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ANTIOXIDANT ACTIVITY ENHANCEMENT OF ICARISIDE II THROUGH COMPLEXATION WITH WHEY PROTEIN CONCENTRATE

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ABSTRACT. Various food supplements provide antioxidant benefits by neutralizing free radicals, thus preventing diseases associated with oxidative stress. The antioxidant properties of pure icariside II (ICS), icariside II whey protein concentrate complex (ICS-WPC), and surfactant-based icariside II whey protein complexes (S-ICS-WPC) were evaluated. The assessment was conducted using the Briggs-Rauscher oscillating system and the 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical scavenging assay, with the latter providing a quantitative analysis of antioxidant capacity. The complexation procedure successfully increased the antioxidant activity of ICS.

Additionally, the inclusion of various surfactants such as Tween 80 and lecithin in the ICS-WPC complex led to further significant advancements.

Keywords: *icariside II, antioxidant activity, whey protein complexes, Briggs-Rauscher oscillating reaction, DPPH· radical scavenging*

INTRODUCTION

Free radicals are formed during regular physiological functions like metabolism, as well as by external factors such as tobacco smoke, UV radiation, chemicals, etc. [1]. The accumulation of free radicals can result in oxidative

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stress, which can damage cells, proteins, and DNA. Thus, antioxidants play an important role in our health by preventing the harmful activity of those free radicals. Antioxidants, based on their origin, can be categorized into synthetic and natural forms. Natural antioxidants have fewer negative effects on the human body, consequently, their demand by the general population has increased in the last decade [2]. Natural flavonoids, such as icariside II (ICS), are essential for various biological processes and responses to environmental factors in plants. These compounds are frequently found in human consumption patterns and exhibit significant antioxidant properties alongside a range of other bioactive functions, including antimicrobial and anti-inflammatory effects, which contribute to the prevention of diseases [3].

Icariside II, also called Baohuoside I, serves as the primary pharmacological metabolite derived from icariin, the principal active ingredient found in the botanical species *Herba Epimedii sp.* [4]. Historically, in ancient China, these plants were utilized as herbal remedies known for their aphrodisiac and antirheumatic properties [5]. Recent investigations have brought to light various other health-enhancing properties of icariside II. Among these, the most noteworthy include its potential as an anticancer agent [6], its efficacy in treating cardiovascular diseases [7], its ability to halt the progression of neurodegenerative disorders [8], and its effectiveness in addressing erectile [9] and testicular dysfunction [10]. ICS exhibits antioxidant effects by effectively reducing the levels of reactive oxygen species. Additionally, it enhances the activities of antioxidant enzymes, thereby demonstrating its great ability to combat oxidative stress [11]. Because of the properties mentioned above, ICS demonstrates considerable promise for utilization as a dietary supplement. Its distinct qualities position it as a favorable choice for integration into dietary regimens to enhance nutritional and wellness advantages.

For the determination of antioxidant activity, numerous tests are documented in the literature: Oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidant capacity (TEAC), 1,1-diphenyl-2-picrylhydrazil (DPPH·), Total oxidant scavenging capacity (TOSC), and Total radical trapping parameter (TRAP) [12]. These methods produce different radicals, using different solvents and different pH values ranging from 3.3 to 10.5. Moreover, a limitation of each assay is their suitability for only hydrophilic or lipophilic substances according to the solvent they use [13].

Another method to determine the antioxidant properties of different natural compounds is the Briggs-Rauscher (BR) oscillating system. It was observed by R. Cervelatti et al. that the addition of antioxidants to the system caused an immediate halt of the oscillations, however, over time, the oscillatory pattern is restored [14]. The duration in which the oscillations cease is referred to as the inhibition time. A linear correlation was found between the duration of inhibition and the quantity of antioxidants incorporated into a BR mixture [15].

