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Variability of some biologically active compounds of *Tribulus terrestris* L.

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Abstract. *Three saponins (prodioscin, prototribestin, dioscin) and one flavonoid glycoside (rutin) were identified and quantified of extracts of T. terestris by means of HPLC. The content of these main compounds was analyzed in 22 samples collected from four floristic regions, growing at localities with different ecological conditions. Significant quantitative differences were detected among the samples examined. Cluster analysis was performed on the basis of the data of the studied compounds. The results showed the presence of two main groups. No similar response in the groups concerning growing conditions or different ecological combinations. Some trends in the accumulation of examined compounds were observed depending of the cultivation of the land (arable or not), but they not clearly expressed.*

Keywords: *Tribulus terrestris,* saponins, flavonoid, ecological conditions

Introduction

The level of secondary metabolites of plants is variable and can be significantly influenced by surrounding environment as well as by the physiological status - stage of growth and individual development. Environmental factors both abiotic and biotic including temperature, soil fertility, the availability of water and light, feeding of phytopathogous or other herbivorous animals, competition with neighboring plants and interactions with pathogens and parasites can be modified the content of flavonoids and saponins of plants (Dixon and Paiva, 1995; Cooper-Driver and Bhattacharya, 1998; Szakiel et al., 2010). Such variation of the content impacts on the quality and properties of plants exploited for pharmaceutical industry.

Tribulus terrestris L. (Zygophyllaceae) is an important feedstock for obtaining of pharmaceutical product Tribestan^{R}. It was found that the plant material collected from different geographical regions have different content of biologically active compounds (Dinchev et al., 2008). Therefore the identification of any correlation among the chemical composition and some environmental characteristics of the place of collection is very important.

The purpose of present study is to evaluate the quantitative variations of three major saponins (prodioscin, prototribestin, dioscin) and one flavonoid glycoside (rutin) in 22 samples of *Tribulus terrestris* depending of growing conditions and stages of development.

Material and methods

Plant material

Plant material of 22 populations of *T. terrestris* was examined. The details of the localities are presented at Table 1. Comparative analysis is made of nine pairs or groups of samples. Each pair or group of samples composed from populations located in close geographic point, but growing under different local conditions - arable land or not, varying degrees of humidity, etc. Voucher specimens were deposited at the Herbarium at the Institute of biodiversity and ecosystem research (SOM), Sofia.

Analytical method

An HPLC system La Chrom Elite consisting of L-2130 pump equipped with gradient controller and UV detector L-2400 was used. The separation was performed on 250 x 4.6 mm i. d., 5 µm, Inertsil ODS-2 column (Tokyo, Japan) with MetaGuard Pursuit direct connect guard column from Varian was used for all separations. The mobile phase which consisted of phosphoric acid buffer with pH-3 (A) and acetonitrile (B) was used for gradient elution. The parameters of the HPLC method utilized for compounds analysis were solvent gradient (%B): 15 at 0 min, 25 at 20 min, 35 at 30 min, 55 at 40 min, 90 at 50 min, 100 at 55 min, 100 at 65 min, 15 at 70 min, 15 at 80 min; run time = 100 min. The flow rate was adjusted to 1.0 ml/min, the detection wavelength was at 203 nm. All separations were performed at ambient temperature.

Sample preparation

Air-dried powdered plant material (1g, leaves and fruits, 1:1) was extracted three times with 5.0 ml of 50% aqueous acetonitrile by sonication for 15 min. The extracts were combined, after filtration in 20.00 ml volumetric flask and the volume was adjusted to 20 µl with the solvent use for extraction. Prior injection, all samples were filtered through a 0.45-m Chromafil 0-45/25 *Machery-Nagel*. Every sample solution was injected in triplicate with injection volume of 20µl.

