdanzi	SOM Co-1175	Aerial parts	Flowering–seeding	24 July 2004
Novo Selo, Belasica mounatin	SOM Co-1176	Aerial parts	Flowering–seeding	23 July 2004
Serbia				
Nish[a]	SOM Co-1177	Aerial parts	Flowering–seeding	August 2003
Georgia				
Tbillissi	SOM Co-1178	Aerial parts	Flowering–seeding	August 2005
Vietnam[b]				
	SOM Co-1180	Aerial parts		2003
		Fruits		2003
India				
Jodhpur, Rajasthan	SOM Co-1181	Fruits	Seeding	09 September 2004
		Leaves		
		Stems		
Bangalore, Karnataka	SOM Co-1182	Fruits		April 2004
Iran				
Mashhad, Khorasan	SOM Co-1183	Aerial parts	All stages of development	July–September 2005

[a] Powdered samples were obtained.
[b] The sample was powdered without estimating of the burr's morphology; collection area not known-the sample was provided by Sopharma J.S.C., Sofia.

Dong et al., 2004). This study is an attempt to correlate the morphology and the chemical composition of the studied samples.

2. Results and discussion

2.1. Quantitative HPLC-ESI/MS determination of compounds 1–7

The first quantitative determination of steroidal saponins in *T. terrestris* originating from Bulgaria, India and China was achieved by HPLC and ELS detection (Ganzera et al., 2001). The marker compound, protodioscin, could be detected by this method at a concentration as low as 10.0 μg/ml. All the samples from Bulgaria were found to contain a rather high percentage (0.245–1.337%) of protodioscin. The samples from China contained either no or only small amounts (0.063% and 0.089%) of this compound. But protodioscin was a minor component (0.024%) in the sample from India. Analysis of market products showed considerable variation in the protodioscin content.

Among the various methods (Gyulemetova et al., 1982; Oleszek, 2002; Liu et al., 2004) that have been applied to the analysis and identification of saponin mixtures LC-MS appears to be the most suitable. Two papers have been already published on the analysis of saponins in *T. terrestris* using LC-MS. The first one developed an HPLC-ELSD-ESI-MS method for separation and identification of steroidal saponins in the aerial parts of *T. terrestris* (Combarieu et al., 2003), the second proposed an HPLC-ESI-MS method in a positive ion mode for qualitative analysis of saponins in dietary supplements of *T. terrestris* (Mulinacci et al., 2003). In the same publication the authors described a semi-quantitative evaluation of the total saponin content by HPLC-MS using protodioscin as external standard.

In the present investigation the steroidal saponins **1–6** and rutin (**7**) (Fig. 1) in various samples of *T. terrestris* (Table 1) were profiled and quantified using reverse phase HPLC with on line photodiode array detector and ESI mass spectrometer.

To obtain correct and reliable results we extracted the saponins with 70% ethanol three times in a ratio 1:50. This procedure provides a good extraction and in the exhausted plant material the analyzed compounds are under the limits of quantification (LOQ). Ethanol was selected as a solvent for extraction in this study because in methanol the furostanol saponins rapidly convert into the corresponding 22-*O*-methyl derivatives (Ganzera et al., 2001; Wang et al., 2003).

Solid-phase extraction (SPE) of the samples on C-18 Sep-Pak cartridges provided samples that did not show many interfering impurities when injected by the syringe pump to the ESI-MS and all the seven investigated compounds were unambiguously and selectively determined among the number of peaks in mass range 400–2000 amu.

Full identification of the saponins **1–6** and rutin (**7**) present in the standard mixture was achieved first by investigation of their mass spectral data and then by co-chromatography with the authentic standards. The retention times and the mass values of the $[M-H]^-$ pseudomolecular ions for **2** and **5** or of the $[M-H+CH_3COOH]^-$ ions for the other standards are shown in Table 2. Under the separation conditions the peaks of **4** and **6** are overlapped but the mass values of their $[M-H+CH_3COOH]^-$ ions are so different, that there was no problem to integrate and to get reliable results.

For the quantitative determination of these seven compounds it was necessary to prepare standard calibration curves. For this purpose the stock solution was diluted to nine different concentrations ranging from 0.03 to 145.00 μg/ml for each individual compound. All standards showed good linearity in the low concentration range between 0.03 and 5.00 μg/ml. For **2** a good linearity was observed in the range of 0.06–145.00 μg/ml, as well. The standard curves showed good linearity, with R^2 values not less than 0.936 for all concentration ranges used.

