



Comparison of antioxidant activity of dihydroquercetin samples with different diastereomeric composition

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Abstract

Introduction. Flavanonol dihydroquercetin (DHQ) is known for its pronounced antioxidant properties, which are associated with a wide range of biological activity of this compound. Due to the presence of two chiral centers at positions 2 and 3 of the benzopyranone ring, this flavonoid is characterized by the phenomenon of *cis-trans* isomerism. However, a comparative analysis of the antioxidant properties of DHQ diastereomers has not been performed before.

Aim. To evaluate the effect of diastereomeric composition on the reducing and radical scavenging properties of DHQ samples.

Materials and methods. The diastereomeric composition of the DHQ samples was controlled under reverse phase chromatography with UV detection. The antioxidant capacity was studied using a model of inhibition of radical cations of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS^{•+}). The reducing ability of the DHQ samples was evaluated using a 5-(2,4-disulfophenyl)-3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2*H*-tetrazolium monosodium salt (WST-8) model. Both tests were performed under the control of UV spectrophotometry.

Results and discussion. The chromatograms of the samples obtained at 288 nm are characterized by two peaks with retention times of 18.79 ± 0.08 and 19.87 ± 0.08 min, which correspond to *trans*- and *cis*-diastereomers of DHQ. After 6 minutes of DHQ samples containing 0.8, 3.7, and 9.5 % of the *cis*-isomer adding to the ABTS^{•+} solution, the optical density at 730 nm decreased by 0.588 ± 0.008 , 0.581 ± 0.017 , and 0.602 ± 0.012 , respectively (α 0.05, p 0.2196, r 0.7857), while in WST-8 solutions at 450 nm for similar samples, an increase in optical density was observed by 0.178 ± 0.030 , 0.222 ± 0.026 , and 0.188 ± 0.036 , respectively (α 0.05, p 0.5157, r 0.0284).

Conclusion. These results indicate that there is no significant effect of the diastereomeric composition on the antioxidant properties of the DHQ samples. Thus, the observed differences in the pharmacology of *cis*- and *trans*-DHQ should be explained by the selectivity of interaction with biological targets, rather than the free radicals scavenging activity.

Keywords: dihydroquercetin, flavonoids, diastereomerism, antioxidants, HPLC

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Сравнение антиоксидантной активности образцов дигидрокверцетина с различным диастереомерным составом

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Резюме

Введение. Флаванол дигидрокверцетин (ДКВ) известен выраженной антиоксидантной способностью, с которой связывают широкий спектр биологической активности данного соединения. Благодаря наличию двух центров хиральности в положениях 2 и 3 бензопиранового кольца, для этого флавоноида характерно явление *цис-транс*-изомерии. Однако сопоставительный анализ антиоксидантной способности диастереомеров ДКВ ранее не осуществляли.

Цель. Оценить влияние диастереомерного состава на восстанавливающие и радикал-связывающие свойства образцов ДКВ.

Материалы и методы. Диастереомерный состав испытуемых образцов ДКВ контролировали в условиях обращенно-фазовой хроматографии с УФ-детектированием. Изучение антиоксидантной емкости осуществляли на модели ингибирования радикал-катионов 2,2'-азино-бис-(3-этилбензотиазолин-6-сульфоновой кислоты) диаммониевой соли (ABTS^{•+}). Восстанавливающую способность образцов ДКВ оценивали на модели 5-(2,4-дисульфобензил)-3-(2-метокси-4-нитрофенил)-2-(4-нитрофенил)-2H-тетразолия моносодиевой соли (WST-8). Оба испытания проводили под контролем спектрофотометрии в УФ-области.

Результаты и обсуждение. Хроматограммы образцов, полученные при длине волны 288 нм, характеризуются двумя пиками с временами удерживания $18,79 \pm 0,08$ и $19,87 \pm 0,08$ мин, которые соответствуют *транс*- и *цис*-диастереомерам ДКВ. При добавлении к раствору ABTS^{•+} образцов ДКВ, содержащих 0,8, 3,7 и 9,5 % *цис*-изомера, оптическая плотность при 730 нм по истечении 6 мин снизилась на $0,588 \pm 0,008$, $0,581 \pm 0,017$ и $0,602 \pm 0,012$, соответственно (α 0,05, p 0,2196, r 0,7857), в то время как в растворах WST-8 при 450 нм для аналогичных образцов наблюдали увеличение оптической плотности на $0,178 \pm 0,030$, $0,222 \pm 0,026$ и $0,188 \pm 0,036$, соответственно (α 0,05, p 0,5157, r 0,0284).

