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Methodological approach to the elaboration of the analytical procedure of the antioxidant activity determination of the *Schisandra chinensis* (Turcz) Baill. extracts

Nataliia Hudz[1]*, Vladimira Horčinová Sedláčková[2], Oksana Yezerska[1], Mykhailo Zhurba[3], Ján Brindza[2]

[1]Danylo Halytsky Lviv National Medical University, Department of Drug Technology and Biopharmaceutics, Lviv, Ukraine
[2]Slovak University of Agriculture in Nitra, Institute of Plant and Environmental Sciences, Nitra, Slovakia
[3]M.M. Gryshko National Botanical Garden of the National Academy of Sciences of Ukraine, Department of Fruit Plants Acclimatization, Kyiv, Ukraine

ORCID
Nataliia Hudz https://orcid.org/0000-0002-2240-0852
Vladimíra Horčinová Sedláčková https://orcid.org/0000-0002-5844-8938
Oksana Yezerska https://orcid.org/0000-0001-8543-2723
Mykhailo Zhurba https://orcid.org/0000-0001-5318-3961
Jan Brindza https://orcid.org/0000-0001-8388-8233

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The DPPH assay provides an easy and rapid way to evaluate the antioxidant activity of herbal preparations. The most important stage of the elaboration of the analytical procedure of total antioxidant activity measured by the DPPH test is to select the volume of an extract or its appropriate dilution for the determination of the reliable total antioxidant activity of the extract. If the concentration of antioxidants is too high in an extract, the kinetic curves of antioxidant activity changes on time are more parallel to axis *X* (time) and what is more, the total antioxidant activity does not depend on the volume of the extract. The analytical procedure of the antioxidant activity determination of the *Schisandra chinensis* (Turcz) Bail. extracts was elaborated, namely a volume and dilution of the extract was selected, rutin was chosen for the calibration curve. The calibration curve was plotted in the concentration range of 95–305 mg/L ($y = 0.228x+7.0992$, $R^2 = 0.9945$). The results suggest that the leaves of *S. chinensis* are a valuable source of antioxidant compounds with significant antioxidant activity. The antioxidant activity was evaluated by the DPPH test. It was equal to 227.2–443.6 mg/L in the extracts or 1.14–2.22 mg/g in the leaves of rutin-equivalents depending on the particle size. Additionally, it was established that particle size in the range of 2 to 3 mm was optimal for the preparation of *Schisandra chinensis* extracts as the antioxidant activity was the highest. The ethanol absorption coefficient is a main technological parameter in the pharmaceutical manufacture of extracts. The absorption coefficient of the *Schisandra chinensis* leaves for 70 % ethanol was in the range of 3.4 to 6.5 ml/g and depended on the particle size.

Keywords: *Schisandra chinensis*, ethanolic extracts, antioxidant activity, DPPH test

***Corresponding Author:** Nataliia Hudz, Danylo Halytsky Lviv National Medical University, Department of Drug Technology and Biopharmaceutics, Str. Pekarska 69, 79010 Lviv, Ukraine
natali_gudz@ukr.net

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Introduction

Schisandra chinensis (Turcz) Bail., Chinese magnolia vine belongs to the family of Magnoliaceae. *Schisandra chinensis* is widely distributed in China, Japan, Korea, and other countries due to its cultivation (Panossian and Wikman 2008; Lai et al., 2015; Qui et al., 2018; Hu et al., 2020). This plant is described in many Pharmacopeias. Among them are the Chinese Pharmacopeia, the Japanese one, the Pharmacopeia of the United State of America, and the State Pharmacopeia of Ukraine (Szopa et al., 2018; Qui et al., 2018; Hu et al., 2020).

The principal active substances of *Schisandra chinensis* are lignans and triterpenes (Song et al., 2016; Szopa et al., 2018; Qui et al., 2018; Hu et al., 2020). Fruits are rich in organic acids, especially citric acid (Hu et al., 2020). According to Hu et al., there existed a strong correlation between the total phenolic content of extracts of fruits and antioxidant activity determined by the DPPH test (Hu et al., 2020).

