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# TOTAL FLAVONOID CONTENT IN THE HERBAL MIXTURE WITH ANTIDIABETIC ACTIVITY

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**Abstract**

Diabetes mellitus is an important social and medical problem, as it causes the development of dangerous complications that lead to disability and mortality. This disease is characterized by a multi-vector pathogenesis that requires a comprehensive approach to treatment. Due to the use of mixtures of medicinal plants in the treatment of diabetes, it is possible to cover all aspects of the development of this disease and its complications. In addition, the niche of the pharmaceutical market of Ukraine with phytomixtures is almost empty.

The aim of the reseach was to study the total flavonoid content in herbal mixture (*Equiseti arvensis herba, Sambuci flores, Inulae rhizomata et radices, Hyperici herba, Tiliae flores, Polygoni avicularis herba, Myrtilli folium, Urticae folia*), which have established hypoglycemic, hypolipidemic and antioxidant activity in previous studies *in vivo* and to validate the method that was used.

The study of the total flavonoid content in herbal sample was carried out by UV-spectrophotometry (λmax 415 nm) with the measurement of the absorption spectrum of flavonoids after complexation with aluminum chloride in terms of rutin. The method used was validated for linearity, sensitivity, precision and was established limit of detection and limit of quantification.

According to the results of UV-spectrophotometric determination, it was found that the total flavonoid content in the herbal mixture was 2.3±0.04 in terms of rutin.

The UV-spectrophotometric method for the analysis of the total flavonoid content was validated and showed good linearity of the standard solution of rutin (0.02-0.1 mg/mL). The calibration curve was expressed by the regression equation y=0.0011x+0.0207, the correlation coefficient was $R^2$=0.9984, the limit of detection – 0.01 mg/mL, and limit of quantification – 0.04 mg/mL. The method used meets the characteristics of precision and accuracy.

The data obtained indicate the presence of a correlation between the phytochemical composition of the studied herbal mixture and their pharmacodynamics, which was previously established.

**Keywords**: *herbal mixture, flavonoids, UV-spectrophotometry, diabetes mellitus, phytotherapy, antioxidant activity, validation, rutin*

## Introduction

Diabetes mellitus is a global social problem in the field of health care, due to rapid spread of this disease and the development of serious complications such as micro- and macroangiopathies, which significantly reduce the quality and life expectancy of patients [1, 2]. According to the official information of International Diabetes Federation (2019) the number of patients is projected to increase to 642 million by 2040 [3]. An important problem of pharmacovigilance is that existing pharmacotherapy is able to reduce hyperglycemia, but it is not always able to stabilize fluctuations of glycemia values during the day and keep it at optimal level [4, 5, 6]. This leads to the formation of a cascade of pathological processes – excessive glycation and inactivation of the body's antioxidant defense system, triggering free radical oxidation of lipids and, as a consequently, the development of oxidative stress, which leads to the development and progression of diabetic complications [7, 8]. In turn, oxidative stress, which is the result of the accumulation of reactive oxygen species (ROS: hydroperoxyl, superoxide, hydrogen peroxide and hydroxyl radicals), nitrogen molecules (RNS: peroxynitrite of active nitrogen) and some derivatives of heavy metals (iron and copper) is the main event for the development of insulin resistance [9, 10]. This may reduce peripheral insulin sensitivity through major molecular mechanisms such as β-cell dysfunction, inflammatory responses, decreased regulation and / or localization of glucose transporter 4 (GLUT-4), mitochondrial dysfunction, and abnormal insulin signaling pathways [1, 4, 11]. Therefore, the optimization of pharmacotherapy, search and study of new remedies with antioxidant activity for the prevention and treatment of this disease and its dangerous complications is a top issue of pharmacy and medicine.