For evaluation of the method precision and extraction/purification repeatability, six samples from the same plant powder were independently extracted and purified with SPE procedure and for each sample three independent LC-MS runs were performed. Relative standard deviations for repetition of one sample ($n = 3$) was ~3% and for six independent extraction ranged from 2.5% to 12.8%. It should be noted that the HPLC-MS method developed by us for the purpose of this investigation allows a good separation and detection of saponins **1–6** in amounts as low as 0.2–0.3 ppm.

The results from the quantitative determination of compounds **1–7** in the analyzed samples are presented in Tables 3 and 4. A general observation is that protodioscin (**1**) was present in all samples under investigation. Its content varied from 2.4 to 10270.3 ppm depending on the plant part studied and the region of collection. All samples from Vietnam and India contained very low amounts (2.4–32.7 ppm) of this saponin. The content of **1** in the remaining samples is higher.

The sulphur containing furostanol saponins prototribestin (**2**) and tribestin (**5**) were not detected in the samples from Vietnam and India. However, they were present in all samples from Bulgaria, Turkey, Greece, Macedonia,

Table 2
Retention times (R_t) and $[M-H]^-$ or $[M-H+CH_3OOH]^-$ ions of compounds **1–7**

Peak no.	Compound	R_t (min)	ESI/MS
1	**7**	14.92	609.20 $[M-H]^-$
2	**2**	16.27	981.70 $[M-H]^-$
3	**1**	18.03	1107.30 $[M-H+CH_3COOH]^-$
4	**3**	20.92	1089.30 $[M-H+CH_3COOH]^-$
5	**5**	24.25	801.50 $[M-H]^-$
6	**4**	32.10	927.30 $[M-H+CH_3COOH]^-$
6	**6**	32.10	1209.20 $[M-H+CH_3COOH]^-$

Table 3
Content of compounds **1–7** in *T. terrestris* from different geographical regions (μg/g of dry wt)

Sample/plant parts	1	2	3	4	5	6	7
Bulgaria							
Haskovo hirsut. fr.							
a.p.	6519.8	2266.0	384.1	100.6	24.1	77.3	1455.2
Fruits	567.9	280.6	442.0	26.1	9.2	26.5	762.9
Leaves	10003.5	7317.5	357.6	895.7	627.3	7.9	2037.3
Stems	193.3	408.8	157.5	8.7	27.7	1.7	92.4
Pomorie hirsut. fr.							
a.p.	1917.2	1346.4	0.7	921.9	2266.4	1.8	41.9
Petrich hirsut. fr.							
a.p.	4305.6	1899.1	41.4	587.1	623.8	1.1	2196.9
Svistov hirsut. fr.							
a.p.	3619.2	3319.1	8.9	201.3	692.6	4.9	450.0
Ropotamo hirsut. fr.							
Fruits	549.9	217.2	78.7	61.2	34.6	–	167.2
Smokinia glabr. fr.							
Fruits	597.5	226.3	44.2	56.1	21.9	–	121.2
Turkey							
Marmaris hirsut. fr							
a.p.	3426.9	3948.4	40.9	129.2	286.1	0.3	170.3
Fruits	254.9	727.0	–	18.6	10.9	–	287.9
Marmaris glabr. fr.							
a.p.	3439.1	2710.4	92.9	66.1	84.0	10.2	84.0
Fruits	170.3	59.5	83.1	21.9	5.5	–	124.4
Ankara							
a.p.	10270.3	4989.1	748.7	93.4	68.9	17.4	349.1
Fruits	652.6	187.3	12.2	21.7	9.5	1.4	240.8
Ayvalak hirsut. fr.							
a.p.	3126.8	3698.2	30.7	111.9	131.7	2.2	524.6
Yatagan glabr. fr.							
a.p.	4649.2	3781.9	38.6	130.5	136.5	–	118.7
Greece							
Rhodes hirsut. fr.							
a.p.	7968.7	3463.2	801.3	311.7	244.3	13.0	1213.6
Kalambaka glabr. fr.							
a.p.	2265.7	1526.7	302.5	260.3	0.4	24.0	1007.7
Macedonia							
Novo Selo glabr. fr							
a.p.	4230.9	1961.9	14.3	135.7	73.8	–	408.6
Bogdanzi glabr. fr.							
a.p.	9996.1	7429.0	1049.3	158.6	106.7	6.8	1620.5
Serbia							
Nish							
a.p.	2033.4	1698.3	198.9	879.5	2151.8	22.4	801.0
Georgia							
Tbillissi hirsut. fr.							
a.p.	5661.9	2419.1	534.3	80.1	67.2	5.6	1287.4
Vietnam							
a.p.	32.7	–	1.3	0.4	–	220.5	4.8
Fruits	13.0	–	3.9	–	–	4196.9	24.1
India							
Rajasthan hirsut. fr.							
Fruits	2.4	–	1.1	–	–	11.2	464.5
Leaves	14.0	–	10.0	–	–	6442.1	76.8
Stems	4.0	–	–	–	–	1850.8	110.7
Bangalore hirsut fr.							
Fruits	6.5	–	–	–	–	10.9	76.2

[a] Not detected.

