STUDIA UNIVERSITATIS BABEŞ-BOLYAI, CHEMIA, XLV, 2, TOM II, 2010

***Dedicated to the memory of Prof. dr. Ioan Silaghi-Dumitrescu marking 60 years from his birth***

# THE STUDY OF POLYPHENOLS FROM *TRIFOLIUM PRATENSE* L. AND *MEDICAGO SATIVA* L. HYDROALCOHOLIC EXTRACTS BY HPLC-UV-MS

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**ABSTRACT.** The polyphenols are widespread compounds from plants having important pharmacological activities. *Trifolium pratense* L. and *Medicago sativa* L. are important cultivated medicinal plants mostly for their content in polyphenols and especially in isoflavons that are also polyphenolic compounds. The identification and quantitative determination of polyphenols from *Trifolium pratense* and *Medicago sativa* hydroalcoholic extracts was performed by a rapid HPLC-UV-MS methods by that can be identified a wide range of polyphenols. There were evaluated the native hydroalcoholic extracts and also the hydrolyzed extracts. There were found in *Trifolium pratense* hyperoside, isoquercitrine – glycosides having as aglykon quercetol, p-coumaric, ferulic, genistic and chlorogenic. In *Medicago sativa* were identified apigenine, p-coumaric, ferulic and genistic acids.

***Keywords:*** *Trifolium pratense, Medicago sativa, polyphenols, HPLC-UV-MS*

## INTRODUCTION

The polyphenols are important active compounds that are present in many plants. The flavonoids, including isoflavons, the polyphenyl carboxylic acids, the tannins, the coumarins take part from polyphenols family.

The polyphenols has important pharmacological properties, one of them being the antioxidant activity. They are important for maintain the health and also as therapeutic compounds in different diseases [1,2].

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Trifolium pratense is a cultivated plant being known for its content in isoflavons as daidzein, genistein and formononetin, but contains also other polyphenols as flavonoids, coumarins and also other compounds like essential oil, cyanogenetic glycosides, etc. [2,3,4,5,6].

Medicago sativa is also a cultivated plant. It contains saponins, carotenoids, and polyphenols including isoflavons like genistein, daidzein and cumestans [2,3,7,8].

The total polyphenols can be evaluated by spectrophotometric methods using Folin-Ciocalteu [9] or Arrnow [10] reagent. The individual polyphenols can be identified and quantitatively determined just by chromatographic methods: TLC [11] and more exactly by HPLC coupled with UV-Vis spectrophotometric detector and mass-spectrometric detector [5,6,12,13,14,15,16,17].

This paper present the evaluation of polyphenolic profile from *Trifolium pratense* and *Medicago sativa* hydroalcoholic extracts using a HPLC-UV-MS method that provides the possibility of identification of a wide range of polyphenols.

## RESULTS AND DISCUSSION

The table 1 presents the retention times found for 16 polyphenol standards respectively the equations for calibration curves obtained for each of 16 standards. In figure 1 can be seen the obtained chromatogram for the standards.

**Table 1.** The retention time and calibration curves equation for polyphenol standards

| Peak no. | Name of standard | Retention time, min, $r_t$ ± SD | Calibration curves equation A = peak area, c = concentration |
|---|---|---|---|
| 1 | Gentisic acid * | 2.15± 0.07 | A = -0,335 + 14,261c |
| 3 | Chlorogenic acid * | 5.62± 0.05 | A = -1,324 + 26,492c |
| 5 | p-coumaric acid | 8.70± 0.08 | A = -0,326 + 33,230c |
| 6 | Ferulic acid | 12.20 ± 0.10 | A = -1,017 + 39,558c |
| 7 | Sinapic acid | 14.30 ± 0.10 | A = -0,237 + 37,103c |
| 8 | Hyperoside | 18.60 ± 0.12 | A = 0,107 + 19,294c |
| 9 | Izoquercitrin | 19.60 ± 0.10 | A = -0,273 + 12,978c |
| 10 | Rutoside | 20.20 ± 0.15 | A = 0,227 + 13,473c |
| 11 | Myricetin | 20.70 ± 0.06 | A = 0,270 + 26,150c |
| 12 | Fisetin | 22.60 ± 0.15 | A = 0,292 + 17,190c |
| 13 | Quercitrin | 23.00 ± 0.13 | A = 0,048 + 10,698c |
| 14 | Quercetol | 26.80 ± 0.15 | A = -1,152 + 36,327c |
| 15 | Patuletine | 28.70 ± 0.12 | A = -0,430 + 31,450c |
| 16 | Luteolin | 29.10 ± 0.19 | A = -0,761 + 28,927c |
| 17 | Kaempferol | 31.60 ± 0.17 | A = -1,271 + 30,152c |
| 18 | Apigenin | 33.10 ± 0.15 | A = -0,909 + 20,403c |

