UDC 615.07
https://www.doi.org/10.34907/JPQAI.2020.60.61.006

DETERMINATION OF ISOFLAVONE CONTENT IN DRY HERBAL EXTRACT OF MEADOW CLOVER (TRIFOLIUM PRATENSE L.) BY HPLC

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Meadow clover, also known as red clover (Trifolium pratense L.), is a natural source of phytoestrogenic isoflavonoids. The purpose of the study is to study the qualitative and quantitative content of individual isoflavones in the dry herbal extract of meadow clover by HPLC in order to expand the range of medicines based on this phytosubstance. In the dry herbal extract of meadow clover, daidzein, genistein, formononetin, prunetin and biochanin A were identified, their content was determined as 0.36±0.01%, 0.84±0.01%, 3.02±0.05%, 0.22±0.01% and 1.62±0.03% respectively. The established significant content in the dry extract of formononetin and biochanin A allows us to consider the dry herbal extract of meadow clover as a promising substance for development of medicines.

Keywords: isoflavone content, dry extract, meadow clover herb (Trifolium pratense L.), HPLC, identification, quantitative determination

Flavonoids are one of the most widely distributed classes of polyphenolic substances in nature. Numerous studies show that preparations based on flavonoids are highly effective antitumor agents, have antioxidant properties, and reduce the risk of cardiovascular diseases.

Plants of the clover species (Trifolium L.), which belong to the legume family (Fabaceae) are of particular interest as sources of flavonoid compounds. In particular, representatives of meadow clover species (Trifolium pratense L.) rich in flavonoids and isoflavonoids of various groups attract interest [1].

In connection with the above, it is relevant to study the qualitative and quantitative determination of individual isoflavones in a dry extract of meadow clover herb by HPLC in order to expand the range of medicinal products based on this phytosubstantiation.

MATERIALS AND METHODS

To obtain a dry extract, the meadow clover herb produced by Company Horst LLC, Barnaul and purchased from a pharmacy chain, was used.

The technology for obtaining a dry extract of meadow clover herb was developed earlier [2]. Content of individual substances of isoflavone groups was determined on the base of the Department of Analytical Chemistry of Saint Petersburg State Chemical and Pharmaceutical University by HPLC method using a HPLC liquid chromatograph Shimadzu SCL – 10A (Japan), software – "MultiChrom" for Windows.

Chromatographic conditions: Selectra C18 column (250×4.6 mm) filled with 5 μm sorbent particles; mobile phase A is 0.1% aqueous solution of formic acid, mobile phase B is acetonitrile; gradient elution mode: linear change of mobile phase B from 20% to 70% within 40 minutes. The speed of the mobile phase is 1 ml/min. Volume of the injected sample is 20 μl. Detection was performed using a UV detector at a wavelength of 270 nm. The temperature of the column was 40°C.

Identification of individual substances was performed by comparing the retention times of peaks obtained on chromatograms of the tested and standard solutions. The content of isoflavones was determined using the standard method.

Standard solutions of daidzein (CAS #486-66-8, 97.8%, HWI group), genistein (CAS #446-72-0, 99%, SIAL), formononetin (CAS #485-72-3, 98.6%, Sigma-Aldrich), prunetin (CAS #552-59-0, 98.2%, Sigma), biochanin A (CAS #491-80-5, 97.4%, Sigma). The initial standard solutions of isoflavones were prepared in 80% ethyl alcohol. Reference Standards of daidzein, genistein, formononetin, prunetin and biochanin A are weighed by 0.005 g each (exact weight), placed in a measuring flask with capacity of 5 ml and brought to the mark with 80% ethyl alcohol, the solutions were mixed. At the same time, solutions with the content of standard substances of 0.001 g/ml (initial solutions) were obtained. Standard solutions of isoflavones were prepared immediately before analysis by diluting the initial solutions with 80% ethyl alcohol up to concentrations of 0.0002 g/ml and stored in a dark place at 4°C during study.

Preparation of a sample of the test sample. 0.125 g of dry extract (exact weight) was placed in a measuring flask with capacity of 25 ml, then, 10 ml of 40% ethyl alcohol was added and the flask was placed in an ultrasonic bath at 35°C for 10 minutes. Then the volume of the solution was brought up to the mark with 40% ethyl alcohol, after that, the solution was mixed and filtered (solution A). From solution A, 5 ml was transferred to a 25 ml measuring flask, brought to the mark with 40% ethyl alcohol and thoroughly mixed (the test solution).

Solutions were passed through a filter with a pore size of 0.45 microns and chromatograms were recorded.

The content of individual isoflavones in the dry extract in terms of reference standard in percents (X) was calculated using the formula:

$$X = \frac{C_{st} \times S_x \times 25 \times 25 \times P \times 100}{5 \times m \times S_{st} \times (100 - W)},$$

where C_{st} – reference standard concentration, g/ml;

S_{st} – area of a standard peak, mV/c;

S_x – area of a peak of substance in a dry extract, mV/c;

P – content of a substance in a reference standard, %;

m – weight of a dry extract, g;

W – weight loss during drying of dry extract, %.