Modern medical researches prove that *S. chinensis* contains multiple active components which can protect liver cells (Ip and Ko, 1996; Ip et al., 2007; Teraoka et al., 2012), restrain oxidation (Jung et al., 2000; Sheng et al., 2011), prevent senility (Nishiyama et al., 1996; Hsieh et al., 1999; Hsieh et al., 2001; Kang et al., 2005), improve human body immunity ability (Mizoguchi et al., 1991). It can have a positive influence on the pulmonary system, kidney, liver, skin, central nervous system, etc. (Kim et al., 2004; Fu et al., 2008; Lai et al., 2015; Gao et al., 2016; Huang et al., 2017; Li et al., 2019; Liu et al., 2019). The renoprotective effects of the *Schisandra chinensis* extract are related to its antiapoptotic and antioxidant abilities, which induced the attenuation of CsA-induced autophagic cell death (Lai et al., 2015). Schizandrin B reduces UVB-irradiation damages of skin fibroblasts and epidermal keratinocytes (Gao et al., 2016).

There are some studies on the anti-inflammatory activity of *Schisandra chinensis* extracts and some individual lignans (Song et al., 2016; Szopa et al., 2018; Qui et al., 2018). The leaves of *Schisandra chinensis* are richer in polyphenolic compounds (Mocan et al., 2014). However, there are few studies related to *Schisandra chinensis* leaves extracts. Some authors indicate that *Schisandra chinensis* leaves are a valuable source of flavonoids with important antioxidants (Yu et al., 2017) and antimicrobial activities (Mocan et al., 2014). The *Schisandra chinensis* leaves extract showed stronger antimicrobial activity compared to the fruit extract (Mocan et al., 2014).

To widen the potential use of *S. chinensis* in antioxidant biomedicine, the present study was carried out to study antioxidant activities of extracts from the leaves of *S. chinensis* of different particle sizes, using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity. Therefore, the purpose of our studies was to evaluate the antioxidant activity of the extracts prepared from the powdered leaves of *Schisandra chinensis* of the different sizes: 1–2 mm, 2–3 mm, 3–4 mm, and 5–7 mm. For the correct evaluation of antioxidant activity by the DPPH method, it is necessary to select an appropriate ratio of a solution of DPPH to an extract. For that reason, one more aim was to elaborate an appropriate analytical procedure for the evaluation of the antioxidant activity of the extracts by the DPPH method.

Material and methodology

While carrying out this research, the following methods were used: analysis, synthesis, systematization, and comparison for processing published scientific data; technological method (remaceration); spectrophotometric method for the elaboration of the analytical procedure of the determination of the total antioxidant activity by DPPH test.

Plant material

Schisandra chinensis leaves were collected in the Arboretum Mlynany (Slovakia) in the middle of July of 2021. The specimen was stored in the herbarium of Institute of Plant and Environmental Sciences, Slovak University of Agriculture in Nitra. The voucher specimen number is Sch-2.

Reagents

The following reagents were used: ethanol 96 % (manufacturer "Centrachem" (Slovakia)), DPPH ("Sigma Aldrich"), and rutin hydrate ("Sigma Aldrich").

Extraction

The dry leaves were ground into powder and fractionated through different sieves (1, 2, 3, 5, and 7 mm) before the preparation of extracts.

