Chavenetidou, A., M., Pankou, I., C., Tziouvalekas, S., M. (2021 A qualitative and quantitative analysis of extractives from the species Trifolium pratense L. in three different solvents. Agriculture and Forestry, 67 (2): 63-73

DOI: 10.17707/AgricultForest.67.2.05

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A QUALITATIVE AND QUANTITATIVE ANALYSIS OF EXTRACTIVES FROM THE SPECIES *Trifolium pratense* L. IN THREE DIFFERENT SOLVENTS

SUMMARY

This research focuses on the relative quantitative and qualitative analysis of extractives from the species *Trifolium pratense* L. samples that were collected from flowers, stems and whole plants. Extractions were carried out with a Soxhlet device and three different solvents (water, ethanol, dichloromethane) were used. Chemical analyses were conducted with gas chromatography and mass spectrometry. The results revealed significant amounts of chemical compounds, such as megastigmatrienone, phytol, squalene, carenol, borneol etc, found in the specimens. The identification of red clover genotypes containing high concentration of these compounds could have multiple applications in chemical, food and pharmaceutical industry.

Keywords: *red clover, extractions, gas chromatography, mass spectrometry*

INTRODUCTION

Red clover (*Trifolium pratense* L.) is a perennial forage legume, mainly used for agriculture (cutting in grass–clover leys of 2–4 years of duration), but also occurring naturally in permanent grasslands and meadows in temperate zones (Figure 1). Although red clover is one of the most important livestock plants, nowadays is showing tremendous potential upon analysis (Ball *et al*., 2015).

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Notes: The authors declare that they have no conflicts of interest. Authorship Form signed online.

Recieved:18/02/2021 *Accepted:10/05/2021*

Species of the genus *Trifolium* have been used in traditional medicine for many centuries. Some *Trifolium* species have biological properties such as antioxidants, anti-inflammatory, antiparasitic (stomach), estrogenic, cytostatic, cytotoxic and are used in cases of cancer or cardiovascular disease. They are also important sources of phytoestrogens in nature, mainly due to isoflavones. (Sabudak and Guler, 2009; Beck *et al*., 2005; Fugh-Berman and Kronenberg, 2001). The high concentration of quercetin, soyasaponin, flavonoids and isoflavones, make the seeds of some *Trifolium* species an ideal source of beneficial phytochemicals for the human diet (Kledjus *et al*., 2001; Polasek *et al*., 2007; Figheiredo *et al*., 2007; Pare, 2000).

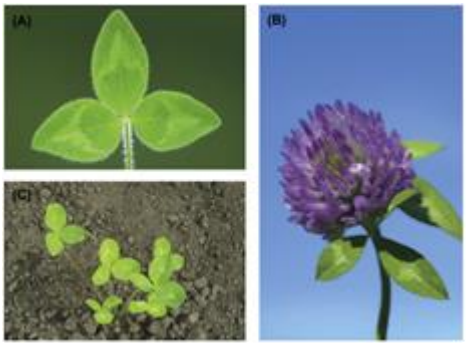

Figure 1. Red clover (A) leaf, (B) flower and (C) a seedling (Ball *et al*., 2015).

A study by Oleszek and Stochmal (2002) found that most species contain quercetin as the main flavonoid (0.05-0.3 mg/g) or with small admixtures of other, unrecognizable flavonoids. The components like biochanin A and isoflavanoides, genistein, daidzein, irilone etc. have been also isolated from the flowering tops, roots and leaves (Rehman, 2019).

In a research conducted by Booth *et al*. (2006), the chemical and biologic profile of red clover extract was studied, by identifying and measuring the major and minor components using visible HPLC-UV chromatogram and evaluating each compound for estrogenic and antioxidant activity. The preformulated extract was approximately composed of 35.54% isoflavones, 1.11% flavonoids, 0.06% pterocarpans, 0.03% coumarins,0.03% tyramine.

Ma *et al*. (2005), at their study, isolated the chemical constituents by chromatography and spectral analysis. The results showed that 8 compounds were obtained and identified as 1-octadecanol, 5-hydroxy-7,4′-dimethoxyisoflavone, biochanin A, prunetin, formononetin, genistein, ononin and daidzein. Compounds 1-octadecanol and 5-hydroxy-7,4′-dimethoxyisoflavone were found in this plant for the first time (Ma et al., 2005).