RESULTS AND DISCUSSION

The proposed HPLC technique was applied to identify and quantify five isoflavones in a

dry extract of meadow clover herb. In Figures 1 and 2 the typical chromatograms of reference standards of isoflavones are shown, and the table shows the results of their identification and determination.

The retention time of the reference standards of daidzein, genistein, formononetin, prunetin, and biochanin A in the conditional analysis was 14.56 minutes, 19.45 minutes, 23.47 minutes, 29.10 minutes, and 29.34 minutes, respectively (Fig. 1). Based on the retention time of standard references in the studied sample of a dry meadow clover extract, the following were identified: daidzein (14.59 min.), genistein (19.47 min.), formonetin (23.46 min.), prunetin (29.11 min.) and biochanin A (29.34 min.) (Fig. 2). As a result of the study, the content of individual isoflavones in the dry extract of meadow clover was determined, which was: daidzein (0.36±0.01%), genistein (0.84±0.01%), formononetin (3.02±0.05%), prunetin (0.22±0.01%) and biochanin A (1.62±0.03%) (see table). The proposed method

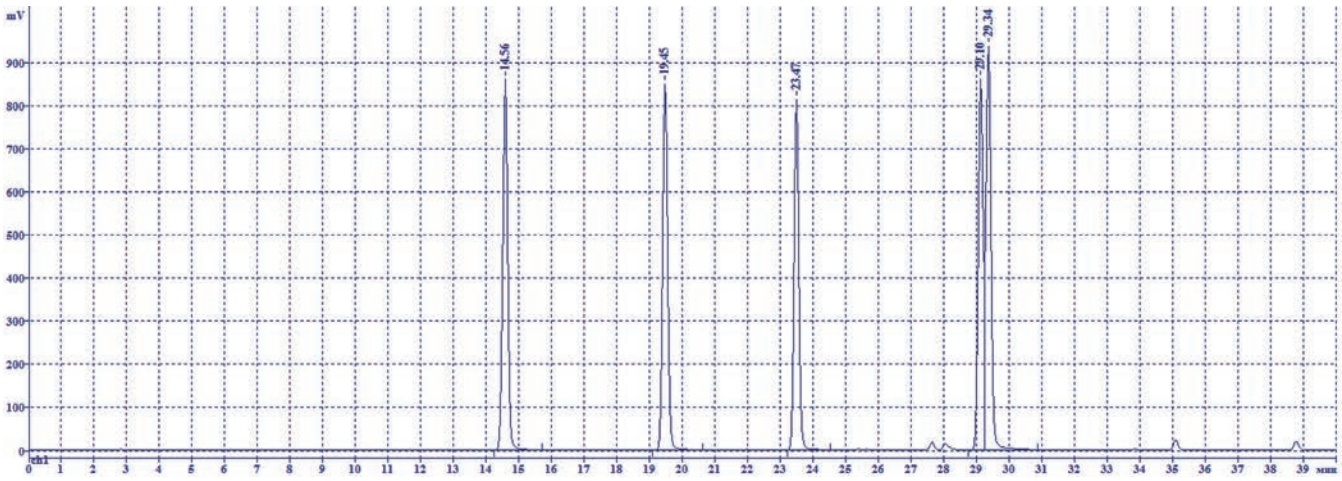

FIG. 1. *Chromatogram of mixture of reference standards of daidzen (0.0002 g/ml), genistein (0.0002 g/ml), formononetin (0.0002 g/ml), prunetin (0.0002 g/ml) and biochanin A (0.0002 g/ml)*