Serbia, Iran and Georgia. In most of the latter samples compound **2** was the second most dominant component after protodioscin. It was the dominating saponin in three samples from Turkey: in two from Marmaris and one from Ayvalak. Tribestin (**5**) was the main component of the aerial parts of *T. terrestris* from Pomorie (Bulgaria) and Nish (Serbia). So far, these two compounds have been reported to occur only in *T. terrestris* from Bulgaria (Kostova et al., 2002; Conrad et al., 2004; Matschenko et al., 1990) and this is the first report for their occurrence in samples from other regions.

With a few exceptions tribulosin (**6**) was found in almost all samples in this study. Its content varied from 0.3 to 6442.1 ppm depending also on the sample origin and the plant part under investigation. It was the main saponin component of the samples from India and Vietnam. So far, this compound has been isolated only from *T. terrestris* from India (Calcutta and Bangalore) (Mahato et al., 1981; Deepak et al., 2002). Its *R* analogue has been found in *T. terrestris* from China (Xu et al., 2000; Wang et al., 1996; Huang et al., 2003). For the purpose of our HPLC-MS investigation we have used authentic sample of tribulosin, provided by Dr. A. Agrawal from Bangalore (Deepak et al., 2002). It is clear that in the absence of a standard of the *R* isomer of tribulosin for HPLC comparison it is difficult to decide about the stereochemistry of this component in the various samples.

2.2. Content of compounds 1–7 in different plant parts

The content of saponins **1–6** and rutin (**7**) in the different plant parts of *T. terrestris* was evaluated for one sample from Bulgaria (Haskovo) and one sample from India (Rajasthan). Table 3 demonstrates that in both cases the total content of all saponins is highest in the leaves, followed by the stems and the fruits. This result is in line with previous findings of the American researchers (Ganzera et al., 2001).

2.3. Content of compounds 1–7 depending on the stage of plant development

The variations in the content of compounds **1–7** in the aerial parts of *T. terrestris* depending on the stage of plant development were also analyzed. Samples from Bulgaria (Plovdiv) and Iran (Mashhad) have been collected for this comparison. The results for these two distant regions are similar (Table 4). A general observation is that the content of saponins **1–5** increases from the pre-flowering to flowering stage, then decreases and reaches its minimum in the flowering–seeding and increases again in the seeding stage. The maximum of the accumulation of these saponins is in the flowering stage for the Bulgarian sample and in the pre-flowering stage for the Iranian sample (a sample from the flowering stage was not available). The accumulation of tribulosin (**6**) in the Bulgarian sample follows a slightly different way with a maximum in the pre-flowering stage and

Table 4
Content of saponins **1–6** and rutin (**7**) in the aerial parts of *T. terrestris* depending on the stage of plant development (μg/g of dry wt)

Sample	Stage	1	2	3	4	5	6	7
Bulgaria, Plovdiv – *glabr. fr.*								
29.05.05	Pre-flowering	3506.2	3547.4	214.4	162.2	211.5	57.9	443.2
28.06.05	Flowering	3749.5	3580.4	144.0	185.5	548.1	15.0	404.2
25.07.05	Flowering–seeding	2240.7	2615.3	89.5	126.7	382.1	2.2	113.5
23.08.05	Flowering–seeding	1956.7	1977.7	4.7	37.7	51.8	11.8	111.5
27.09.05	Seeding	2069.0	2713.7	61.1	70.6	86.4	14.7	118.0
Iran, Mashhad – *hirsut. fr.*								
02.07.05	Pre-flowering	5090.3	3645.7	1.5	30.4	45.6	–	106.9
01.08.05	Flowering–seeding	1060.1	874.5	1.4	10.4	13.9	–	92.3
20.09.05	Seeding	2270.3	2614.5	7.9	43.7	63.6	2.0	23.3
20.11.05	Seeding	268.3	1663.3	–	30.3	167.1	–	–

a minimum in the flowering–seeding stage. In the sample from Iran saponin **6** was detected only in the seeding stage.