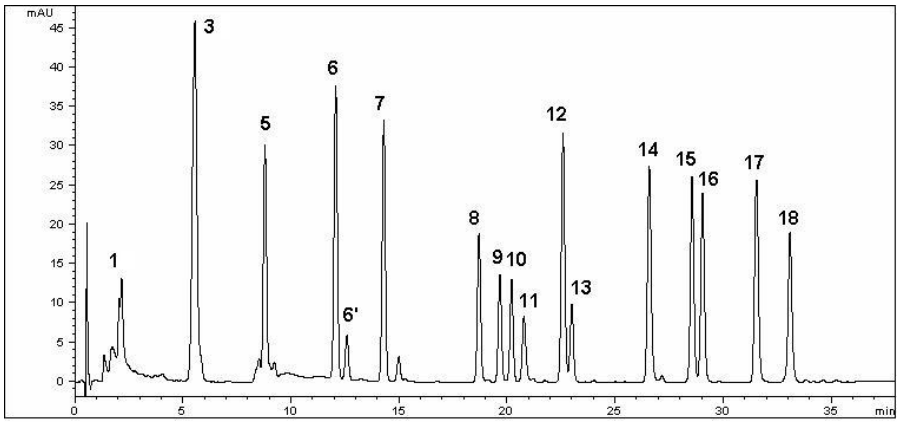


**Figure 1.** The chromatogram of polyphenol standards

In figures 2 and 3 can be seen the HPLC-UV chromatograms for the native hydroalcoholic and hydrolyzed *Trifolium pratense* extract. It can be identify in native extract the hyperoside and isoquercitrin from flavonoids group respectively in small amount, the free p-coumaric and ferulic acids. The identification of flavonoids was made based on comparison of retention time and MS spectra with those of the studied standards. These compounds can be also quantified by HPLC-UV. The small amount from p-coumaric and ferulic acids were identified based on them MS spectra. Because of small amount these compounds can not be exactly quantified by HPLC-UV.

The hyperoside and isoquercitrin free during the hydrolysis quercetol, they aglykon. These could be demonstrated by HPLC-UV-MS evaluation of hydrolyzed extract in that there were identified quercetin, p-coumaric, ferulic, chlorogenic and genistic acids. The higher amounts from p-coumaric and ferulic acids respectively the presence of other acids that can not be identified in the native extract, can be explained by the hydrolysis of the compounds in that they are naturally bounded. The quercetol, p-coumaric and ferulic acids were identified based on the comparison of retention times and MS spectra with those of the studied standards.

The chromatogram of native *Trifolium pratense* hydroalcoholic extract shows a main peak X at 21-22 minute that can not be identified as being one of the studied standards. The evaluation of MS spectra (figures 4 and 5) show that this compound has the molecular weight 448 (= 447 + 1 – the signal is for deprotonated molecule) and has a main fragment with molecular weight 286 (= 284 + 1 + 1 – the signal is for deprotonated fragment having a radical to a phenol position). The kaempferol and luteolin, both being flavonoid aglykons, has the molecular weight 286. This indicates that the unidentified can be glycosides of kaempferol or luteolin.