Cluster analyses

An unsupervised technique was applied, cluster analysis, to discover similarities within the obtained concentration data of the main compounds – protodioscin, prototribestin, dioscin and rutin. Squared Euclidean distance was always used as the interval measure for clustering using distinct linkage methods: between groups linkage, within groups linkage and Ward's method. Used method is standard in the study of plant populations. All statistical manipulation and graphical display was performed on STATISTICA 7 for Windows (StatSoft, Inc., Tulsa, OK, USA).

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Table 1. Samples of *T. terrestris* used in present study – location, ecological conditions, features in the development

№	Name of the samples	Floristic region	Characteristics of the locality	Stage, length of individuals, cm
1	Kozlodui 1	Danube Plain	abandoned fields	stage of fruit formation, to 150
2	Kozlodui 2	Danube Plain	coastal area, dry soil, moist air, trampling soil by people	stage of fruit formation, to 70
3	Kozlodui 3	Danube Plain	corn field treated with herbicides	stage of flowers formation, to 40
4	Kozlodui 4	Danube Plain	corn field treated with herbicides	stage of fruit formation, to 100
5	Aheloi 1	Black Sea coast	well moist black soil, near the beach	stage of fruit formation, to 100
6	Aheloi 2	Black Sea coast	dry land, around road with heavy traffic	stage of flowers formation, to 30
7	Pirne1	Tundża hilly region	abandoned fields	stage of flowers formation,to 40-60
8	Aitos 2	Tundża hilly region	vineyards	stage of fruit formation, to 70
9	Kamen 1	Tundża hilly region	abandoned farm buildings	stage of fruit formation, to 111
10	Kamen 2	Tundża hilly region	orchards	stage of fruit formation, to 100
11	Zhelju voivoda 3	Tundża hilly region	peach garden, treated with herbicides	stage of fruit formation, to 100
12	Mamarchevo 1	Tundża hilly region	deserted places	stage of fruit formation, to 100
13	Bolyarevo 2	Tundża hilly region	plantings of watermelon	stage of fruit formation, to 100
14	Hadzhi dimitrovo 3	Tundża hilly region	old vines	stage of fruit formation, to 100
15	Chukarevo 1	Tundża hilly region	in fields with mint (Mentha)	stage of early fruit formation, to100
16	Ovchi kladenets 2	Tundża hilly region	abandoned agricultural buildings	stage of early fruit formation, to 70
17	Sandanski 1	Struma valley	old vines	stage of fruit formation, to 90
18	Sandanski 2	Struma valley	vineyards	stage of fruit formation, to 50
19	Petrich 1	Struma valley	ruderal place	stage of fruit formation, to 100
20	Petrich 2	Struma valley	around road with heavy traffic	stage of fruit formation, to 50
21	Rupite 1	Struma valley	around the church, sandy, trampling soil by people	stage of fruit formation, to 100
22	Rupite 2	Struma valley	around wells trampling of cars and people	stage of fruit formation, to 100

Results and discussion

Accumulation of flavonoids

The main flavonoid in the aerial parts of *T. terestris* is rutin. The highest levels of rutin were established in the samples 2, 9 - 4,02 mg/g, and the lowest 0,22 mg/g in the sample 15 (Table 2). Analyzing the data obtained it was noted that often the amount of rutin was lower in the samples collected from area with crops – for example - sample 15 <sample 16; sample 4 <sample 1; samples 10,11 <sample 9; sample 18 < sample 17 (Figure 1). Our results confirms the experimentally obtained data that with increasing N-fertilization of soil the concentration of rutin or flavonoids as whole decreased (Court et al., 1978; Stewart et al., 2001; Grevsen et al., 2008)

Accumulation of saponins

Three main saponins - protodioscin, prototribestin and diostsin were quantified of the extracts of *T. terestris* (Figure 2; Table 2)

Protodioscin. The highest levels of protodiocin were established in the samples 1,4 - 22 mg/g, and the lowest in the sample 16 - 3,79 mg/g. Relatively high content of protodioscin was established in the samples 8, 2, 10, 13, 14, 19. Samples with low content of protodioscin were 15,16. The analysis of the data revealed that often individuals in the early stage of development with small size have a low content of protodioscin - sample 3 < sample 4; sample 20 <19. Conditions such as arable or deserted areas, herbicides and others have a mixed impact.