2.4. Chemotypes

2.4.1. TLC comparison of the investigated samples

A TLC comparison exhibited distinct differences in the furostanol saponin profile of the investigated samples. The samples from Bulgaria, Turkey, Greece, Serbia, Macedonia, Georgia and Iran contained protodioscin (**1**) and prototribestin (**2**) as main components, while in those from Vietnam and India a more polar saponin was dominating, which correlates with the LC-MS findings.

A TLC comparison of the analyzed samples revealed that their flavonoid profiles were also distinctly different. However, of the flavonoids only rutin (**8**) has been included in present comparison. The data in Tables 3 and 4 show that rutin is present in all samples. The Vietnamese samples contain the least amount of this compound. Its content varies depending on the stage of plant development (Table 4). The distribution of the flavonoids in the studied samples is expected to provide important information about the chemotaxonomy of *T. terrestris* and will be subject of our future investigations.

2.4.2. Differences in the LC-MS profiles

The LC-MS chromatograms of the aerial part and fruit samples analyzed in this study reveal that the samples from Bulgaria, Turkey, Greece, Serbia, Macedonia, Georgia and Iran have similar profiles. This is demonstrated by the chromatograms of the samples from Haskovo (Bulgaria) and Rhodes (Greece) (Figs. 2 and 3). In this group only quantitative differences in the content of compounds **1–7** are observed (Tables 3 and 4). These samples are rich in protodioscin and prototribestin, while tribulosin is either absent or present in very low amounts (0.3–73.0 ppm).

As seen from Figs. 2 and 3 the Vietnamese and Indian samples exhibit totally different chemical fingerprintings. The chemical profile of the sample from Bangalore is different from that of Rajasthan, as well as from that of Vietnam. These samples lack protodioscin and prototribestin, while tribulosin is present in good amounts (Table 3). It is also evident from the chromatograms that compounds different from **1** to **7** are dominating in these 3 samples.

These observations give arguments to propose the existence of one chemotype common to the East South European and West Asian regions. Prototribestin (**2**) and tribestin (**5**) could be used as chemotaxonomic markers of this chemotype, as they are present in all samples from this group and totally lack in the samples from India and Vietnam (Tables 3 and 4). Most probably, the Vietnamese and Indian samples belong to other chemotypes which are still to be studied and characterized. Samples from China were not available for the present study. However, previous publications clearly show the great differences in the saponin composition of samples from China, India and Bulgaria (Kostova and Dinchev, 2005; Ganzera et al., 2001).

The present research showed significant differences in chemical fingerprinting and in the content of the saponins **1–6** in the investigated samples from the various geographical regions. Several factors could be responsible for these differences. The first factor could be the influence of the climate which is so different in the studied geographical regions – monsoon in South India and Vietnam, arid in Rajasthan (India) and Mash had (Iran), subtropical in Greece, Turkey and Georgia, and temperate in Bulgaria, Serbia and Macedonia. However, the observed differences do not correlate with the samples' origin based on the climate zones. The second factor could be the possible divergence caused by isolation of the plant populations in the geographical regions eastern from Iran. The probable border between them could be in the region East from Iran, as Tar desert, and the mountains in Pakistan and Afghanistan.

The saponin fraction of *T. terrestris* is included in many food supplements with a claim of a general stimulating action on motor activity, muscle tone and restorative tonic for vigour, as well as in preparations (Tribestan, Libilov) used for treatment of male infertility and libido disorders in men and women (Mulinacci et al., 2003). The furostanol saponins and protodioscin (**1**) and prototribestin (**2**) in particular have been found to be responsible for the general stimulating activity with emphasis upon sexual system of the saponin fraction of *T. terrestris* of Bulgarian origin and Tribestan. (Tomova et al., 1981; Kostova et al., 2002; Kostova and Dinchev, 2005). Clinical trial proved

that Libilov and protodioscin (**1**) are effective in treatment of male infertility and increase the level of dehydroepiandrosterone in infertile men (Adimoelja and Adaikan, 1997; Adimoelja, 2000). It is suggested that this hormone participates in improvement of cell membrane integrity and function of cellular level, in improvement of circulation, health and sense of well being. This indirectly results in improved sex drive. Because of these activities *T. terrestris* extracts find a wide application and in connection of quality assurance of the various market products it is important to measure their protodioscin content.