Four extracts were prepared, using the different fractions of the powdered leaves. The remaceration consisted of maceration for 24 hours and the following two macerations for a period of 3 h for each one. The filtration was performed after each maceration and the obtained extracts were combined. Therefore, the total maceration accounted for 30 hours (24 hours +

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Table 1 Characterization of the obtained extracts

Number of the extracts	Particle size (mm)	Mass of the leaves (g)	Added volume of ethanol/obtained volume of an extract		Total volume of the extract (µL)
			main maceration	additional two macerations	
Extract 1	1–2	1.54	10/0	5.0/4.2 5.0/3.3	7.5
Extract 2	3–4	2.50	15/3.3	5.0/4.5 5.0/5.0	12.5
Extract 3	2–3	2.50	15/2.4	5.1/3.6 7.0/7.0	12.5
Extract 4	5–7	2.50	15/6.6	3.2/2.4 5.0/2.8	12.5

2 macerations × 3 hours = 30 hours). Features of the preparation of the extracts are provided in Table 1.

DPPH free radical scavenging activity assay

The two described analytical procedures were put in base of the evaluation of the extracts of *Schisandra chinensis* (Hudz et al., 2017; Hu et al., 2020), where authors used ascorbic acid as a positive control. In our study we used rutin. Additionally, Mocan et al. (2014) used quercetin as a positive control.

DPPH was dissolved in 96 % ethanol to a final approximate concentration of 0.003 %. In the free radical scavenging activity assay, 1 950 µL of the DPPH solution was added to 2 000 µL tubes, followed by 50 µL of the extract or its dilution. The solutions were mixed and incubated in the dark at room temperature. The absorbance was recorded at a wavelength of 515 nm (As). The mixture of 1 950 µL of 96 % ethanol solution with 50 µL of the extract was used as a blank for the extract. The value A0 was recorded when 1 950 µL of DPPH solution was mixed with 50 µL of ethanol after incubation under the former conditions. 96 % ethanol solution was used as a blank for the DPPH solution.

To compare the free radical scavenging activity of the four extracts, the rutin equivalents of each sample were calculated. Rutin was dissolved in 50 % ethanol solution and diluted to different five concentrations. Then 50 µL of each solution was mixed with DPPH solution. The reaction mixtures were incubated for 40 min. Their absorbance was recorded at a wavelength of 515 nm each 10 min. The mixture of 1 950 µL of 96 % ethanol solution with 50 µL of each rutin solution was used as a blank for each rutin solution.

In addition, we studied the stability of reaction mixtures or, in other words, we studied the kinetics of the reaction of DPPH with the extracts and rutin depending on the time.

Statistical analysis

All the analyses of the DPPH test for each extract, its dilution, and solutions of rutin were carried out in triplicate and the results were expressed as a mean value ± standard deviation (SD).

Results and discussion

Herbal preparations are used as an alternative source of medicines to mitigate the diseases associated with oxidative stress (Priya and Nethaji, 2015). The free-radical scavenging activity of the extracts was evaluated using the widely used 2,2-diphenyl-1-picrylhydrazyl (DPPH) test (Sheng et al., 2011; Mocan et al., 2014; Ivanišová et al., 2017; Vergun et al., 2018, 2021; Grygorieva et al, 2020; Shelepova et al., 2020; Tvrdá et al., 2020; Mňahončaková et al., Vinogradova et al., 2021). The DPPH assay provides an easy and fast mode to estimate antioxidant activity. This test is based on electron-transfer. DPPH produces a violet solution in ethanol or methanol. The reduction of DPPH in the presence of an antioxidant or mixture of antioxidants leads to the formation of non-radical form DPPH-H of yellow or yellowish colour (Sheng et al., 2011; Mocan et al., 2014; Rachman et al., 2015). According to our studies, the colour of final mixtures can be light purple or yellow depending on the concentration of antioxidants.

Various modifications and optimizations of DPPH assay are described for their adaptation to tested extracts or are the invention of researchers (Sheng et al., 2011; Mocan et al., 2014; Hu et al., 2020).