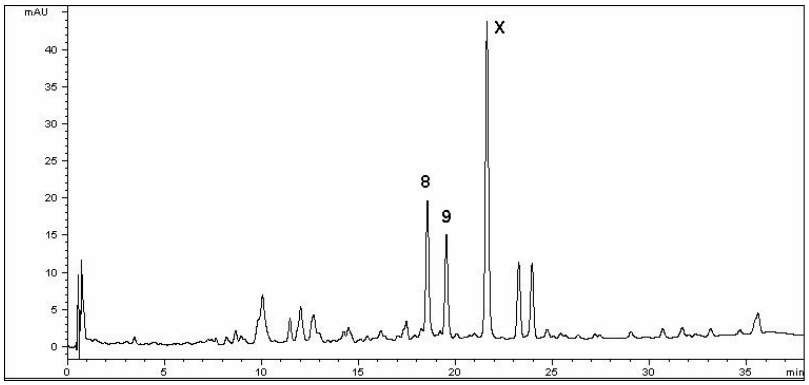


**Figure 2.** The chromatogram of native *Trifolium pratense* hydroalcoholic extracts

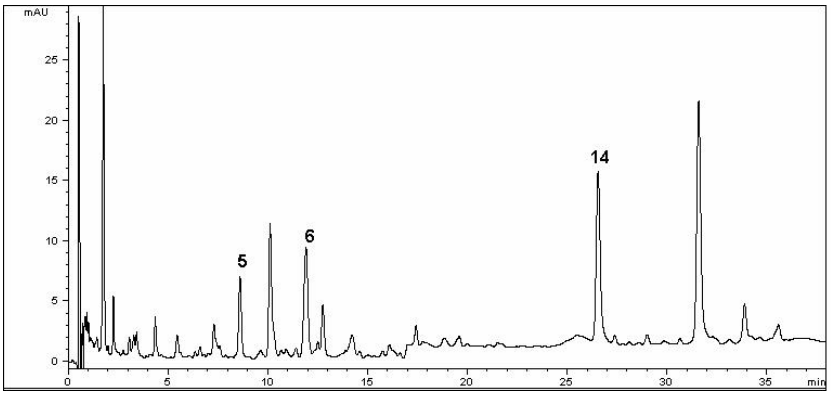


**Figure 3.** The chromatogram of hydrolyzed *Trifolium pratense* hydroalcoholic extracts

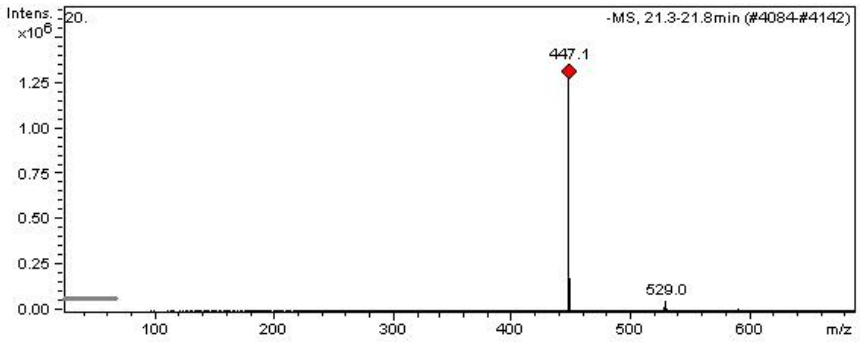


**Figure 4.** The MS spectra for compound X with molecular signal

In figures 6 and 7 can be observed the HPLC-UV chromatograms for native respectively hydrolyzed *Medicago sativa* hydroalcoholic extract. It can be identify in native extract the apigenin based on comparison of retention time and MS spectra with those of standard apigenin respectively the p-coumaric acid based on MS spectra. This result shows that the apigenin can be found in *Medicago sativa* also as free aglykon, in significant amount to be quantified. The p-coumaric acid is present in small amount as free acid in native extract.