One of these areas is phytotherapy, as it has a number of advantages over traditional therapy with using oral synthetic agents, namely, it is low-toxic, has a mild pharmacological effect and possibility to be used for long periods of time without significant side effects, is well combined with synthetic drugs, has a complex activity through a number of biologically active compounds [12, 13, 14, 15]. Particular attention deserve the combinations of different medicinal plants [16, 17], because such herbal mixtures are expected to have more biologically active substances that influence on all links of the pathogenetic mechanism of development of diabetes mellitus and its complications [18, 19]. Plant biocompounds have a wide range of pharmacological action and a variety of mechanisms of influencing on the development of diabetes (pathogenesis of which involves the development of insulin resistance, relative insulin deficiency, which leads to the decrease of secretory activity of β-cells of the pancreatic gland) and diabetic angiopathies (inactivation of antioxidant protection system, activation of lipid peroxidation and development of oxidative stress) [4, 9].

In this regard, the important biologically active substances are hydroxylated polyphenolic compounds, namely flavonoids. Therefore, the aim of study was to investigate the total flavonoid content in herbal mixture (*Equiseti arvensis herba, Sambuci flores, Inulae rhizomata et radices, Hyperici herba, Tiliae flores, Polygoni avicularis herba, Myrtilli folium, Urticae folia*) with previously studied antidiabetic activity *in vivo* [20, 21, 22] and validate this method of determination.

## Methods

**Plant materials:** It was used the herbal raw materials harvested in June – August 2017 in Ternopil region and Charpathians (*Vaccinium myrtillus* leaf) (Ukraine) during the study. After harvesting, the raw materials were dried, crushed and brought back to standard according to the general GACP requirements [23]. The plants were identified by Department of Pharmacognosy with Medical Botany, I.Horbachevsky Ternopil National Medical University, Ternopil, Ukraine. Samples of herbal raw materials have been deposited in Departmental Herbarium for future record.

**Chemicals and standards:** Chemical reference substance (CRS) of rutin was of primary reference standard grade (≥ 95 % purity HPLC) and was purchased from Sigma-Aldrich Chemical Company (Germany). Absolute ethanol, aluminium chloride and acetic acid was purchased from Sfera Sim LTD (Ukraine). Water used in the studies was produced by MilliQ Gradient water deionizaton system (USA).

**Instrumentation and conditions:** spectrophotometric measurements were performed on spectrophotometer UV-1800 Shimadzu (Japan) equipped with 1 cm quartz cells. Determination of the total flavonoid content was performed at a maximum absorption of 415 nm after complexation with aluminum chloride ($AlCl_3$). The measurements were performed 40 min after the addition of $AlCl_3$.

**Exrtact:** 1.00 g (accurately weight) of each powdered herbal raw materials was placed into a 150 mL flask. Then was added 30 mL of ethanol 50 % (v/v) and was heated on a water bath under reflux for 30 minutes. The hot extract was filtered in cotton to a volumetric flask of 100 mL. The cotton and raw materials residue were returned to the flask and a new extractive cycle was performed by 15 min with 30 mL of ethanol 50 % (v/v). This procedure was repeated two times. The filtrates were collected in the 100 mL volumetric flask, and the volume made up with ethanol 50 % (v/v).

**Stock solution:** the solution resulting from the reflux was taken as stock solution.

**Test solution:** 1 mL of stock solution was placed into 25 mL volumetric flask and 2 mL of 3 % $AlCl_3$ in 95 % ethanol (w/v) solution was added. Then volume was completed to the mark by 95 % ethanol.

**Reference solution:** 1 mL of stock solution was placed into 25 mL volumetric flask, then 1 drop of dilute acetic acid solution was added and volume was completed to the mark by 95 % ethanol.

**Standard solution:** 0.05 g (accurately weight) of CRS of rutin was dissolved in 100 mL of 95 % ethanol in a water bath. Then 1 mL of rutin solution was taken and placed in a 25 mL volumetric flask, 1 drop of dilute acetic acid solution was added to it and was completed to the mark by 95 % ethanol.

**Method validation:** the method was validated for linearity, sensitivity and precision (intra-assay and intermediate precision), accuracy, LOD, and LOQ [24].