Moreover, Vlaisavljević *et al*. (2014), investigated the chemical constituents of the essential oils coming from *Trifolium pratense* in three different growth stages to test them for their antioxidant and antimicrobial activities, with the use of gas chromatography-mass spectrometry. As it is shown in Table 1,

numerous chemical compounds were found, mainly consisted of monoterpenes, sesquiterpenes, diterpenoids, aliphatic compounds and aromatic compounds.

At another research, Saviranta et al (2010), Vlaisavljević *et al*. (2017), aimed to investigate the phenolic content of red clover and its biological activity at various growth stages (30 cm growth, 50 cm growth and bud phase) of the plant. Isoflavonoids, genistein, and daidzein, as well as other phenols, p-hydroxybenzoic and caffeic acids, kaempferol 3-O-glucoside, quercetin 3-O-glucoside, and hyperoside were found in all the extracts, but the content of these compounds was the highest in the extract of the plant at the lowest growth stage (30 cm, vegetative). These results indicated that red clover has potential health benefits, and that growth phase affects its biological activity. The extract of red clover at the growth stage of 30 cm is a great source of bioactive compounds and could be used in phytotherapy and nutrition.

Table 1. Chemical composition of Trifolium pratense L. essential oil (Vlaisavljević *et al*., 2014)

No	Component	No	Component
1	Hexane	19	Decane
2	2-Pentanone	20	Undecane
3	Methylbenzene	21	Dihydrocarvone
4	1,3-Dimethylbenzene	22	Beta-ionone
5	1,4-Dimethylbenzene	23	10-Methylnonadecane
6	Pentanoic acid	24	Megastigmatrienone
7	7-Octen-4-ol	25	Hexadecane
8	Beta-myrcene	26	Dodecanoic acid
9	Cyclopropane	27	2,6-Diisopropylnaphthalene
10	Nonanal	28	Tetradecane
11	2,4-Heptadienal	29	Pentadecane
12	1-Bromocycloexane	30	Isopropyl myristate
13	Fenchyl alcohol	31	Tetrahydroionone
14	1,2,6-Hexanetriol	32	Hexahydrofarnesyl acetone
15	p-Cymene	33	Ocenol
16	L-Limonene	34	Phytol
17	Benzaldehyde	35	n-Hexadecanoic acid
18	Isobornyl thiocyanoacetate	36	Pentacosane

Tundis *et al*. (2015), studied the chemical profile and health properties of *T. pratense* (red clover) and *T. repens* (white clover). Furthermore, edible flowers were investigated for quercetin, kaempferol, luteolin, rutin, and myricetin that were used as markers and quantified by HPLC. The results support the use of *Trifolium* flowers as healthy food ingredients. Rehman (2019), at his review about *T. pratense* and its biological activities, indicated that red clover is a rich source of plant secondary metabolites isoflavonoids, which belong to the group of phenylpropanoids.

Saviranta *et al*. (2008) conducted at their study the quantification of three isoflavones in various clover species and their aerial plant parts by a high-

performance liquid chromatography. Isoflavone contents were quantified in the plants of 11 perennial and 4 annual species of genus *Trifolium*, among them the species *T. pratense*. HPLC revealed the concentration of biochanin A, isoflavones, daidzein, genistein, coumarin and other cardiac glycosides was high in *T. pratense*, among the rest of *Trifolium* species.

The aim of this research was to analyze the extractives of the species *Trifolium pretense* of Greek origin, in quantity as well as in quality, in order to obtain data that can promote the multiple uses of this species in various industrial sectors. The methodology applied differed from other studies, since Soxhlet device was used for the extractions. The interest of pharmaceutical and food industries for substances originating from plants is constantly rising and this essay wishes to contribute to that field.

MATERIAL AND METHODS

The material under investigation originated from Northern Greece (Holomontas, Chalkidiki) (Figure 2). The research took place during the period 06/2015-11/2015.

Figure 2. Map of the area where sample collection was conducted.

At first, the plant samples were carefully cleaned to remove soil and foreign particles, then the roots were removed and finally left to dry. (Figure 3). The flowers were separated from the stems at most samples. Then, all samples were cut into smaller pieces by hand with a sharp blade and were grinded with a mill (Wiley's mill) (Figure 3), in order to create particles with approximately the same dimensions, smaller than 0.1mm. The analysis was conducted on two samples in each case and a third one when needed, according to the standards. The total number of the collected plants was approximately 80-100 plants. Their average height was 31.32 cm (22,5-43,5 cm).