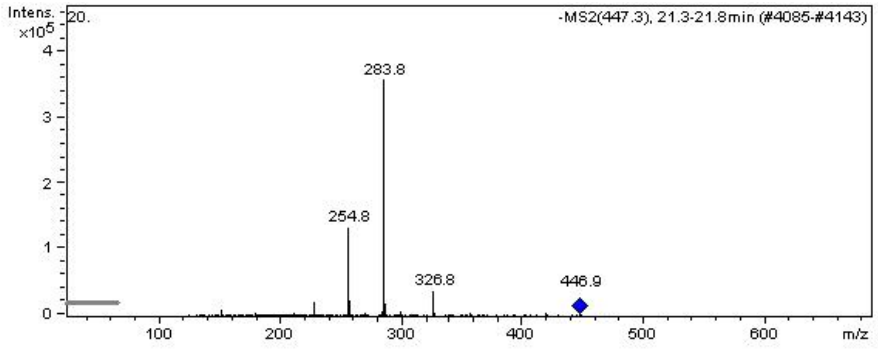


**Figure 5.** The MS spectra for compound X with fragmentation

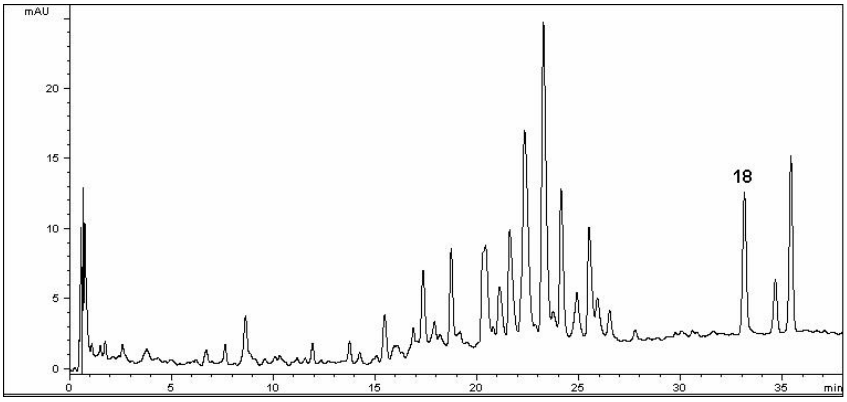


**Figure 6.** The chromatogram of native *Medicago sativa* hydroalcoholic extracts

The chromatograms show also a lot of significant compounds separated at 15-27 minute that can not be identified as being one of the studied standard compounds. After hydrolysis the chromatogram shows a lower level for apigenin and the unidentified compounds from 15-27 minutes. That indicated that also the unidentified compounds can be flavonoids, probably C-glycosides. The

lower level of these compounds and of apigenin can be explained by the degradation of some flavonoidic compounds like apigenin in the hydrolysis condition.

In the hydrolyzed extract can be identified based on retention time and MS spectra in comparison with those of standards the following polyphenols: apigenin, ferulic and p-coumaric acids respectively based just on MS spectra the genistic acid. These results show that the polyphenolic acids are present in *Medicago sativa* mostly in bounded form. The apigenin, p-coumaric and ferulic acids can be quantified.

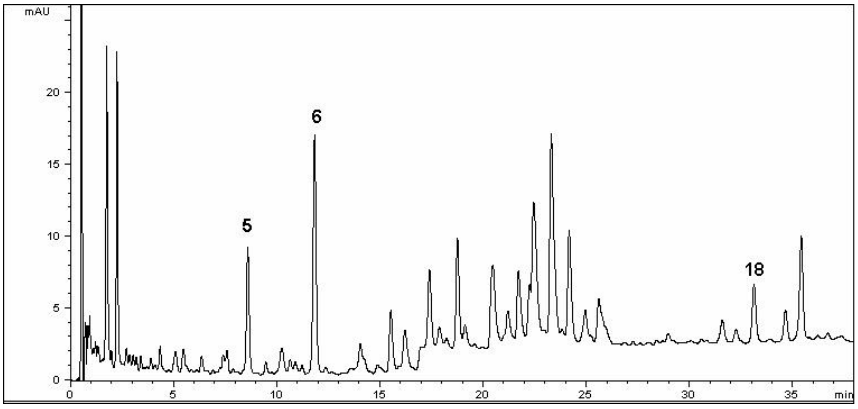


**Figure 7.** The chromatogram of hydrolyzed *Medicago sativa* hydroalcoholic extracts

In table 2 can be observed the retention times and MS spectra data of identified compounds that can be compared with those of the standards.