2.5. Burr morphology – saponin composition correlation

According to the botanists the morphology of *T. terrestris* is extremely variable and the species is divided into 2–3 subspecies or varieties based on the degree of hairiness and presence or absence of basal spines (Boissier, 1867; Hayek,

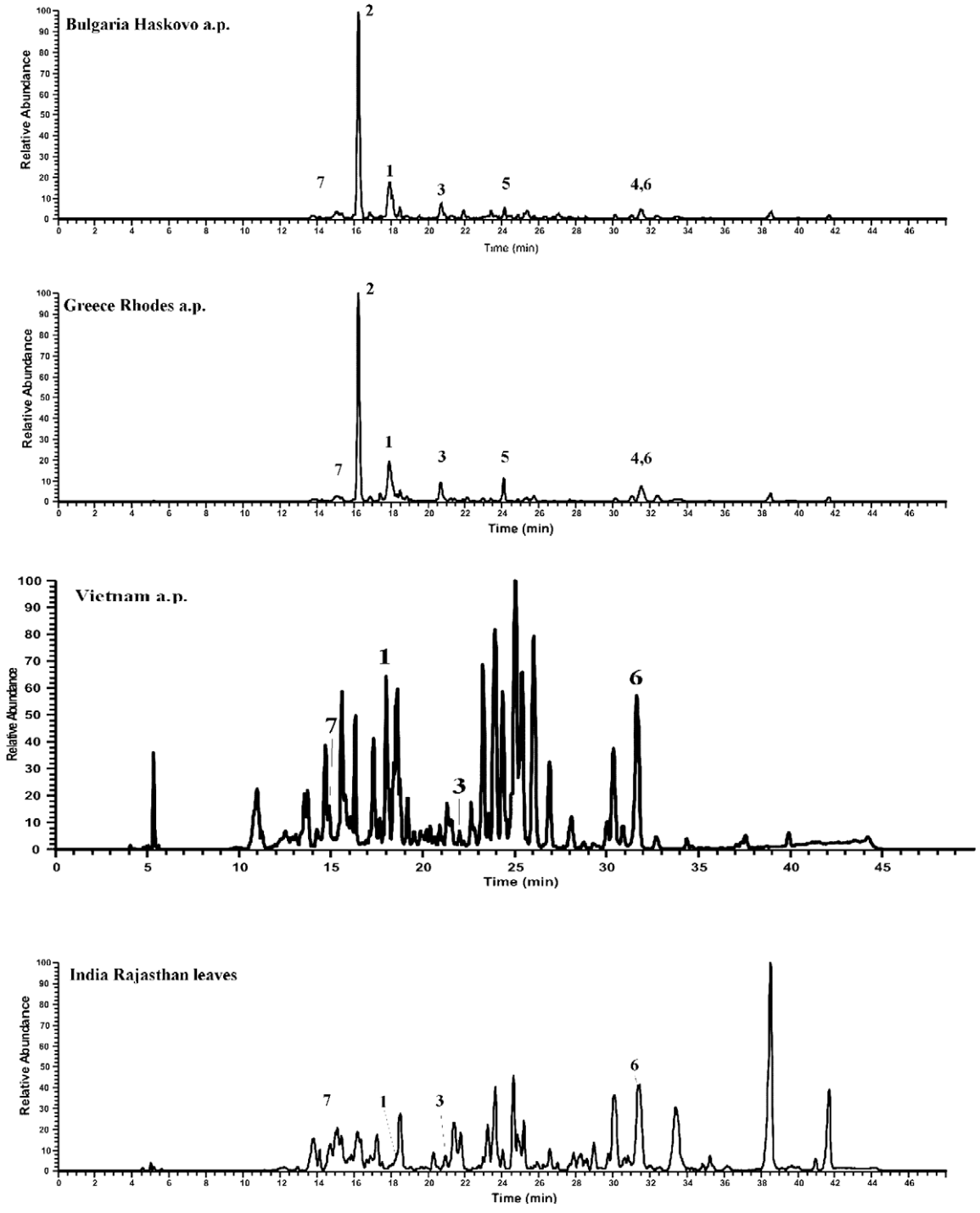


Fig. 2. LC-MS profiles of selected aerial parts and leaves samples from Bulgaria, Greece, Vietnam and India. **1** – protodioscin; **2** – prototribestin; **3** – pseudoprotodioscin; **4** – dioscin; **5** – tribestin; **6** – tribulosin; and **7** – rutin.