**Calibration curve and linearity:** standard solution of rutin in 95 % ethanol (0.5 mg/mL) was prepared in two replicate and diluted to concentrations of 0.02 mg/mL, 0.04 mg/mL, 0.06 mg/mL, 0.08 mg/mL and 0.1 mg/L. The absorbance of diluted rutin solutions was measured at 415 nm 40 min after adding of $AlCl_3$ against the blank solution. The calibration curve was constructed by plotting the measurements of mean absorbance versus the concentration of the standard solutions. The results were analyzed by linear regression and the correlation coefficient ($R^2$).

**Sensitivity:** the values for limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on the data obtained during linearity testing in the low concentration range of the working in the test solution, using the following formulas: LOD = 3.3 * s / Slope; LOQ = 10 * s / Slope.

**Precision and accuracy:** precision was determined through the determination of intraassay and intermediate precision (41). Intra-assay precision was determined by measuring three different concentrations of each standard (prepared as two replicates, each measured three times) in one laboratory by one person. Intermediate precision was carried out by measuring the same concentrations of standards (prepared as two replicates, each measured three times) by two people in the same laboratory. Precision was expressed as percentage coefficient of variation (CV), using the following formulas: CV = standard deviation / mean x 100. Accuracy is expressed through recovery, which was determined by measuring known concentrations of standard (in three concentration levels, in triplicate).

**Statistical Analysis:** MS Excel (Microsoft, Redmond, WA) was used for data analysis. The results for the calibration curves were based on two replicate samples of each standard concentration, each measured twice (n = 4). Regression was used to obtain the linear equation and determine $R^2$. Precision and recovery studies were based on the measurement of three different concentration levels, each as two replicates and each measured three times (n = 6). Total flavonoid content in the herbal mixtures was the results of two replicate samples, each measured twice (n = 4) and were presented as mean value ± SD.

## Results and Discussion

The results of spectrophotometric determination of the total flavonoid content in terms of rutin are presented in Figure 1. The method of determination was based on the reaction of complexation of flavonoids with $AlCl_3$. The maximum absorption of herbal sample was at λmax = 415±0.5 nm.

According to UV-spectrophotometric

determination, it was found that the total flavonoid content was 2.3±0.04 % in the herbal mixture (Fig. 1).

The analytical procedure has been validated to confirm its reliability. Calibration solutions were prepared in triplicate and analyzed under the optimal conditions as described above. The calibration curve was found to be linear in the range 0.02-0.1 mg/mL for CRS of rutin (Fig. 2). Regression equation (y=0.0011x+0.0207) and $R^2$=0.9984 revealed a good linearity response for spectrophotometric method developed for the determination of total flavonoid content.

The results of validation showed that the LOD and the LOQ of CRS of rutin was 0.01 mg/mL and 0.04 mg/mL, respectively, indicating that the sensitivity of the method was satisfactory (Table 1).

In the table 2 represents the precision and accuracy of spectrophotometric method for the determination of total flavonoid content. CVs for the spectrophotometric method were between 1.4 % and 3.9 % and between 0.7 and 5.5 % for intra-assay and intermediate precision, respectively. Precision was studied through intra-assay and intermediate precision. Accuracy was expressed through the determination of recovery, which was between 96.4 % and 106.2 %.

The chemical structure of flavonoids is based on fifteen-carbon skeleton consisting of two benzene rings linked via a heterocyclic pyrane ring [25]. The pharmacological activities of this biocompounds depend on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization. In particular, functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions [26, 27].

Antioxidant activity of flavonoids in the treatment and prevention of diabetes and its complications is important because they can include suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation [28]; scavenging ROS; inhibition the enzymes involved in ROS generation – microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, nicotinamide adenine dinucleotide phosphate (NADH) oxidase, and so forth [29].