This discovery [14] has paved the way for the development of a straightforward, consistent, swift, and cost-effective analytical method for assessing the efficiency of antioxidant agents in neutralizing free radicals. The greatest advantage of this method is that it operates at pH≈2, which is in the vicinity of the acidic environment of stomach fluids [16]. Given that ICS food supplements are ingested orally, it is plausible that they demonstrate a significant portion of their antioxidant properties in combating the free radicals generated within the stomach. Additionally, certain antioxidants exhibit higher levels of activity and stability in an acidic solution as opposed to an alkaline one [17]. It is important to note that the free radicals found in the BR system, hydroperoxyl (HOO·) and hydroxyl (HO·) are identical to those naturally produced in the human body [18]. Providing an advanced representation of physiological processes in terms of antioxidant activity, the BR method is particularly valuable in assessing the *in vivo* effects of digested antioxidants.

The DPPH· radical scavenging assay is the most widely recognized, popular, and commonly used method to quantitatively determine antioxidant activity [19]. The mechanism of this approach lies in the modification of the solution's color as it interacts with the antioxidant substances. The antioxidants cause a hydrogen atom transfer, leading to the reduction of DPPH· to DPPH-H, resulting in the solution changing color from violet to pale yellow [20]. This color shift can be easily monitored with UV-VIS spectroscopy. Consequently, the DPPH· radical scavenging assay is commonly employed to evaluate the antioxidant capabilities of various substances, including pure antioxidant compounds, herbal extracts, and phenolic compounds such as icariside II. While this technique may not be as accurate in representing the physiological processes as the BR, but it offers improved quantitative analysis capabilities.

The main objective of this research was to assess the changes in antioxidant properties of the products in relation to the pure material, demonstrating that the production process improved antioxidant activity. The Briggs-Rauscher oscillation technique provided qualitative insights into the antioxidant activity occurring in the human digestive system, especially in the stomach, whereas the DPPH· method facilitated a quantitative evaluation.

RESULTS AND DISCUSSION

Antioxidant activity measurements with Briggs-Rauscher oscillating system

The Briggs-Rauscher oscillation method provided a close insight into the antioxidant activity in the human digestive system, specifically the stomach.

Distilled water was employed in this setup to closely mimic the circumstances of the orally consumed ICS food supplements. From each sample, based on their molecular ratio, an equal amount of active agent was measured.

At the 3rd oscillation, the antioxidants were added to the system, which led to the immediate halt of the oscillations. The measurement data were acquired, and analyzed by utilizing the OriginPro 2019b software. The obtained result is given in Figure 1.

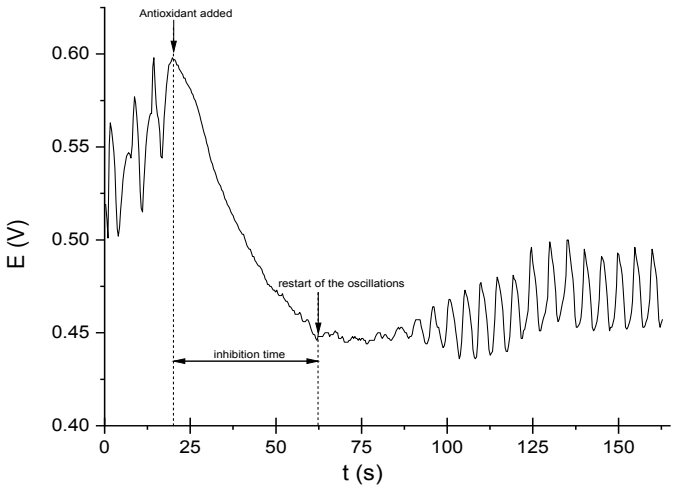

Figure 1. The effect of 2-1-3-3 molar ratio ICS-WPC-Tween 80-Lecitin on the BR mixture. The addition of antioxidant, and the restoration of the oscillations are marked with arrows.

The interval between the cessation and resumption of the oscillations, known as the inhibition time, was calculated and resumed in Table 1.