In table 3 are presented the results of quantitative determination on the identified compounds. It can be seen that the *Trifolium pratense* extract contains mostly flavonoids – hyperoside and isoquercitrin and smaller amount of bounded acids: p-coumaric acid and ferulic acid. The *Medicago sativa* extract contains apigenin in higher amount and in smaller amount bounded p-coumaric acid and ferulic acid.

The quercetol identified in this paper in the *Trifolium pratense* extract was found also by Booth in different commercial *Trifolium pratense* products. The result of quantitative determination for quercetol (5,581 μg/ml extract meaning 1,86 % reported to the dry plant) shows a higher value that found by Booth (1 %). Booth identified in *Trifolium pratense* also the kaempferol (0,07 %) and fisetin (0,02 %), in small amounts [4]. The kaempferol glycoside can not be certainly identified in the studied Romanian *Trifolium pratense*, neither the kaempferol in the hydrolyzed sample.

**Table 2.** The retention time and MS spectra data for identified compounds

| Name of sample | Name of identified compound | Retention time, min, $r_t$ | MS spectra data for identified compounds / standards |
|---|---|---|---|
| *Trifolium pratense* native extract | Hyperoside | 18,6 | 463> 254.9, 270.9, 300.7 |
| | | | 463> 254.9, 270.9, 300.7 |
| | Isoquercetin | 19,6 | 463> 254.9, 270.9, 300.7, 342.8 |
| | | | 463> 254.9, 270.9, 300.7, 342.8 |
| | p-coumaric acid | 8,7 | 163> 118.7 |
| | | | 163> 118.7 |
| | Ferulic acid | 12,2 | 193.2> 133.7, 148.7, 177.6 |
| | | | 193.2> 133.7, 148.7, 177.6 |
| *Trifolium pratense* hydrolyzed extract | Quercetol | 26,8 | 301> 150.6, 178.6, 272.7 |
| | | | 301> 150.6, 178.6, 272.7 |
| | p-coumaric acid | 8,7 | 163> 118.7 |
| | | | 163> 118.7 |
| | Ferulic acid | 12,2 | 193.2> 133.7, 148.7, 177.6 |
| | | | 193.2> 133.7, 148.7, 177.6 |
| | Chlorogenic acid | 5,62 | 353.5>178.7, 190.7 |
| | | | 353.5>178.7, 190.7 |
| | Genistic acid | 2,15 | 153>108.7 |
| | | | 153>108.7 |
| *Medicago sativa* native extract | Apigenin | 33,1 | 269.2>148.6, 150.6, 224.7, 226.7 |
| | | | 269.2>148.6, 150.6, 224.7, 226.7 |
| | p-coumaric acid | 8,7 | 163> 118.7 |
| | | | 163> 118.7 |
| *Medicago sativa* hydrolyzed extract | Apigenin | 33,1 | 269.2>148.6, 150.6, 224.7, 226.7 |
| | | | 269.2>148.6, 150.6, 224.7, 226.7 |
| | p-coumaric acid | 8,7 | 163> 118.7 |
| | | | 163> 118.7 |
| | Ferulic acid | 12,2 | 193.2> 133.7, 148.7, 177.6 |
| | | | 193.2> 133.7, 148.7, 177.6 |
| | Genistic acid | 2,15 | 153>108.7 |
| | | | 153>108.7 |

**Table 3.** The results of quantitative determination of identified compounds

| Sample | Compound | Concentration, □g/ml |
|---|---|---|
| *Trifolium pratense* native extract | Hyperoside | 9,766 |
| | Isoquercetin | 11,206 |
| *Trifolium pratense* hydrolyzed extract | Quercetol | 5,581 |
| | p-coumaric acid | 1,900 |
| | Ferulic acid | 3,074 |
| *Medicago sativa* native extract | Apigenin | 11,206 |
| *Medicago sativa* hydrolyzed extract | Apigenin | 2,667 |
| | p-coumaric acid | 2,387 |
| | Ferulic acid | 4,424 |

The apigenin identified and quantified in the *Medicago sativa* extract was found in this plant by Stochmal also [8].