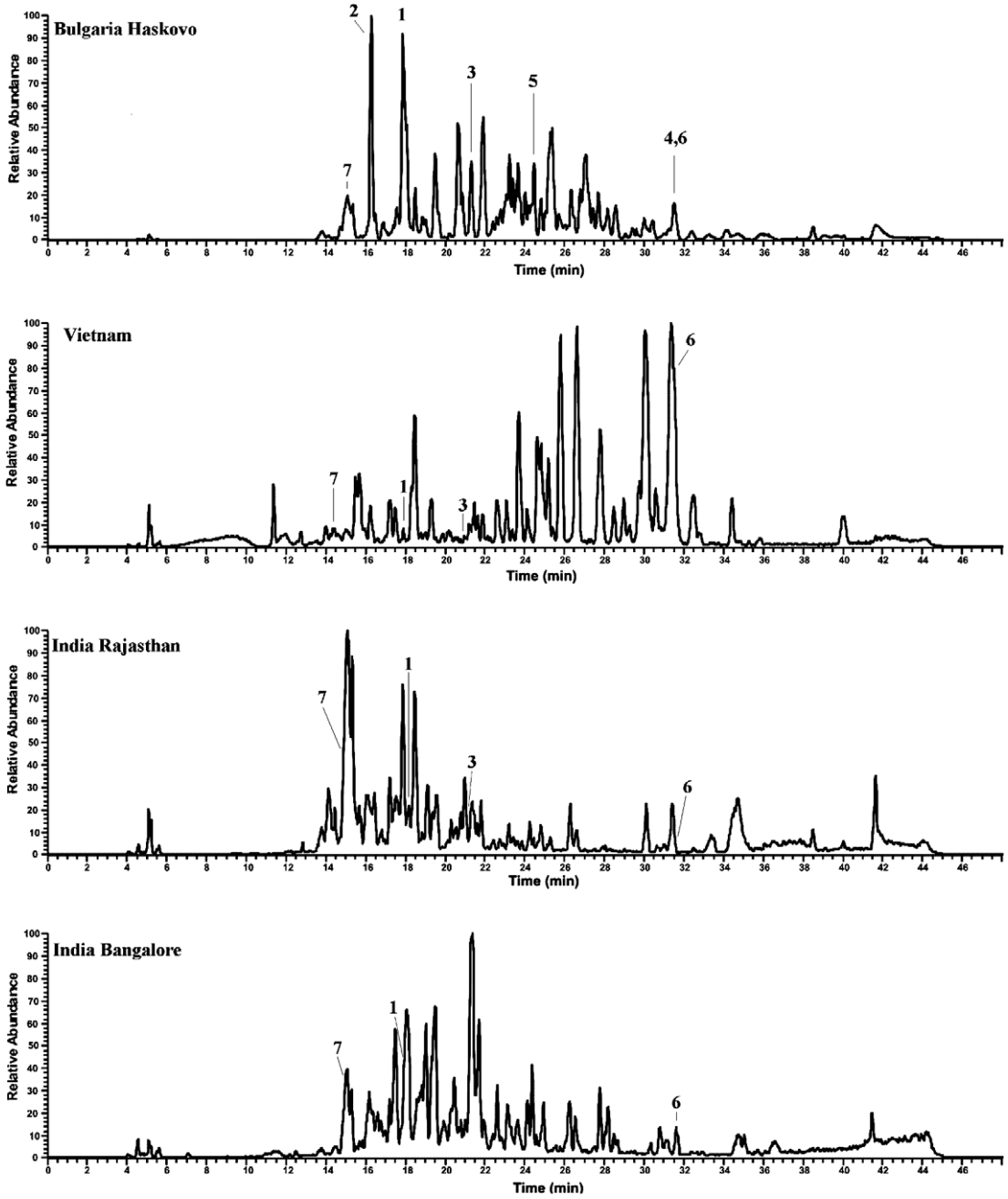


Fig. 3. LC-MS profiles of selected fruit samples from Bulgaria, Vietnam and India. **1** – protodioscin; **2** – prototribestin; **3** – pseudoprotodioscin; **4** – dioscin; **5** – tribestin; **6** – tribulosin; and **7** – rutin.

1925; Tutin, 1968; Petrova, 1979). Variations in burr morphology have been also reported (Scott and Morrison, 1996). They measured four size variables, four spine angles and the number of the seeds in each burr from 31 Australian and overseas collection sites and identified four groups of burrs.