Prototribestin. The highest level of prototribestin was established in samples 4 - 13,89 mg/g, and the lowest in the sample 15 - 0,84 mg/g. Rich in prototribestin were the extracts of the samples 8,10,19. Samples with low content of prototribestin were 21,20,16. The results showed that the synthesis of prototribestin has positive influence by soil use - arable or not. The amount of prototribestin is higher in samples collected from area with crops - sample 8> sample 7; sample 10> sample 9; sample 4> samples 1,2; sample 13> samples 12,14. There are data in literature that concentration of saponins are improved with treatment with fertilizer (Vijay et al., 2009)

Dioscin. The highest level of dioscin was established in samples 3 - 2,38 mg/g, and the lowest - below the limit of detection - in the samples 5,6,7,9,17,18,19,21. The synthesis of diostsin not shows depending on growing conditions.

Total synthesis of saponins. The highest content of total saponins was established in the samples 1 and 4 respectively 37,65 and 36,18 mg/g (Figure 1). The both samples are from the area of Kozloduy, growing at abandoned and cultivated cornfields. The samples - 8,10,13,14,19 - are rich in total saponins too. They have developed at areas with fruit garden (10), vineyards (10,14), plantings of watermelon (13), wet place (19). These results show that fertile soils is factor, that in most cases leads to a higher level of saponins, that is confirmation of a trend already observed (Šalamon et al., 2006).

Comparative analysis of flavonoid and saponin accumulation

Comparing the results obtained for the content of flavonoids and saponins in the examined samples makes an impression, that in some cases there is correlation in the accumulation of flavonoids and saponins - samples (12,13,14); (15,16); (19,20) but in other no such connection exists (Figure 1). In sample 21 the flavonoid content is higher in comparison with sample 22 but the accumulation of saponins has opposite meaning. A similar trend was observed for the samples (9,10,11) and (1,2,4). The observed opposite trend in the accumulation of flavonoids and saponins shows that both groups of substances are influenced differently by growing conditions. In

Table 2. Concentration of the main compounds of studied 22 samples of *Tribulus terestris*

№ of samples	Flavonoids [mg/g DW]		Saponins [mg/g DW]		
	Rutin±SD	Protodioscin±sd	Prototribestin±sd	Dioscin±sd	Total saponins
1	3.56±0.08	22.08±0.09	12.06±0.13	2.04±0.04	36.18
2	4.02±0.14	16.23±0.10	10.08±0.11	1.68±0.03	27.99
3	2.37±0.06	9.14±0.13	4.92±0.04	2.38±0.07	16.44
4	2.82±0.09	22.13±0.16	13.89±0.04	1.63±0.01	37.65
5	1.54±0.06	8.42±0.41	6.10±0.27	LOD	14.52
6	0.58±0.03	6.89±0.02	6.13±0.04	LOD	13.02
7	0.92±0.00	4.75±0.03	3.01±0.00	LOD	8.68
8	1.13±0.04	13.62±0.00	11.69±0.29	1.72±0.02	28.16
9	4.02±0.09	12.77±0.06	6.15±0.04	LOD	18.92
10	2.58±0.11	14.24±0.18	10.39±0.29	1.62±0.01	26.25
11	1.65±0.09	7.23±0.21	6.81±0.06	1.31±0.03	17.00
12	1.46±0.01	5.32±0.18	6.38±0.13	1.48±0.00	14.64
13	2.41±0.03	8.39±0.17	7.62±0.22	1.98±0.00	20.40
14	2.57±0.13	10.27±0.19	5.92±0.01	1.30±0.02	20.06
15	0.22±0.00	2.38±0.05	0.84±0.00	0.88±0.00	4.32
16	1.31±0.00	3.79±0.07	2.52±0.01	1.52±0.00	9.14
17	2.22±0.02	8.47±0.22	5.06±0.16	LOD	15.75
18	1.66±0.04	8.29±0.00	6.20±0.21	LOD	16.15
19	2.28±0.05	10.47±0.33	11.07±0.24	LOD	23.82
20	0.82±0.00	5.00±0.07	3.04±0.05	2.31±0.12	11.17
21	1.94±0.06	4.39±0.27	2.31±0.11	LOD	8.64
22	1.44±0.05	9.17±0.30	5.21±0.16	1.62±0.00	17.44