Заключение. Полученные результаты свидетельствуют об отсутствии статистически значимого влияния диастереомерного состава на антиоксидантные свойства образцов ДКВ. Таким образом, наблюдаемые различия в фармакологии *цис*- и *транс*-ДКВ следует объяснять селективностью взаимодействия с биологическими мишенями, а не способностью связывать свободные радикалы.

Ключевые слова: дигидрокверцетин, флавоноиды, диастереомерия, антиоксиданты, ВЭЖХ

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INTRODUCTION

Flavonoids are widespread in medicinal plants and their content is often used as a quality indicating parameter of active pharmaceutical ingredients of plant origin [1–3]. The wide range of biological activity of this natural polyphenol group is usually explained by their pronounced antioxidant properties [4]. However, the characteristics of the stereochemical structure of various flavonoids are rarely taken into account by researchers.

One of the representatives of this group of compounds is the flavanone dihydroquercetin (DHQ) – 2,3-dihydro-2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one. The industrial source for obtaining this compound is larch wood, but it can also be extracted from other plant objects: *Artemisia martjanovii* Krasch [5], *Dracocephalum jacutense* P. [6], *Rhododendron mucronulatum* Turcz. [7], etc. Recently, biotechnological approaches to obtaining DHQ have been actively developed [8–9].

Due to the presence of two chiral centers in positions 2 and 3 of the benzopyranone ring, this flavonoid is characterized by the phenomenon of *cis-trans* isomerism. On the one hand, this leads to the need of introducing new quality indicators for drugs based on it [10]. On the other hand, there are data in the scientific literature reporting differences between DHQ diastereo-

mers in pharmacokinetic [11–12] and pharmacodynamic profiles [13–14]. One of the possible reasons for the emergence of these differences may be the non-equivalence of the antioxidant properties of diastereomers, as one of the most important types of biological activity of flavonoids. However, according to our data, a comparative analysis of the antioxidant capacity of DHQ diastereomers has not been carried out previously.

Thus, the aim of the study is to evaluate the influence of diastereomeric composition on the reducing and radical scavenging properties of DHQ samples.

MATERIALS AND METHODS

Materials

The objects of the current study were three samples of DHQ: sample I – LLC "Robios", Russia; sample II – JSC "Ametis", Russia; sample III was obtained by spray drying from sample II according to the previously described conditions [15].

The model compounds employed to evaluate the antioxidant properties were 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Acros Organics, Belgium) and 5-(2,4-disulfophenyl)-3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl)-2H-tetrazolium monosodium salt (WST-8) in a cell counting kit

(CCK-8, Selleck Chemicals, USA). Potassium peroxydisulfate (Acros Organics, Belgium) was also used as a reagent. Methanol (for HPLC, LLC «Aquametriya», Russia), denatured ethyl alcohol (99.8 %, Carl ROTH GmbH, Germany), purified and deionized water, phosphate buffered saline system, pH 7.4 (PBS, Sigma-Aldrich, USA), Dulbecco's modified Eagle's medium (DMEM) – 1X, Gibco, USA – acted as solvents, and acetic acid (96 %, "for analysis", Merck KGaA, Germany) was applied as an acid modifier in chromatography.

Chromatography

The LicArt 62 chromatographic system (LLC "Labconcept", Russia) consisted of a QP-62d pump, T-85 thermostat, UV-62 spectrophotometric detector, 7725i injector, and Zorbax 3.5 μ m SB-Phenyl 4.6 $\times$ 150 mm column (Agilent Technologies, USA). Mobile phase: A – acetic acid solution in water (pH 2.7), B – methanol. Elution was carried out in a gradient mode: from 71 % (0 min) to 55 % (30 min) of component A. The column temperature varied from 30 to 60 °C (step – 15 °C), and the flow rate from 0.2 to 0.8 ml/min. The UV detector recorded data at the absorption maximum of DHQ at a wavelength of 288 nm. The test sample was a water-methanol (1:1, vol.) solution of DHQ with a concentration of 0.05 mg/ml.