The results presented in this paper show that near isoflavons both plants contain also other flavonoids and polyphenolic acids. The polyphenolic acids (p-coumaric, genistic, chlorogenic and ferulic acids) from *Trifolium pratense* and *Medicago sativa* were studied in detail for first time in this paper.

## CONCLUSIONS

Using the proposed HPLC method it can be separated a wide range of polyphenols from complex samples as the plant extracts. Using UV and MS detection can be identify also the compounds in small amounts due by the high sensitivity and more precise data that can be obtain with the MS detector.

In the studied plant extracts can be identified the main flavonoidic compounds and polyphenolic acids, both in free and bounded forms. Those existing in significant amounts could be also quantified by HPLC-UV. The present study confirm the presence of some flavonoids in both plants, compounds identified also by other researchers, but the detailed qualitative and quantitative study of polyphenolic acids in these plants is made for first time in this paper.

The identification and quantification of polyphenols from *Trifolium pratense* and *Medicago sativa* extracts is important for the study of therapeutically active compounds from these plants.

## EXPERIMENTAL SECTION

There were used the aerial part of fresh plant from *Trifolium pratense* and *Medicago sativa*, both harvested from organic culture near Cluj, in 2008. The plants were botanical identified to PlantExtrakt, Radaia.

The hydroalcoholic extracts were obtained from fresh plant (70% moisture) using 90 % vol. ethanol (pharmaceutical grade) by cold maceration at room temperature, in 10 days. After 10 days of maceration by repetitive mixing the extracts were decanted and the plant residue was pressed. The extracts obtained from decantation and pressing were mixed and filtered. The fresh plant-extract ratio was 1:1 for both extracts.

The hydrolysis of extracts was made using 2N hydrochloric acid (Merck, Germany), 1 ml to 1 ml extract. The hydrolysis was performed at boiling temperature in 40 minutes.

There were used HPLC grade methanol (Merck, Germany) and analytical grade acetic acid (Merck, Germany) to perform the HPLC analysis. As standards were used genistic acid, chlorogenic acid, p-coumaric acid, ferulic acid, sinapic acid, hyperoside, isoquercitrin, rutoside, myricetin, fisetin, quercitrin, quercetol, patuletine, luteolin, kaempferol and apigenin purchased from Merck, Fluka, Sigma or LGC.

The experiment was carried out using an Agilent 1100 HPLC Series system (Agilent, USA) equipped with degasser, binary gradient pump, column thermostat, autosampler and UV detector. The HPLC system was coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). For separation purpose, a reverse-phase analytical column was employed (Zorbax SB-C18 100 x 3.0 mm i.d., 3.5 µm particle); the working temperature was 48 °C. The detection of the compounds was performed on both UV and MS mode. The UV detector was set at 330 nm until 17.5 min, then at 370 nm. The MS system operated using an electrospray ion source in negative mode. The chromatographic data were processed using ChemStation and DataAnalysis software from Agilent, USA.

The mobile phase was a binary gradient prepared from methanol and solution of acetic acid 0.1% (v/v). The elution started with a linear gradient, beginning with 5% methanol and ending at 42% methanol, for 35 minutes; isocratic elution followed for the next 3 minutes with 42% methanol. The flow rate was 1 ml $min^{-1}$ and the injection volume was 5 µl.

The MS signal was used only for qualitative analysis based on specific mass spectra of each compound. The MS spectra obtained from a standard solution of compounds were integrated in a mass spectra library. Later, the MS traces/spectra of the analysed samples were compared to spectra from library, which allows positive identification of compounds, based on spectral mach. The UV trace was used for quantification of identified compounds from MS detection. Using the chromatographic conditions described above, the polyphenols eluted in less than 35 minutes. The acids were identified by MRM mode and the other polyphenols by AUTO MS mode. The detection limits were calculated as minimal concentration producing a reproductive peak with a signal-to-noise ratio greater than three. Quantitative determinations were performed using an external standard method. Calibration curves in the 0.5–50 µg $ml^{-1}$ range with good linearity (R2 > 0.999) for a five point plot were used to determine the concentration of polyphenols in plant samples.

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