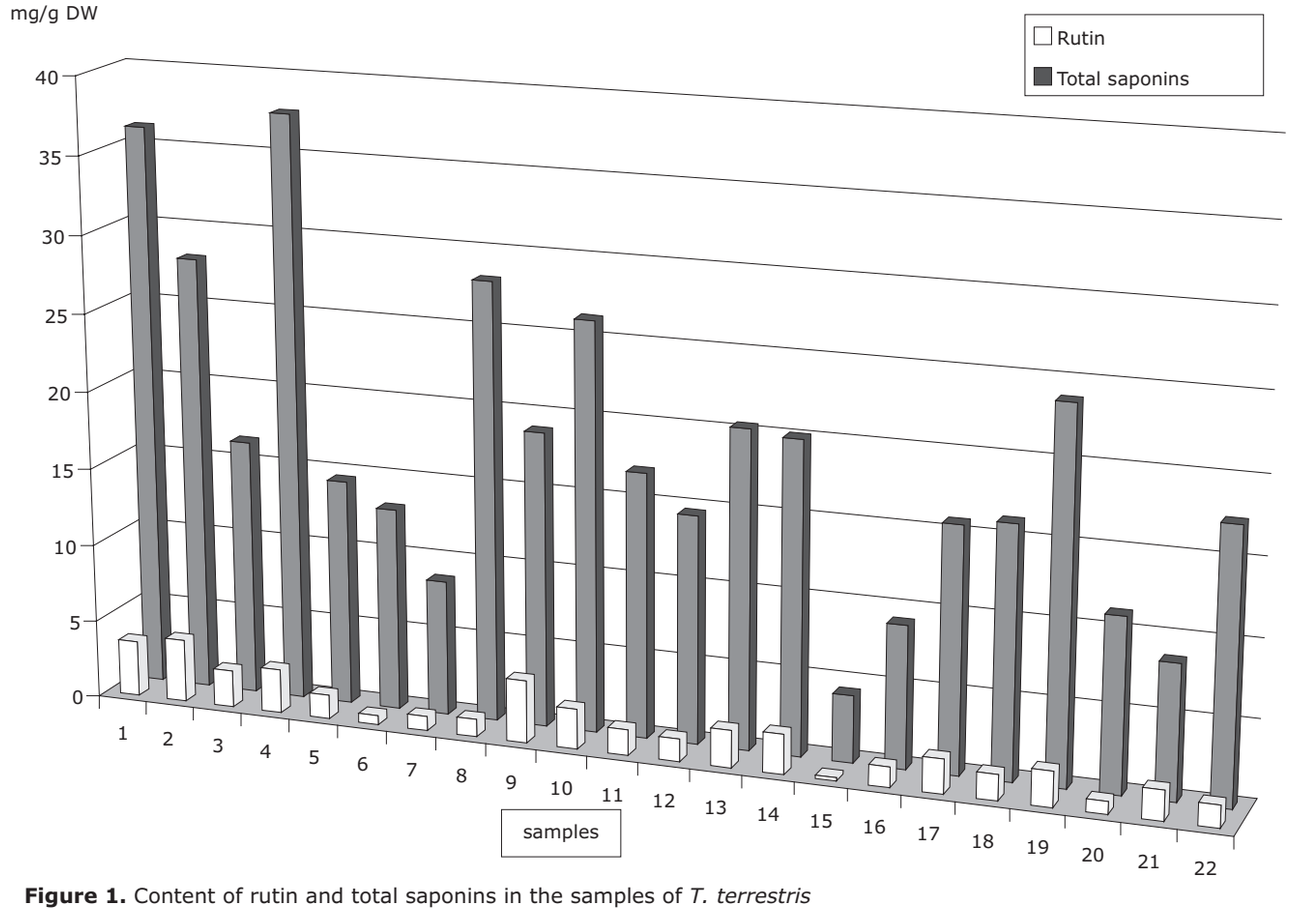

Figure 1. Content of rutin and total saponins in the samples of *T. terrestris*

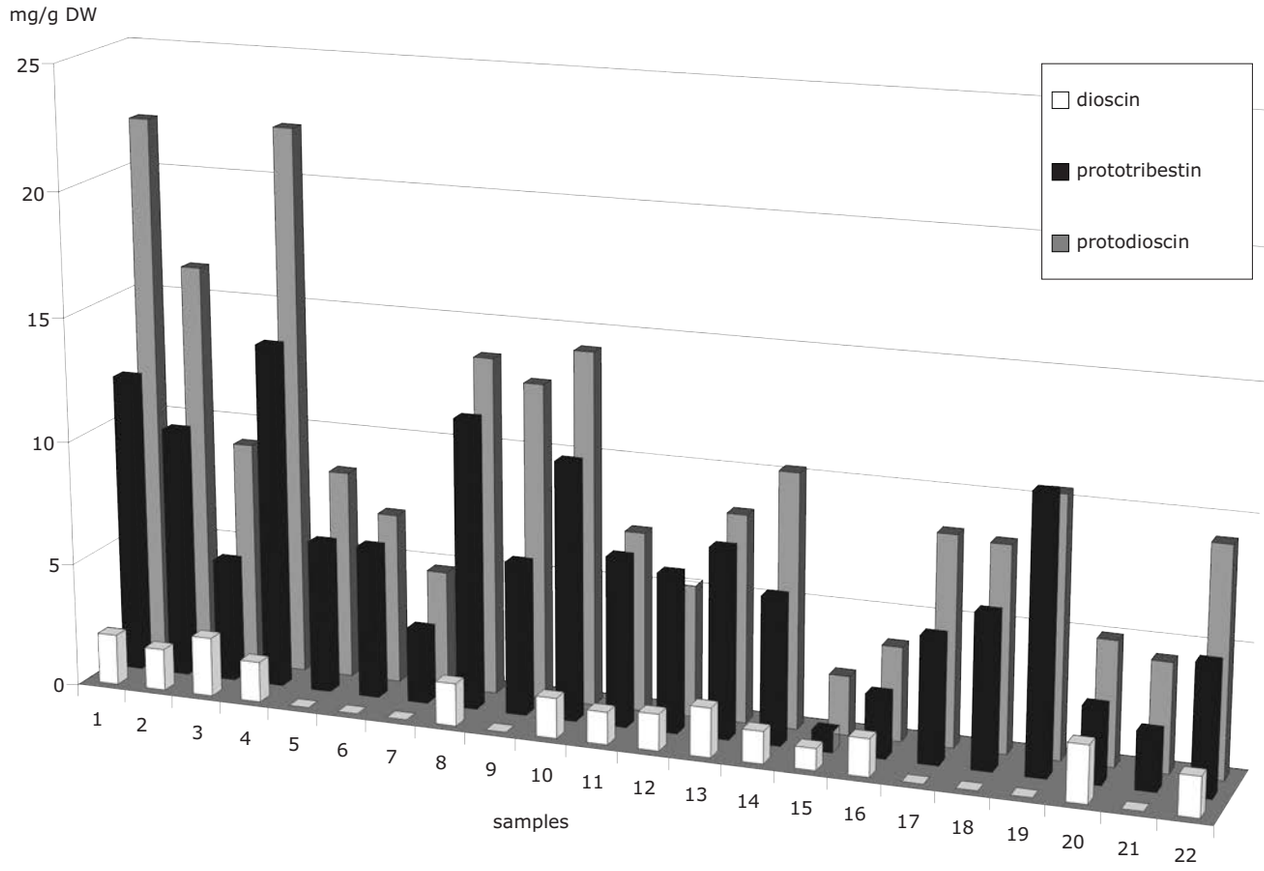

Figure 2 . Content of saponins in the samples in the samples of *T. terrestris*

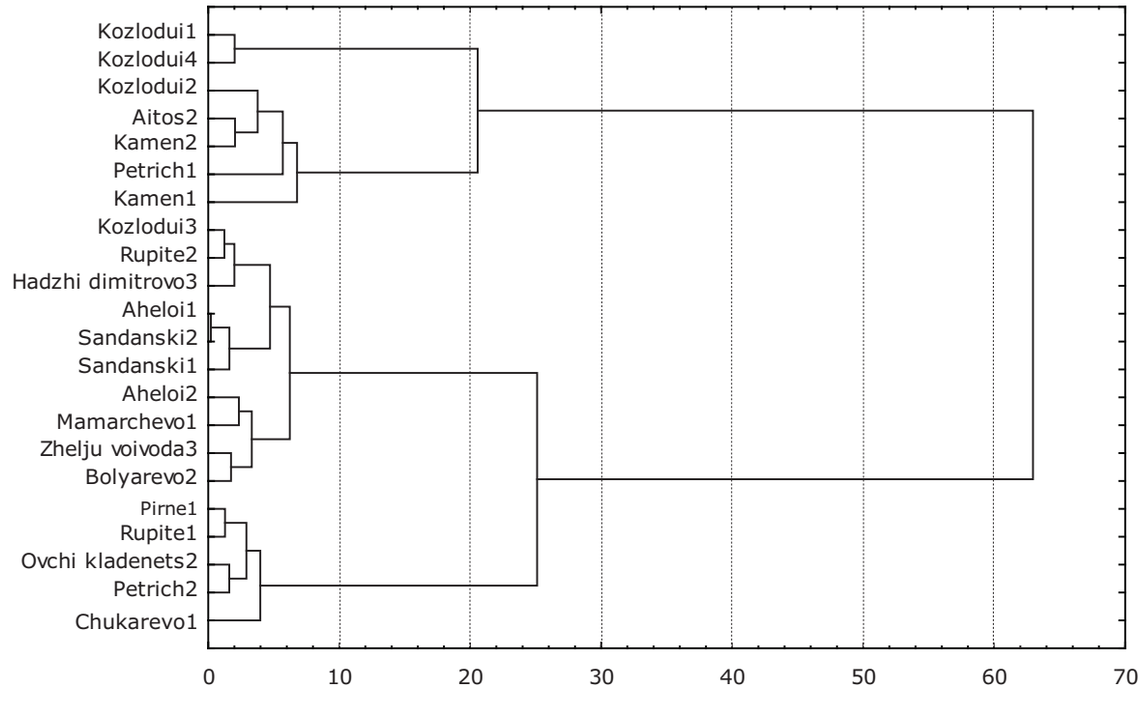

Figure 3. Cluster analysis of 22 *T. terrestris* samples according to the four studied phytochemical comppounds

confirmation of this observation is the study of Georgiev et al., (2010) presenting experimental data that *soil fertilized plants of T. terrestris contained* more saponins *but less of flavonoid glycoside - rutin.*

Cluster analysis

Cluster analysis was performed of the main compounds of 22 samples (Figure 3). Two main groups were emerged. Not a leading factor (altitude, geographical region, nature of soil, habitat conditions, etc.) into the groups.

Conclusion

Significant quantitative differences in the contents of three saponins and one flavonoid glycoside were detected among the 22 samples of *Tribulus terrestris.* In the cluster analysis is not shown correlation between content of examined chemical compounds and growing conditions or other ecological parameters. Some not very clear trends in the accumulation of examined compounds were observed but further analysis is needed to clarify and confirm the dependences outlined. It is known that *T. terrestris* forms panmictic populations from that point of view we can accept presence of modification variability.

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