The efficiency of the chromatographic system was assessed by the resolution value (R_s) between the peaks of the diastereomers of DHQ, determined in accordance with GPM.1.2.1.2.0001 "Chromatography" (State Pharmacopoeia of the Russian Federation XV) using the formula:

$$R_s = \frac{1.18 (RT_1 - RT_2)}{(W_1^{50\%} + W_2^{50\%})},$$

where RT_1 and RT_2 are retention times of peaks of DHQ diastereomers (min), and $W_1^{50\%}$ and $W_2^{50\%}$ are the widths of these peaks at half their height (min). The asymmetry factor (A_s) was determined as follows:

$$A_s = \frac{W^{5\%}}{2 \cdot f},$$

where $W^{5\%}$ is the width of these peaks at 5 % of their height (min), f is the distance from the ascending side of the peak to the perpendicular dropped from its top (min).

The content of *trans*-DHQ was determined using an external standard. Quantitative analysis of the *cis*-diastereomer was performed using the internal normalization method.

The quantitative method for determining the *cis*-DHQ impurity was validated in accordance with the recommendations of GPM.1.1.0012 «Validation of analytical methods» for the characteristics «Specificity», «Accuracy», «Repeatability», «Analytical range», «Linearity», and «Limit of quantification» (State Pharmacopoeia of the Russian Federation XV).

Moisture assessment

The test object weighing 1.0 g was placed on the stage of an i-Thermo GA64M (BEL Engineering s.r.l., Italy), after which the object was dried to constant mass at a temperature of 105 °C.

Antioxidant capacity

To measure the antioxidant capacity, an approach based on the preliminary generation of ABTS^{•+} radical cations was used [16] with a number of subsequent modifications [17–18]. Briefly, a solution of potassium peroxydisulfate with a concentration of 2.5 mmol/l in the same buffer was added to a solution of ABTS with a concentration of 7 mmol/l in PBS solution (pH 7.4) and placed in a dark place. The maximum concentration of ABTS^{•+} is achieved 6–8 h after the addition of the initiator. Spectrophotometric analysis was performed on a Cary 1C (Varian, USA), / 1.0 cm.

Working solutions of ABTS^{•+} were prepared in such a way that the absorption at the maximum at 730 nm was 1.00 ± 0.20 units of optical density. For this purpose, 240–260 μ l of the stock solution of pre-generated ABTS^{•+} were added to 7.6 ml of the PBS solution. For each measurement, the absorption spectrum of the working solution of ABTS^{•+} was recorded in the range of 680–780 nm, then 3.3 ml of this solution was immediately taken and 20 μ l of the working stock solution of the sample under study (solution B) were added to it and incubated in the dark at 30 °C for 30 min, after which the spectrum was recorded again in the same wavelength range.

Working stock solutions of the provided DHQ samples were prepared by dissolving 0.5 g (precisely weighed amount) in 20 ml of ethanol (solution A), then 7 ml of ethanol were added to 1 ml of the resulting solution (solution B).

The radical-scavenging capacity (N) was calculated using the formula:

$$N = \Delta A / (\epsilon \cdot C_{DHQ} \cdot l),$$

where ΔA is the difference in optical densities of the ABTS^{•+} solution at a wavelength of 730 nm before adding the test sample of DHQ and 30 min after adding, ϵ is the molecular extinction coefficient of ABTS^{•+} solution at a wavelength of 730 nm (15 000 l/mol·cm), C_{DHQ} is the molar concentration of solution B, and l is the optical path length (1.0 cm).

Reducing ability

DMEM was distributed at 100 μ l per well in a 96-well plate, after which 100 μ l of the analyzed samples, dissolved in the same solvent at a concentration of 0.15 mg/ml, were added. Then 10 μ l of CCK-8 were added to each well and kept for 1 hour at 37 °C in an atmosphere containing 5 % carbon dioxide and at a relative moisture of 95 %.

The resulting solutions were analyzed on an Eon microplate spectrophotometer (BioTek, USA) at the absorption maximum of the reduced form of WST-8 at 450 nm.

The reducing ability (R) was calculated using the formula:

$$R = (A_n - A_0)/A_n \cdot 100 \%,$$

where A_n is the optical density of WST-8 solution at a wavelength of 450 nm 6 min after adding the test sample of DHQ, A_0 is the optical density of WST-8 solution at a wavelength of 450 nm without the addition of the test sample of DHQ (0.400).

Statistical processing of data

All tests were performed three times. The results are presented as mean $\pm$ confidence interval (α 0.05). To assess the statistical significance of the difference in the results obtained for different study objects, a one-way analysis of variance (α 0.05) was performed, the results of which are presented as a p -value. To assess the strength of the correlation between the content of the *cis*-diastereomer and the observed values, the correlation coefficient r was calculated.