The most important stage of the development of the analytical procedure of the total antioxidant activity measured by the DPPH test is to select the volume of an extract or its appropriate dilution for the determination of the reliable total antioxidant activity of the extract. From our experience, if the concentration of antioxidants is too high in an extract,

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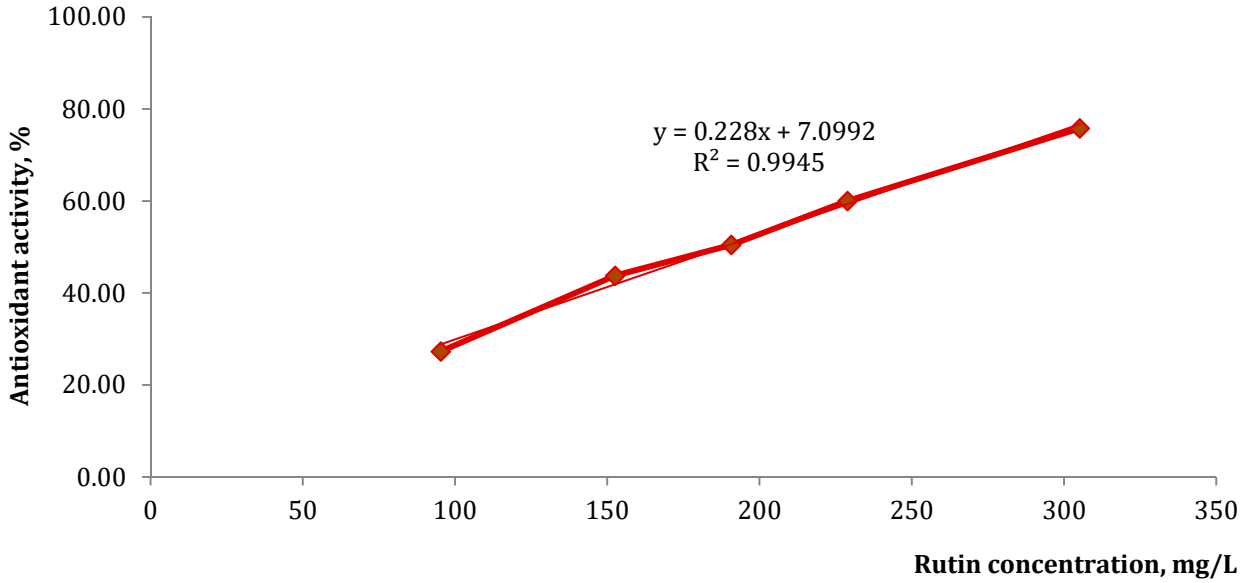