In order to follow the dependence of the saponin content on the degree of hairiness the samples of *T. terrestris* with hairy and glabrous fruits from the region of Marmaris (Turkey) were analyzed. We observed that prototribestin (**2**) is the main component in the hairy samples, while protodioscin (**1**) is dominating in the glabrous ones (Table 3). The same observation was valid for the Turkish samples from Ayvalak and Yatagan, while the European samples did not follow this tendency. Furthermore, a great variability was observed in the content of **1** and **2** in samples from the regions of Plovdiv (Bulgaria) and Mashhad (Iran) collected in different stages of plant development (Table 4). These data ruled out any correlation between the saponin content and the degree of hairiness.

In the present study the base length of the burrs and the length of the abaxial and basal spines of all Asian locations (Turkey, Iran, Georgia and India) are approximately identical. The burrs of all European origins also have identical

base length and length of abaxial and basal spines but we found them to be 1.2–1.5 times shorter than those from Asia. However, the saponin composition of the European samples is very similar to that of the samples from West Asia (Turkey, Georgia and Iran). All this suggests the absence of a correlation between the saponin composition and these morphological characteristics of the studied samples.

Present investigation of the angles between abaxial and basal spines showed high variability not only between the different origins, but also in the individual plants in one population. That is why these data were not discussed.

All the plants from the population from Rajasthan (North India) are characterized with the absence of basal spines, while the plants from the population of Bangalore (South India) have 2.2 ± 0.13 mm length of basal spines. The chemical fingerprinting of these two samples is different (Figs. 2 and 3), although the observed similarity in the content of the saponins **1–7** (Table 3). In this case, our results point to some correlation between this morphological characteristic (presence/absence of basal spines) and the chemical composition of the samples that suggests the existence of different chemotypes. However, a final conclusion could be made only after a careful examination of more samples from different origins.

3. Concluding remarks

The LC-ESI/MS analysis of samples of *T. terrestris* from different geographical regions revealed great differences in their chemical composition and content of the steroidal saponins **1–6** and rutin (**7**) depending on the plant part studied, stage of plant development and the region of sample collection. The data suggested the existence of one chemotype common to East South Europe and West Asia. The high content of protodioscin (**1**) and the presence of the sulphur containing saponins prototribestin (**2**) and tribestin (**5**) is a characteristic feature of this chemotype. The samples from India and Vietnam belong to other chemotypes. They all contain negligible amounts of protodioscin (**1**) and lack prototribestin (**2**) and tribestin (**5**). The use of (**2**) and (**5**) as chemotaxonomic markers to differentiate between the East South European – West Asian chemotype and those in Vietnam and India is proposed.

Our results imply great product-to-product variations in the saponin composition and the saponin content of market products based on extracts of *T. terrestris* depending on region of plant collection. Therefore, a quality evaluation of these herbal products and a proper analysis of their main biologically active components are necessary.

The LC-ESI/MS method developed for the purposes of this study allows a rapid, accurate and reliable quantitative determination of 6 steroidal saponins and rutin in extracts of *T. terrestris* from the South European – West Asian chemotype. The method could be used for evaluation of protodioscin (**1**) in commercial products – plant materials, crude extracts, pharmaceutical preparations and food supplements.

4. Experimental

4.1. Plant material

Aerial parts, stems, leaves and fruits of *T. terrestris* L. (Zygophyllaceae) were collected from different geographical regions at the stage of flowering–seeding or seeding (Table 1). In two cases (the regions of Plovdiv, Bulgaria and Mashhad, Iran) samples were collected during different stages of plant development. The plant materials were identified by Assoc. Prof. L. Evstatieva and voucher specimens of these samples deposited at the Herbarium of the Institute of Botany, Bulgarian Academy of Sciences, Sofia. Some of the samples were received in a powdered state. The plant material was dried at room temperature, finely powdered and used for extraction.

The morphological data about the base length of the burrs, the abaxial and basal spine length, the abaxial and basal spine angle, and the degree of hairiness were determined for all samples using fifty fruits from each of all studied locations excluding those from Vietnam, Ankara and Serbia.

4.2. Standards

The protodioscin (**1**), prototribestin (**2**), dioscin (**4**) and tribestin (**5**) were earlier isolated in our laboratory (Kostova et al., 2002; Conrad et al., 2004). Pseudoprotodioscin (**3**) was isolated from the aerial parts of *T. terrestris* of Bulgarian origin. Its 1D, 2D NMR and ESI mass spectra were in good agreement with those reported for the same compound (Ju and Jia, 1992). This is the first report for the presence of **3** in the genus Tribulus. Tribulosin (**6**) was provided by Dr. A. Agrawal from Bangalore, India. An authentic sample of rutin (**7**) was obtained from Merck (Darmstadt, Germany).