RESULTS

The development of the analytical method based on HPLC was carried out according to the principle of maximizing the R_s value depending on the chromatography conditions (Figure 1). An inverse linear relationship was established between the temperature and the resolution between the peaks of the DHQ diastereomers (r -0.9882). At the same time, the flow rate had a more complex effect on the R_s value: Initially, as the flow rate increased, an increase in the resolution between the peaks of the DHQ diastereomers was observed according to a parabolic law (r 0.9833), which then turned into a linear decrease in this characteristic (r -0.9699).

As a result, the temperature of 30 °C and the flow rate of 0.464 ml/min were recognized as the most optimal conditions (R_s 2.5), the chromatogram for these

parameters is shown in Figure 2. The peaks with the retention times of 17.935 ± 0.320 min and 19.038 ± 0.339 min were identified as the *trans*- and *cis*-isomers of DHQ, their asymmetry factors were 1.122 ± 0.006 and 0.999 ± 0.013 , respectively. The high R_s value and the reproducibility of the peak retention times determine the specificity of the developed method. In the range of DHQ sample concentrations from 0.01 to 0.20 mg/ml, the determination coefficient R^2 was 0.9993, and the repeatability was ± 1.3 %. The reference method for assessing the "Accuracy" characteristic was ^1H NMR spectroscopy data [19], compared to which the results of the current analysis deviated by 3.4 ± 0.1 %. Limit of quantification was 0.003 % of *cis*-DHQ amount.

Information on the quantitative content of diastereomers in the study objects is summarized in Table 1. The content of *cis*-DHQ in samples 1, 2, and 3 was 0.8, 3.7 and 9.5%, respectively.

Table 1. Content of diastereomers of DHQ in the objects of study

Sample	Moisture, %	Content of DHQ, %		
		total amount	<i>trans</i> -isomer	<i>cis</i> -isomer
I	8.5 ± 0.3	90.4 ± 1.3	89.6 ± 1.3	0.8 ± 0.1
II	7.3 ± 0.1	101.9 ± 1.5	98.2 ± 1.5	3.7 ± 0.1
III	5.0 ± 0.2	85.3 ± 1.3	75.8 ± 1.1	9.5 ± 0.1

The results of the evaluation of the radical-scavenging capacity and reducing ability of the analyzed objects on the ABTS^{•+} and WST-8 models are presented in Table 2. In both cases, no significant difference was found in the values of the optical density change for samples with different *cis*-diastereomer content: On the ABTS^{•+} and WST-8 models, the p -values were 0.2196 and 0.5157, respectively. At the same time, a strong positive correlation was established between the proportion of *cis*-DHQ and the N value (r 0.7857), while there was virtually no relationship with the R value (r 0.0284). In addition, there was a moderate inverse correlation between the values of radical-scavenging capacity and reducing ability (r -0.5627).

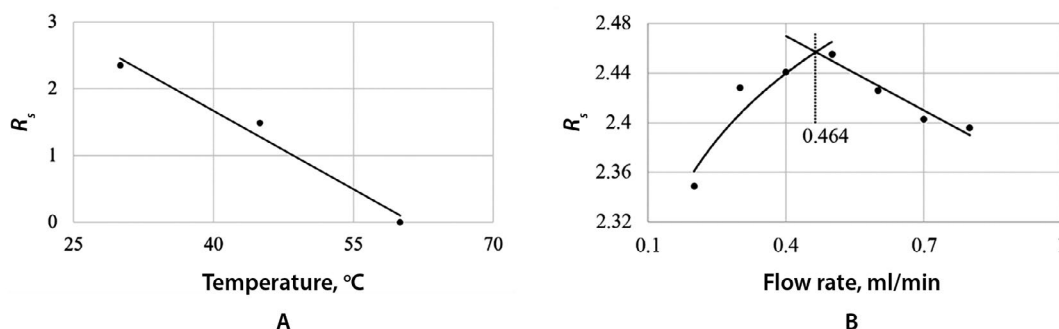


Figure 1. Optimization of chromatographic separation conditions based on R_s value:

A – temperature; **B** – flow rate

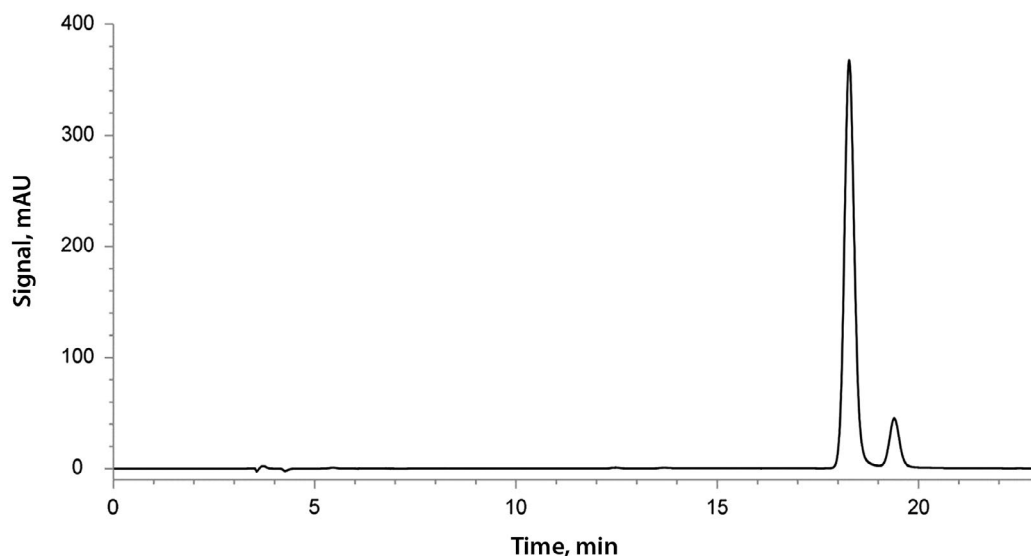


Figure 2. Separation of diastereomers of DHQ: *trans*- and *cis*-isomer

Table 2. Results of tests with ABTS^{•+} and WST-8

Sample	Antioxidant capacity		Reducing ability	
	ΔA	N	ΔA	$R, \%$
I	0.588 ± 0.008	6.27 ± 0.09	0.178 ± 0.030	30.8 ± 5.1
II	0.581 ± 0.017	6.20 ± 0.18	0.222 ± 0.026	35.6 ± 4.2
III	0.602 ± 0.012	6.42 ± 0.13	0.188 ± 0.036	32.0 ± 6.1

Thus, all analyzed samples of DHQ were characterized by pronounced antioxidant properties.

DISCUSSION

This study was conducted to fill the knowledge gap regarding the influence of diastereomerism on the antioxidant properties of the natural flavanone DHQ.

Due to two chiral centers, the DHQ molecule can exist in two pairs of diastereomers: *cis*- and *trans*-isomers. Because of the similarity of the physicochemical properties of the DHQ diastereomers, their chromatographic separation under achiral conditions remained a non-trivial task. Therefore, the implementation of this scientific project required establishing the content of the *cis*-diastereomer in the analyzed DHQ samples. According to the results of the literature analysis performed earlier, quantitative methods based on HPLC are the most promising for further implementation in routine pharmaceutical practice [10]. The quantitative analysis method presented in this work is a modification of a previously developed technique [20]. The stationary phase is silica gel with attached phenyl groups, the mobile phase is a water-methanol mixture with an acid modifier (acetic acid), the elution mode is gradient. The acid modifier (pH 2.7) was added to suppress the ionization of flavanone molecules in accordance with the established practice of chromatographic separation of

flavonoids [21], preventing peak blurring, which was successfully achieved: The asymmetry factor was in the range from 0.8 to 1.8, which met the requirements of GPM.1.2.1.2.0001 "Chromatography": (State Pharmacopoeia of the Russian Federation XV).

The main parameter by which the chromatography conditions were optimized was the resolution between the *trans*- and *cis*-DHQ peaks, the value of which, according to regulatory documentation, should be no less than 1.5.