The quantitative estimation of extractives soluble in hot water, ethanol and dichloromethane were conducted according to ASTM Standards (ASTM D1107-

96, D1108-96, D1110-84). The specimens used for the extractions originated from the flowers, the stem or the whole plant. The stages of the extraction of the samples are illustrated in Figure 4.

For the extractions, a glass Soxhlet type device with the appropriate size was used, so as a 2,0g specimen and glass filter with medium porosity to be fit. Each hot water extraction lasted almost 6 hours and almost 4 hours for each of the other two solvents and 4 cycles of the solvent were repeated per hour.

Figure 3. Samples of the T. pratense plants collected, left to dry and grinded with Wiley's mill.

Figure 4. T. pratense samples after grinded in the mill and stages of extraction.

After the procedure, the specimens were removed from the Soxhlet device and left at normal conditions of temperature and humidity (approximately 25^{o}C and 55%) for 24h, before they were put in the oven at 103±2^{o}C for another 24 hours, until the total drying of the material. In the end, they were weighed to determine the dry weight of the extracted (wood) material, after the removal of the extractives (Chavenetidou, 2009).

Qualitative analysis of extractives was conducted with gas chromatography and mass spectrometry. The solvents containing the extractives after extraction was reduced with the use of rotary evaporator up to 1-2mL, in order to trace very small amounts of the chemical compounds of interest. In most cases, at the final stage of the procedure the reduction was reached with the use of nitrogen gas stream. In all cases, specimens were filtered in a chromatographic column (clean-up step) with the use of the following materials: Florisil (MgO_3Si) 2.5g, Al_2O_3 3.5g and Na_2SO_4 1.5g to absorb moisture or other materials that would damage the chromatographic column.

For the identification of the compounds and the quantification of the results, gas chromatograph Agilent 7890A was used, provided with non-polar capillary column DB-5ms, 30m length and 0.25mm internal diameter, film thickness 0.25μm and as a filler 5% phenyl polysiloxane, 95% methyl polysiloxane, using Helium as a carrier gas (flow 0.99333 mL/min, pressure 11.656 psi) and mass spectrometer with quadrupole Agilent 5975C). Finally, the mass spectrometer with quadrupole Agilent 5975C was also implemented and 1-bromo-2-nitrobenzene was used as internal standard for the estimation of the quantity (Tziouvalekas, 2011).

Two temperature programs were applied, in order to succeed better analyses. The temperature programs which were applied where:

1.Initial temperature: 60^{o}C – for 4 minutes. Final temperature: 240^{o}C for 5 minutes. Raising rate 50^{o}C/min

2.Initial temperature: 70^{o}C – for 4 minutes. Final temperature: 280^{o}C for 10 minutes. Raising rate 50^{o}C/min.

RESULTS AND DISCUSSION

In the following tables 2 and 3 the results from the statistical analysis are presented. According to the analysis of variance (Table 3), statistically significant differences were revealed between the amount of extracts released in the three different solvents that were used. Moreover, the part of the plant that was used for the analysis also played a crucial role as the differences between them were also statistically significant, although there was also statistically significant interaction between the two factors evaluated. The mean percentages of the extracts are presented in table 3.

Table 2. Analysis of variance for the three solvents and samples that was used for the analysis.

Source	df	F	Sig.
Solvent	2	384.800	0.000
Sample	2	13.571	0.010
Solvent * Sample	3	6.463	0.036
Error	5		
Total	12		

Table 3. Mean extract percentages of T. pratense samples soluble in water, ethanol and dichloromethane

Solvent	Mean %Extracts	Sample	Mean %Extracts
Dichloromethane	10.797a	Stem	18.707a
Ethanol	16.392b	Flower	20.539a
Water	30.433c	Whole Plant	26.704b

Means that differ statistically significantly ($p<0.05$) according to Tukey's multiple comparison test are followed by different letters.

As figure 4 shows, the percentage of the extractives was higher in the case of water used as a solvent, then ethanol and at last dichloromethane. In the cases that the whole plant was used for the extraction, the percentages of the extractives' content were higher than the rest. Both flowers and stems contain significant amounts of extractives, in all cases.