Thus, the high content of flavonoids in the studied herbal mixture (Fig. 1) may indicate the ability of these mixtures to prevent the development of oxidative stress, which is the main pathogenic mechanism of diabetic angiopathies [30]. In addition, the obtained results are reliable because the method to determine the total flavonoid content in the studied object was validated for linearity, sensitivity and precision (intra-assay and intermediate precision), accuracy, LOD, and LOQ (Fig. 2, Table 1-2).

The obtained data testify to the expediency of using the studied herbal mixture in order to optimize antidiabetic pharmacotherapy.

## Conclusions

The results of UV-spectrophotometric analysis indicate the high content of flavonoids in the studied herbal mixture (*Equiseti arvensis herba, Sambuci flores, Inulae rhizomata et radices, Hyperici herba, Tiliae flores, Polygoni avicularis herba, Myrtilli folium, Urticae folia*) that provides the powerful antioxidant activity, which is an important factor in the treatment of diabetes and in preventing the development of diabetic angiopathies. The total flavonoid content was (2.3±0.04 %) in the herbal mixture.

UV spectrophotometric method to determine the total flavonoid content was validated and showed good linearity of the standard solution of rutin in the range of 0.02-0.1 mg/mL, high sensitivity of the method, precision (intra-assay and intermediate precision) and accuracy.

The obtained phytochemical studies may indicate a correlation between the component composition and total flavonoid content in the sample of studied mixture and its effectiveness in the treatment and prevention of diabetes mellitus and its complications.

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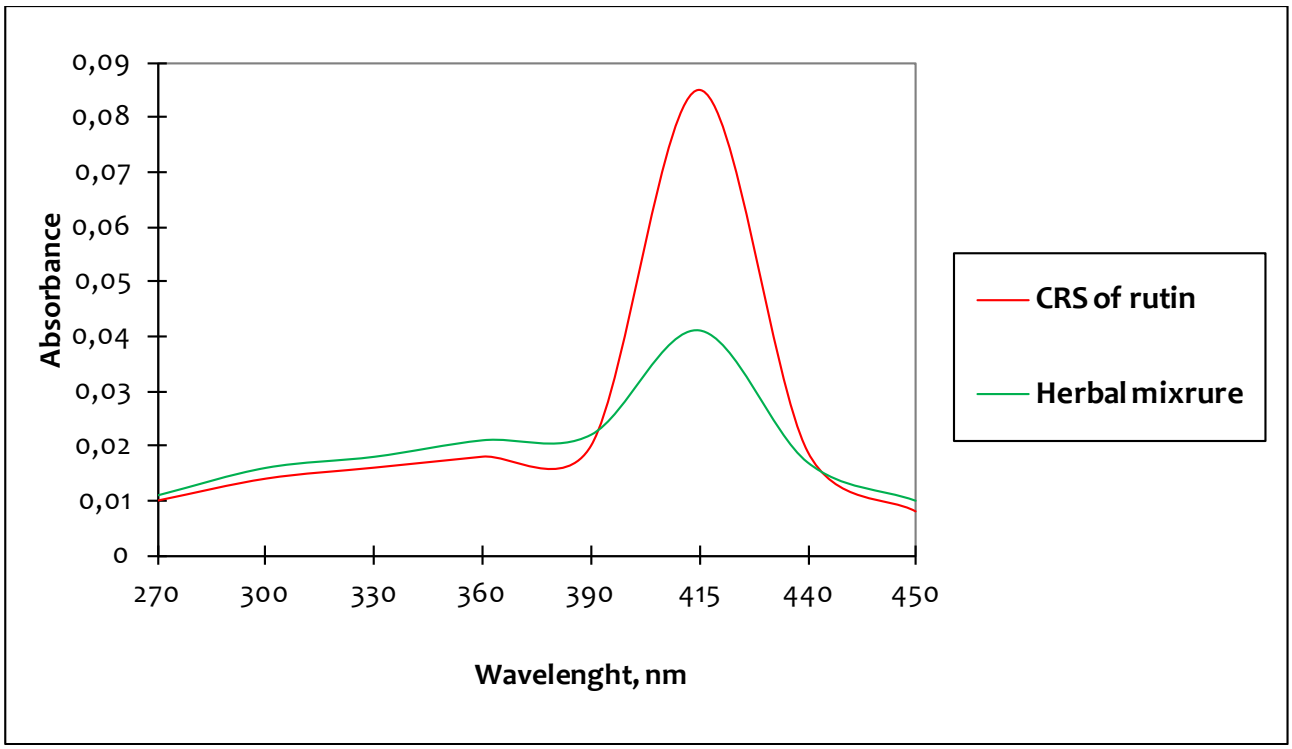