The relationship between R_s and flow rate found in this study resembles the inverted curve described by the van Deemter equation [22]:

$$H = A + \frac{B}{v} + C \cdot v,$$

where H is the plate height (cm), v is the flow rate (cm/min), and A , B и C are constants describing dispersion by flow (cm), dispersion by axial molecular diffusion (cm²/min), and slow mass transfer (min), respectively. The H value is calculated using the formula:

$$H = \frac{L}{N},$$

where L is the column length (cm), and N is the number of theoretical plates. At the same time, the relationship between the values of N and R_s submits the following equation:

$$N = 16 \cdot R_s^2 \cdot \left(\frac{K' + 1}{K'} \right)^2 \cdot \left(\frac{\alpha}{\alpha - 1} \right)^2,$$

where K' is the retention factor, and α is the separation selectivity. Combining the three expressions above, we obtain the following equation:

$$\frac{L}{16 \cdot R_s \cdot \left(\frac{K' + 1}{K'} \right)^2 \cdot \left(\frac{\alpha}{\alpha - 1} \right)^2} = A + \frac{B}{v} + C \cdot v.$$

The column length remained constant under the experimental conditions. The retention factor depends on the temperature, as well as on the nature of the sorbent, solvent and sorbed substance, but not on the flow rate [23]. The separation selectivity, on the contrary, depends on v value, however, Peristyy et al. showed, as the flow rate increases, this characteristic reaches a plateau and, starting from 0.2 ml/min, its changes become negligible [24]. Thus, under the conditions of the current experiment, the influence of all three variables described above can be taken into account in the format of the constant δ (cm):

$$\delta = \frac{L}{16 \cdot \left(\frac{K' + 1}{K'} \right)^2 \cdot \left(\frac{\alpha}{\alpha - 1} \right)^2}.$$

In this case, the general equation is simplified to the following form:

$$\frac{\delta}{R_s} = A + \frac{B}{v} + C \cdot v,$$

which clearly explains the discovered dependence (see Figure 1, B). We did not find the last equation in previously published literature, but it may be of practical importance for future developers of analytical methods based on chromatography.

With increasing temperature, a linear decrease in R_s value was observed. Earlier, Jánovová & Hroboňová found a similar relationship between temperature and peak resolution in the chromatographic separation of lactic acid enantiomers [25], while Yadrova et al., on the contrary, found a direct relationship during the analysis of benzimidazoles [26]. Such a multidirectional effect can be explained by the influence of temperature on various factors, such as the solubility of the analyzed substance, the degree of its ionization and the distribution coefficient between the mobile and stationary phases, and the viscosity of the solvent [27], each of which can be decisive for a specific compound under the conditions of a particular chromatographic system.

The developed method of quantitative analysis based on HPLC was validated in accordance with the requirements of the State Pharmacopoeia of the Russian Federation XV for the characteristics "Specificity", "Accuracy", "Repeatability", "Analytical range", "Linearity", and "Limit of quantification", which allows us to recommend it for the needs of pharmaceutical analysis.

To assess the antioxidant capacity of the analyzed objects, one of the most common analytical approaches based on the model of inhibition of pre-generated ABTS^{•+} was chosen [28]. The N value of the analyzed

objects varied from 6.20 to 6.42, i.e. each DHQ molecule inhibited more than 6 molecules of ABTS^{•+}. Thus, all samples had a high antioxidant capacity: At a fairly low concentration of 1.9 µg/ml, they caused suppression of ABTS^{•+} by approximately 48 %, which is in good agreement with the results of tests on this model conducted earlier [29–30]. In similar experiments assessing diastereomers of silybin and dihydrosilybin these antioxidant properties differed insignificantly [31]. At the same time, theoretical calculations allow for the existence of discrepancy between diastereomers in the ability to scavenge free radicals [32]. In any case, there was no significant difference between the analyzed samples, and the sample size of the objects of study was too small for unambiguous statements.

The chemical process between DHQ and WST-8 is based on the reduction of the tetrazolium salt according to chemical reaction equation shown in Figure 3.

This chemical transformation is accompanied by a change of solution color. The pronounced reducing properties of DHQ have been repeatedly described previously using such models as FRAP, CUPRAC, and others [33–37]. Our results obtained using the WST-8 model confirm these data: All samples demonstrated a high R value at microgram concentrations. Differences in the R values of the analyzed objects were insignificant. In contrast to the ABTS^{•+} test, no correlation was found between the *cis*-diastereomer content and the reducing ability of the DHQ samples studied using the WST-8 model, which generally corresponded to our expectations. Nevertheless, the results of the current study indicate the need to take into account the reducing properties of DHQ when assessing its cytotoxicity using CCK-8.