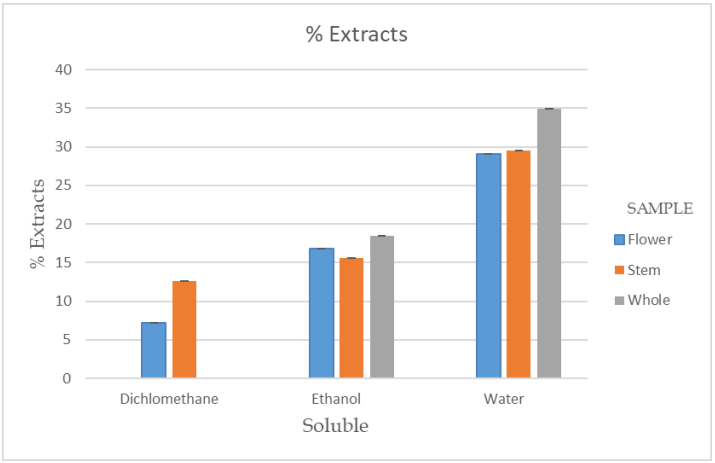

Figure 4. Extract percentage in three solvents (water, ethanol & dichlomethane).

The results of the qualitative analysis are presented in detail in the following tables (Tables 4, 5 and 6), which contain data from all the three solvents applied. The approximate amount of each substance was calculated by the fraction Integration area/internal standard area, with the use of **Benzene-1-bromo-2-nitro-** used as a standard area. From the detailed processing of the data it is obvious that:

-Specimens from different parts of the plant appeared to contain a few widely used chemical compounds, some of them in significant amounts, such as megastigmatrienone (stem-0.155), phytol (stem-3.324, flower-.0.72), squalene (stem-0.842, flower-0.140), linoleic acid (stem-0.783, flower-0.296), carenol (stem-0.107), borneol (stem-0.236), tetradecane (stem-0.587, flower-0.445), pentadecane (stem-0.094, flower-0.205), hexadecane (stem-0.151, flower-0.763), 7-tetradecene (stem-1.846, flower-0.123), hinesol (stem-0.575).

-Stem contained greater amount of pyrrolidine, 1-acetyl-, benzyl alcohol, tributyl acetylcitrate, squalene, 7-tetradecene, tetradecane and linoleic acid than flower

-Phytol appeared in much larger quantity at the stem than the flower

-Flower contained larger amount of benzeneamine, 4-bromo-, phenol, 2,5-bis (1,1-dimethylethyl) and bacchotricuneatin C than the stem

-Specimens appeared to contain some widely used chemical compounds, some of them in significant amounts, such as phytol (0.503), butyl citrate (0.792), oleanitrile (0.120), tetradecane (0.587), 7-tetradecene (1.846) and benzenamine-bromo (0.198)

-The samples extracted with the use of C_2H_5OH contained significantly larger amounts of phytol, 7-tetradecene and squalene than those extracted with the use of CH_2Cl_2

-Samples extracted with the use of CH_2Cl_2 contained, in most cases, different chemical compounds than those extracted with the use of C_2H_5OH

Table 4. Chemical compounds at stem after extraction with C2H5OH

Trifolium pratense stem			
Chemical compounds	Integration area/internal standard area	Chemical compounds	Integration area/internal standard area
Pyrazine, methyl	0.020	Megastigmatrienone	0.155
Benzaldehyde	0.135	Diethyl phthalate	2.464
Benzyl alcohol	0.137	n-capric acid	0.047
.gamma.dodecalactone	0.057	Hinesol.beta.panasinsene	0.575
Carenol	0.107	Isopropyl myristate	0.191
Pyrrolidine, 1-acetyl-	0.262	Thiophene	0.224
Benzeneamine, 4-bromo-	0.086	Phytol	3.324
benzothiazole	0.214	Linoleic acid	0.783
Benzene-1-bromo-2-nitro-	**1.000**	Tributyl acetylcitrate	1.047
2-(3-hydroxypropyloamino) pyrimidine	0.019	Triphenylene	0.484
Benzoic acid	0.016	Squalene	0.842
Phenol, 2,5-bis (1,1-dimethylethyl)	0.240	Piperidine	0.622
Bacchotricuneatin C	0.148	Benzopyranone	2.312
Borneol	0.236	Silane	0.183
1-Heptanol	0.244	7-Tetradecane	1.846
1-Octen-3-ol	0.137	Hexadecane	0.151
1-Octanol	0.176	Pentadecane	0.094
Hinesol	0.575	Heptadecane	0.153
Tetradecane	0.493	Nonedecane	0.307