Figure 1 Calibration curve of rutin

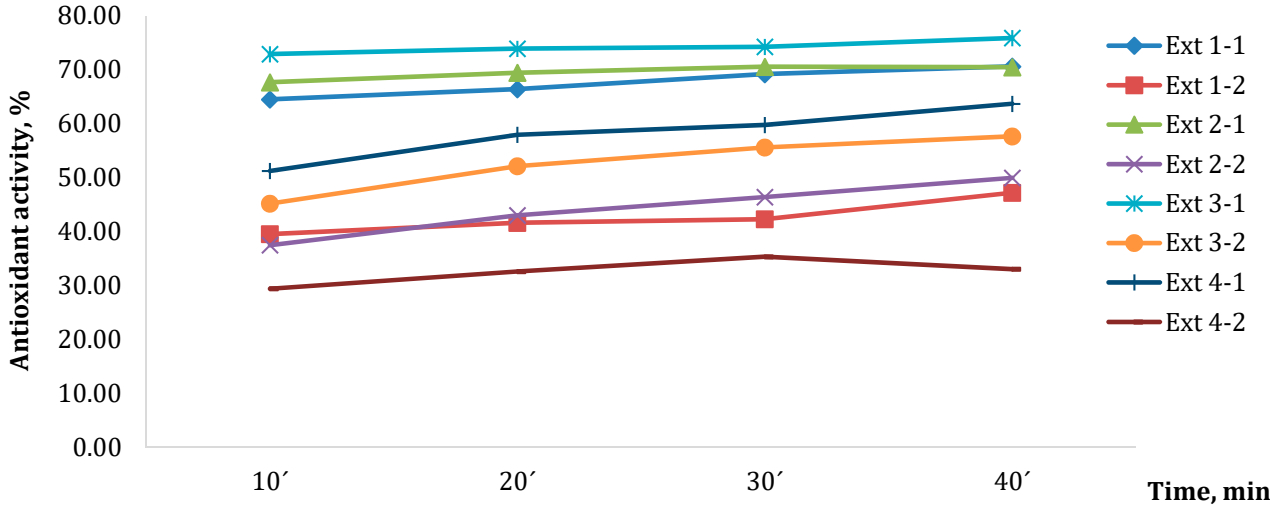

Figure 2 Dependence of the antioxidant activity of the extracts of *Schisandra chinensis* (Turcz) Bail. on time
Ext 1-1, Ext 2-1, Ext 3-1, Ext 4-1 – extracts 1, 2, 3 and 4 without dilution and Ext 1-2, Ext 2-2, Ext 3-2, Ext 4-2 – extracts 1, 2, 3 and 4 diluted twice by 70 % ethanol

namely if the content of biologically active substances is very large and an appropriate dilution was not selected, then DPPH is reduced very quickly without dependence on dilution and final mixtures get yellow. In that case, the kinetic curves of antioxidant activity changes on time are more parallel to axis *X* (time), and, what is more, the total antioxidant activity is equal to 80–85 % and did not depend on the volume of the extract of beebread (20–50 µl) (Hudz et al., 2017). In other words, the higher the rate of DPPH consumption is, the more powerful the antioxidant potential (Mocan et al., 2014). Therefore, it is necessary to decrease the volume of the sample or dilute it.

According to the literature, DPPH has an absorption maximum at a wavelength of 514–517 nm (Ivanišová et al., 2017; Shelepova et al., 2019; Vergun et al., 2019, 2021; Grygorieva et al, 2020; Hu et al., 2020; Tvrdá et al., 2020). Reducing a colour intensity of a reaction mixture is carried out by various authors at the time range of 4–15 minutes to 1 hour (Sheng et al., 2011; Mocan et al., 2014; Hu et al., 2020).

The results obtained by the DPPH test were presented as rutin equivalents, mg/L and mg/g (Table 2). The percentage of DPPH consumption was converted to rutin equivalents by using a calibration curve ($y = 0.228x + 7.0992$, $R^2 = 0.9945$) with the rutin

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Table 2 Antioxidant activity of the *Schisandra chinensis* (Turcz) Bail. extracts expressed in %, mg/L and mg/g of rutin-equivalents

№	Antioxidant activity (%), mean ± SD		Concentration of rutin-equivalents (mg/L) in the extract*	Concentration of rutin-equivalents (mg/g), in the leaves*
	native extracts	diluted extract twice		
1	70.6 ±0.1	47.2 ±0.8	351.8 ±6.0	1.76
2	70.5 ±1.3	50.0 ±1.3	376.3 ±9.8	1.88
3	75.9 ±0.9	57.7 ±0.9	443.6 ±6.9	2.22
4	63.7 ±1.5	33.0 ±0.6	227.2 ±4.1	1.14

Notes: * – calculations were performed on the values of AA of the diluted extracts

Table 3 Calculations of the experimental determination of the absorption coefficient of 70 % ethanol

№	Particle size (mm)	Calculations
1	1–2	X1 = (10–0) : 1.54 = 6.5 ml/g
2	3–4	X2 = (15–3.3) : 2.5 = 4.7 ml/g
3	2–3	X3 = (15–2.4) : 2.5 = 5.0 ml/g
4	5–7	X4 = (15–6.6) : 2.5 = 3.4 ml/g

solutions in the concentration range of 95–305 mg/L (Figure 1).

From Figure 1 we can observe that the higher the rate of DPPH consumption is, the more powerful is the antioxidant potential. For undiluted *Schisandra chinensis* extracts the antioxidant activity was in the range of 70–76 % independing on particle size in the case of extracts 1, 2, and 3. This pointed to an inappropriate ratio of antioxidants content and DPPH. Therefore, firstly we diluted our extracts twice and measured the antioxidant activity.