4.3. Extraction

One gram of the plant material (aerial parts, fruits, leaves and stems) was extracted with 50 ml $CHCl_3$ for 1 h, by shaking at room temperature. The extract was filtered and the plant material was extracted again, three times by refluxing with 50 ml of 70% EtOH for 1 h. The extracts were combined, and the solvent was removed under reduced pressure.

4.4. Purification

The crude extract was dried at 70 °C in the oven to remove water entirely then was powdered and 10 mg of it was suspended in 5 ml water and passed through a C_{18} Sep-Pak cartridge (Waters Associates) preconditioned with

water. The cartridge was washed with water to remove sugars and tannins. Saponins and rutin were eluted with 70% EtOH, evaporated and dissolved in 50% CH_3CN (1 ml) for HPLC and MS analyses.

4.5. Thin layer chromatography

Aluminium sheets of silica gel $60F_{254}$ (Merck) were used. The chromatograms were developed 2 times in the mobile phase *n*-BuOH:AcOH:H_2O – 4:0.5:1, dried and sprayed with Ehrlich reagent and reagent for flavonoids. Following amounts per spot were applied: fruit extracts – 200 μg; stems, leaves and aerial parts extracts – 100 μg; standards – 10 μg.

4.6. High-performance liquid chromatography

An LC system consisting of a Finnigan Surveyor pump equipped with a gradient controller, an automatic sample injector, and a PDA detector was used. The separation was performed on 250 × 4 mm i.d., 5 μm, Eurospher 100 C_{18} column (Knauer, Germany). A mobile phase consisted of 0.025% acetic acid in water (B), and 0.025% acetic acid in acetonitrile (A) was used for the separation. The flow rate was kept at 0.5 ml/min for a first 40 min, then increased to 1.0 ml/min, and kept constant for the rest time of the run (50 min). The system was run with the following gradient program: from 10% A to 90% A in 40 min, from 90% A to 10% A for the next 5 min, then was kept at 10% A for 5 min. The sample injection volume was 25 μl. Column temperature was ambient.

4.7. Mass spectrometry

A Thermo Finnigan LCQ Advantage Max ion trap mass spectrometer was coupled to column via an automatic sampler injection or direct injection by a syringe pump at a flow rate of 5 μl/min. The spray voltage was set to −33 V. All spectra were acquired at a capillary temperature of 220 °C. The calibration of the mass range (250–2000 Da) was performed in negative ion mode. Nitrogen was used as sheath gas, and the gas flow rate was 0.9 l/min. The maximum ion injection time was set to 200 ms.

4.8. Identification, standards preparation, and quantitative analysis

Three independent chromatographic runs were performed for each extract, and saponins and rutin were identified through comparison with authentic standards. These included retention times, co-chromatography, and mass spectra. Quantitation was based on external standardization by employing calibration curves constructed by injecting the standard solution across 9 different concentrations in the range:

(0.008 μg/ml–17.143 μg/ml) $R^2 = 0.936$ for rutin; (0.067 μg/ml–145.714 μg/ml) $R^2 = 0.998$ for prototribestin; (0.038 μg/ml–18.254 μg/ml) $R^2 = 0.964$ for protodioscin; (0.025 μg/ml–161.429 μg/ml) $R^2 = 0.953$ for tribestin; (0.024 μg/ml–5.926 μg/ml) $R^2 = 0.950$ for dioscin; (0.022 μg/ml–5.344 μg/ml) $R^2 = 0.974$ for tribulosin; (0.024 μg/ml–17.460 μg/ml) $R^2 = 0.976$ for pseudoprotodioscin. Microsoft Excel 2003 was used for the statistical analysis.

Acknowledgements

We thank Prof. I. Saracoğlu, Dept. of Pharmacognosy, Faculty of Pharmacy, Hacettepe Univ. Ankara, Turkey, Prof. S. Miloslavljevic, Faculty of Chemistry, Univ. of Belgrade, Serbia, Prof. L. N. Gvazava, Institute of Pharmacochemistry, Acad. of Sci. of Georgia, Dr. A. Kackar, Plant Biotechnology Unit, Dept. of Botany, Univ. of Jodhpur, India and Dr. A. Amit, Bioassay Unit, R&D Centre, Natural Remedies Pvt. Ltd, Bangalore, India for providing us with samples of *T. terrestris*.

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