It is also interesting to note that an inverse correlation was observed between the results of the ABTS^{•+} and WST-8 models. Inconsistency in the results of antioxidant properties assessment is widely presented in the literature [38–40]. Moreover, the results of assessment *in vitro* models may not coincide with *in vivo* experiments [41]. Therefore, the design of studies including several models seems to be a source of more relevant data for healthcare professionals. However, it can be concluded that the studied samples demonstrated high antioxidant activity, the value of which did not differ significantly in the studied range of *cis*-isomer of DHQ.

CONCLUSION

This study was conducted to investigate the antioxidant properties of DHQ samples with different diastereomeric compositions. A new analytical method based on HPLC characterized by good resolution between the peaks of *trans*- and *cis*-isomers was proposed for these purposes. The obtained results indicate the absence of a significant effect of the diastereomeric composition on the antioxidant properties of the studied DHQ samples. Thus, possible differences in the pharmacology

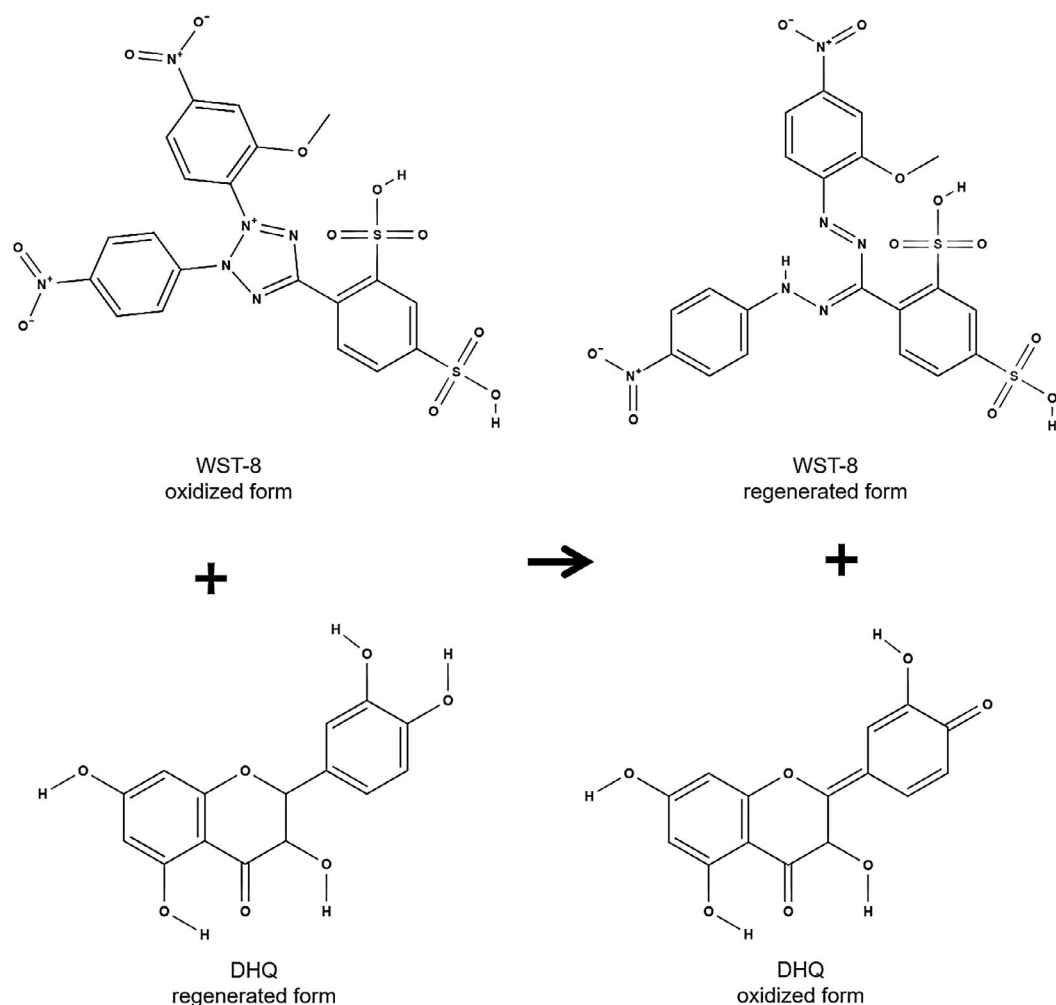


Figure 3. The chemical equation of the process between DHQ and WST-8 is based on the reduction of tetrazolium salt

of *cis*-isomer-enriched DHQ should be explained by the selectivity of interaction with biological targets, rather than the ability to scavenge free radicals. This hypothesis will need to be tested in future studies.

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