The previous tables show that compounds mainly consisted of acids, phenols, aldehydes, ketones, alcohols, esters and hydrocarbons, findings in agreement with those of Kami (1978) and Vlaisavljevic *et al*. (2014). Moreover,

the chemical compounds found at this research agree with those of Vlaisavljević *et al*. (2014) as it is shown in Table 1. Compounds such as limonene, phytol, megastigmatrienone benzaldehyde, undecane, hexane, tetradecane, pentadecane, hexadecane were found in both essays.

Table 5. Chemical compounds at flowers after extraction with C2H5OH

Trifolium pratense flower			
Chemical compounds	Integration area/internal standard area	Chemical compounds	Integration area/internal standard area
Benzaldehyde	0.050	**Benzene-1-bromo-2-nitro-**	**1.000**
Piperidinone,1-methyl-	0.145	Phenol, 2,5-bis (1,1-dimethylethyl)	0.343
Silane	0.207	Benzophenone	0.196
Benzyl alcohol	0.081	Triallysilane	0.229
Phenylethyl alcohol	0.034	Phytol	0.072
Benzeneamine, 4-bromo-	0.379	Phthalic acid	0.046
Benzothiazole	0.083	Linoleic acid	0.296
Caprolactam	1.394	Tributyl acetylcitrate	0.099
Bacchotricouneatin c	0.964	Squalene	0.140
Pyrrolidine, 1-acetyl-	0.061	Undecane	0.042
Hexadecane	0.763	7-Tetradecane	0.123
Pentadecane	0.205	Carbonic acid	0.180
Hexane	0.288	1-Octanol	0.075
Oleic acid	0.067		

Table 6. Chemical compounds at stem after extraction with CH2Cl2

Trifolium pratense stem			
Chemical compounds	Integration area/internal standard area	Chemical compounds	Integration area/internal standard area
D- Limonene	0.009	Squalene	0.019
Limonene	0.032	Benzophenone	0.050
Piperazineethinamine	0.053	Isopulegol	0.004
Benzenamine-bromo	0.198	Oleic acid	0.007
Benzene-1-bromo-2-nitro-	**1.000**	Oleanitrile	0.120
Vanillin	0.010	Phytol	0.503
Naphthaelne,2,6-dimethyl-	0.006	Butyl citrate	0.792
m-Nitroaniline	0.012	Hexadecane	0.744
Tetradecane, 2,5-dimethyl	0.016	Tetradecane	0.587
3-Tetradecene	0.006	Dodecane	0.014
Hexadecanoid acid	0.330	N-Acetylpyrrolidone	0.032

CONCLUSIONS

Overall, results demonstrate a strong effect of the solvent that was used on the percentage of the extractives. As depicted in figure 4, the percentage of the extractives was higher when water was used as a solvent, followed by ethanol and at last dichloromethane. Additionally, when the whole plant was used for the extraction, the percentages of the extractives' content were higher than the rest.

Both flowers and stems contain significant amounts of extractives, in all cases. The findings of the chemical analysis demonstrated that specimens from different parts of the plant appeared to contain some widely used chemical compounds, some of them in significant amounts, such as megastigmatrienone, phytol, squalene, linoleic acid, carenol, caprolactam, bacchotricuneatin C and borneol. Furthermore, samples appeared to contain some widely used chemical compounds, some of them in significant amounts, such as phytol, linoleic acid, butyl citrate, oleanitrile and benzenamine-bromo, hexadecane. Overall, results show a great potential of the species *Trifolium pratense* for the use of their chemical compounds by various industrial sectors.

ACKNOWLEDGEMENTS

This research was part of the action "Research & Technology Development Innovation Projects - AgroETAK", MIS 453350, in the framework of O.P. "Human Resources Development", co-funded by ESF and National funds (NSRF 2007-2014), coordinated by the Hellenic Agricultural Organization "DEMETER".

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