**Figure. 1.** *UV-spectrum of $AlCl_3$-flavonoid complexes content in the herbal mixture (Values are expressed as mean ± SD (n=4)*

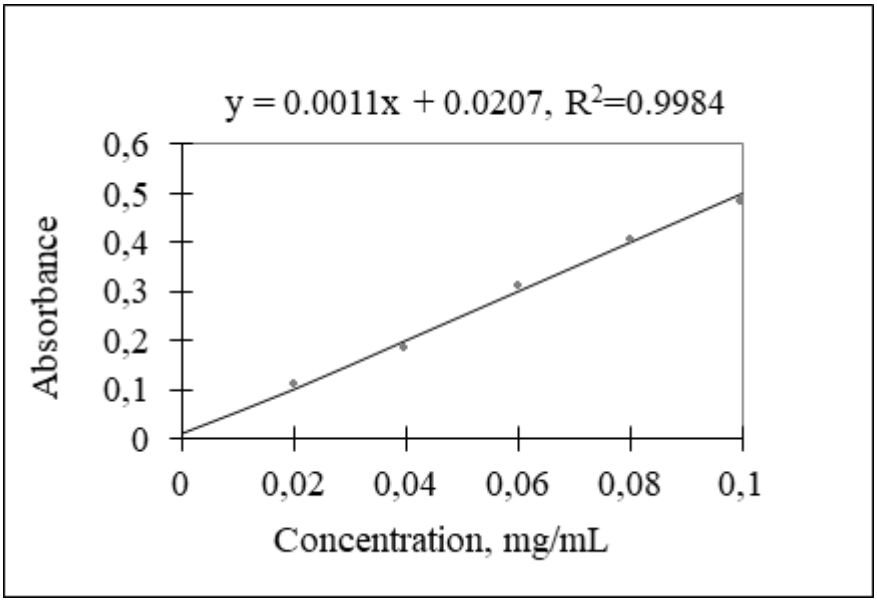


**Figure 2.** Calibration curve of standard solution of rutin-$AlCl_3$ complex

**Table 1.** Linearity, LOD and LOQ of the spectrophotometric method for the determination of total flavonoid content

| Standard | Range, mg/mL | Regression curve | $R^2$ | LOD, mg/mL | LOQ, mg/mL |
|---|---|---|---|---|---|
| **Rutin** | 0.02-0.1 | y =0.0011x+0.0207 | 0.9956 | 0.01 | 0.04 |

Note: Results are based on two replicate samples of each standard concentration, each measured twice (n = 4).

**Table 2.** Precision and accuracy of the spectrophotometric method for the determination of total flavonoid content

| Standard | Concetration, mg/mL | Intra-assay precision*, % | Intermediate precision**, % | Recovery, % |
|---|---|---|---|---|
| **Rutin** | 0.25 | 1.4 | 0.7 | 96.4 |
| | 0.50 | 3.9 | 5.5 | 106.2 |
| | 1.00 | 2.6 | 2.4 | 105.5 |

Note:

***** intra-assay precision – one analyst in one laboratory, with each concentration made as two replicates and each measured three times (n = 6);

****** intermediate precision – two analysts on different days in the same laboratory, with each concentration made as two replicates and each measured three times (n = 6).