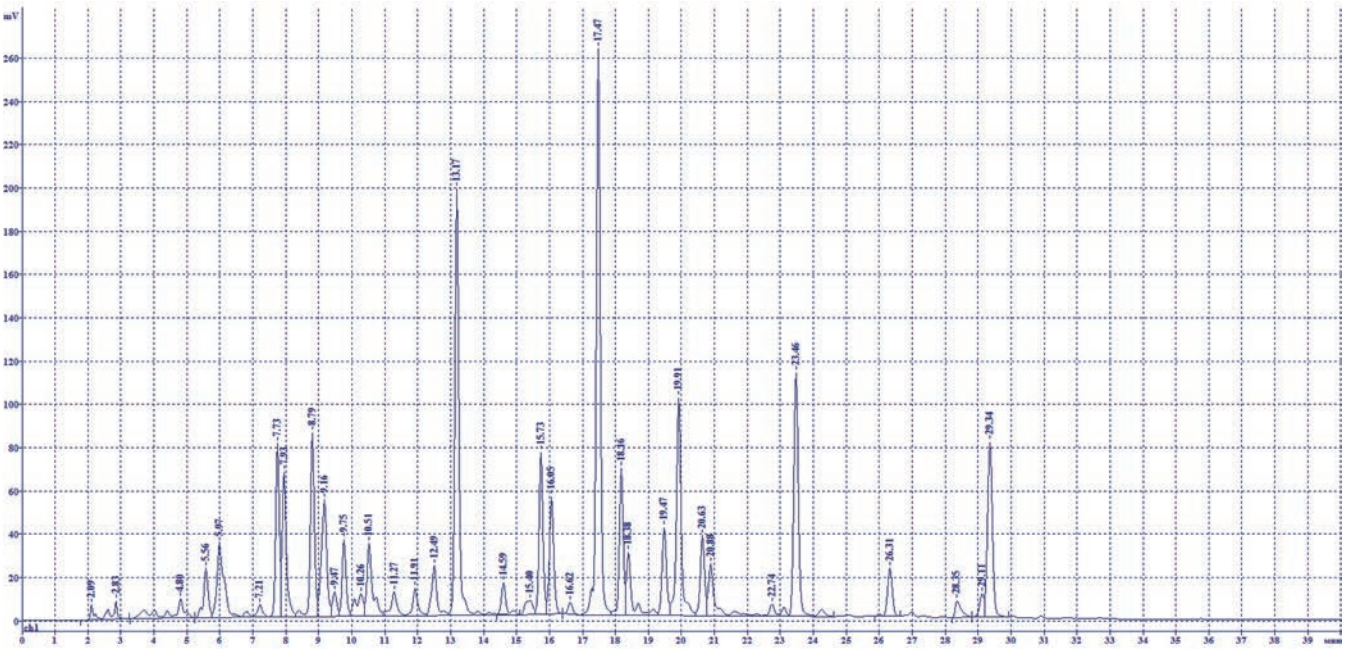

FIG. 2. *HPLC- chromatogram of a dry extract of the meadow clover herb*

Table

IDENTIFICATION AND DETERMINATION OF INDIVIDUAL ISOFLAVONES IN DRY MEADOW CLOVER EXTRACT BY HPLC, N=3, F=2, P=95%, T (P, F) =4,30

№	Name of a substance	t_{st}, min	t_x, min	S_{st}, mV/s	S_x, mV/s	Substances content in dry extract, %	Metrological characteristics of results
1	Daidzen	14.56	14.59	9197.614	145.397 149.710 140.837	0.3505 0.3601 0.3601 $\bar{X}$ = 0.36±0.01	S^2 = 0.000031 S = 0.0055 $S\bar{x}$ = 0.0032 ε, % = 2.8
2	Genistein	19.45	19.47	9153.454	388.630 387.539 380.939	0.8506 0.8462 0.8353 $\bar{X}$ = 0.84±0.01	S^2 = 0.00015 S = 0.012 $S\bar{x}$ = 0.0069 ε, % = 1.19
3	Formononetin	23.47	23.46	8210.147	1145.592 1172.919 1135.778	3.0074 3.0716 2.9863 $\bar{X}$ = 3.02±0.05	S^2 = 0.0017 S = 0.041 $S\bar{x}$ = 0.024 ε, % = 1.7
4	Prunetin	29.10	29.11	8878.792	92.041 95.113 87.596	0.2266 0.2335 0.2123 $\bar{X}$ = 0.22±0.01	S^2 = 0.00014 S = 0.012 S = 0.0069 ε, % = 4.6
5	Biochanin A	29.34	29.34	10426.282	835.150 833.634 806.617	1.6422 1.6353 1.5875 $\bar{X}$ = 1.62±0.03	S^2 = 0.0012 S = 0.035 $S\bar{x}$ = 0.020 ε, % = 1.85

Note: t_x – retention time of the substance in the dry extract, minutes; t_{st} – retention time of the reference standard, minutes; S_{st} – peak area of the reference standard, mV/s; S_x – peak area of the substance in the dry extract, mV/s.

is reproducible, the results obtained are reliable, and metrological characteristics are given.

Scientific studies have shown that biochanin A has significant lipid-lowering effect [3]. It was shown that in mammalian cell culture, biochanin A reduced the amount of binding of [3H] benzopyrene to DNA by 37–50% in a dose of 25 μg/ml and reduced the metabolism of [3H] benzopyrene by 54% compared to control cultures [4]. Biochanin A has cardioprotective, antitumor activity, antioxidant properties, and anti-inflammatory effects [5].

Formononetin has a potential anti-cancer effect in vitro and in vivo. When using formononetin in combination with other chemotherapeutic medicines such as bortezomib, LY2940002, U0126, sunitinib, epirubicin, doxorubicin, temozolomide and metformin, the anti-cancer potential of both formononetin and the corresponding pharmaceuticals is enhanced due

to the synergistic effect [6]. The neuroprotective effect of formononetin has been demonstrated in patients with Alzheimer's disease in both in vivo and in vitro studies [7]. In addition, it has been shown that formononetin exhibits vasorelaxant, antiapoptotic, cardioprotective, proliferative antioxidant, antimicrobial, and anti-inflammatory activities [8].

CONCLUSIONS

In a dry extract of meadow clover, five isoflavone compounds such as daidzein, genistein, formononetin, proetin, biochanin A were detected by HPLC. It was found that the highest content is characteristic for isoflavones – formononetin (3,02±0,05%) and biochanin A (1,62±0,03%), and that fact allows to consider the dry extract of meadow clover herb as a promising substance for medicine development

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