In Figure 2 we presented the dependence of the antioxidant activity of the undiluted and diluted extract on the time. The ratio of an extract to a final dilution was 1 to 2.

Our studies are in line with studies performed by Sheng et al. (2011) and Rachman et al. (2015). Increasing the concentration of compounds with antioxidant activities enhances the antioxidant activity of a reaction mixture (Sheng et al., 2011; Rachman et al., 2015). Moreover, such an increase in the concentration of compounds with antioxidant activities leads to that the kinetic curves of antioxidant activity changes on concentration are parallel to axis *X* (Sheng et al., 2011; Rachman et al., 2015).

From Table 2 we can observe that the total antioxidant activity of the native extracts is equal to 63.7–75.9 %. This antioxidant activity did not correlate with the values of the antioxidant activity of the same diluted extracts except for extract 4. We can suppose that it is necessary to select the appropriate dilution of an extract of the total antioxidant activity of this undiluted extract exceeds 64 %. Moreover, the kinetic curves of the dependence of antioxidant activity on time are more parallel to axis *X* for extracts 1–3 that is in line with the studies performed for the extracts of beebread (Hudz et al., 2017).

Furthermore, it was established that particle size in the range of 2–3 mm is optimal for the preparation of *Schisandra chinensis* extracts. We observed in our study that an increase in the surface area available for molecular transport contributes to a more extensive mass transfer of compounds with antioxidant activity into an extract if not considering extract 1.

Additionally, rutin was selected as a marker for the DPPH test as such flavonoid glycosides as rutin, hyperoside, quercitrin, and isoquercitrin and flavonoid aglycones as myricetin, kaempferol, and quercetin were identified in the extract of the *Schisandra chinensis* fruits Bail (Mocan et al., 2014; Tvrdá et al., 2020). Moreover, rutin was dominated flavonoid among glycosides in the extract (Tvrdá et al., 2020).

The DPPH assay showed that the free-radical scavenging activity of the extract from fruits was 5.93 mg of Trolox equivalents/g d.w. (Tvrdá et al., 2020). The antioxidant activity of *S. chinensis* leaves was 26.87 ±0.84 mg QE/g of plant material, while the antioxidant activity of *S. chinensis* fruits was 7.80 ±0.55 mg QE/g of plant material (Mocan et al., 2014).

We cannot compare our results with published data as antioxidant activity was expressed in rutin equivalents in our studies.

The ethanol absorption coefficient is a main technological parameter in the pharmaceutical manufacture of extracts (Yezerska et al., 2021). The results of the technological studies are provided in Table 3. It was revealed that the coefficient of alcohol absorption of the crushed leaves depended on the size of particles.

We observed such regularity: the more particle size, the less was the absorption coefficient (Table 3).

Conclusion

The analytical procedure of the antioxidant activity determination of the *Schisandra chinensis* extracts by the DPPH test was developed from a point of view of choosing a volume and dilution of the extracts, marker for the calculation of the antioxidant activity of the extracts. The calibration curve was plotted in the concentration range of 95 to 305 mg/L of rutin ($y = 0.228x + 7.0992$, $R^2 = 0.9945$). The results suggest that the leaves of *Schisandra chinensis* are a valuable source of antioxidant compounds with significant antioxidant activity. The antioxidant activity of the *Schisandra chinensis* extracts (1 : 5) was equal to 227.2–443.6 mg/L rutin-equivalents depending on the particle size. Additionally, it was established that particle size in the range of 2–3 mm was optimal for the preparation of *Schisandra chinensis* extracts as the antioxidant activity was the highest. This study established the basis for future research into the elaboration of the *Schisandra chinensis* extracts from leaves.

Conflicts of interest

The authors declare no conflict of interest.

Ethical statement

This article does not contain any studies that would require an ethical statement.

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