Table 1. Inhibition time of the different samples

Sample		Inhibition time (s)
Pure ICS		3
Molecular ratio of ICS-WPC-Tween 80-Lecithin	2-1-0-0	20
	2-1-3-0	22
	2-1-0-3	25
	2-1-3-3	42

It can be observed that the pure ICS displayed a negligible to non-existent antioxidant impact. This result can be attributed to the fact that this material is insoluble in water [21]. The complexation with whey protein

concentrate led to a significant enhancement in antioxidant activity. This outcome can be attributed to the antioxidant capacity of the β-lactoglobulin [22], which is the main component of the whey protein. Also, the complexation results in a higher water solubility which contributes to enhancing antioxidant activity. Furthermore, it can be observed that the addition of one of the surfactants didn't result in a significant antioxidant increment. In case when both surfactants were added to the mixture a synergetic effect took place and significantly increased the antioxidant activity of the final product.

Antioxidant activity measurements with DPPH• free radical scavenging

To quantitatively determine the antioxidant activity of the samples the value of IC_{50} was calculated. By definition, the IC_{50} value represents the concentration of antioxidants needed to decrease the initial free radicals found in the DPPH· solution by 50% [23]. Therefore, this signifies that the value of IC_{50} is inversely proportional to the antioxidant activity. The measured data can be interpreted with the following equation:

$$C_{sample} = a \times A + b$$

where the $\mathbf{C_{sample}}$ represents the concentration of the sample, **A** the measured decrease in absorption, while **a** and **b** are the slope of the line and its y-intercept. From those values, the IC_{50} can be calculated with the following equation:

$$IC_{50} = \frac{(50 - b)}{a}$$

The obtained IC_{50} values are presented in Table 2.

Table 2. Half-maximal inhibitory concentrations (IC_{50}) and standard error of the mean

Sample		IC_{50} (μg/mL)
Ascorbic acid		5.0 ± 1.1
Pure ICS		3567± 300
Molecular ratio of ICS-WPC-Tween 80-Lecithin	2-1-0-0	2650 ± 615
	2-1-3-0	1635 ± 353
	2-1-0-3	1210 ± 63
	2-1-3-3	856 ± 178

As can be observed, the antioxidant activity of pure ICS is exceedingly low compared to the ascorbic acid. However, the complexation with WPC led to an increase in antioxidant capacity. Furthermore, the addition of both surfactants (Tween 80 and lecithin) resulted in a 4.16-fold antioxidant activity increase. The progression of each product demonstrated a rise in antioxidant activity. The initial formation of complexes between ICS and WPC led to a 1.34-fold increase, possibly due to the antioxidant capabilities of β-lactoglobulin [22] and the increased solubility of the complexes in comparison to pure ICS. Subsequently, the incorporation of surfactants, such as Tween 80 and lecithin, further enhanced the final products' antioxidant activity.

CONCLUSIONS

The primary aim of this study was to investigate the impact of complexation and the addition of surfactants on the antioxidant activity of icariside II. Two methods were employed for this purpose: the BR technique and the DPPH· method. The first was applied for qualitative studies, replicating the antioxidant effects of the samples within the human gastric environment using distilled water as a solvent. Secondly, the DPPH· method was utilized for quantitative measurements of antioxidant properties. The BR method closely imitates antioxidant reactions related to free radicals in the human body, while the DPPH· method provides a more precise quantitative evaluation of antioxidant activity, especially given the limited solubility of the samples in water.

The results from these measurements demonstrated that the antioxidant activity of pure ICS was significantly enhanced through complexation with whey protein concentrate. Furthermore, the addition of surfactants resulted in additional increases in antioxidant activity. The similarity in trends between the two measurement methods is evident in how various preparation methods impacted antioxidant activity.

The BR method revealed that orally administered pure ICS would not exhibit antioxidant effects in the human body due to its insolubility in water. To address this limitation, complexation with WPC and the addition of surfactants were necessary.

Quantitatively, the final product, 2-1-3-3 ICS-WPC-Tween 80-Lecithin, showed the most significant improvement, with a remarkable 4.16-fold increase in antioxidant activity compared to pure ICS.

EXPERIMENTAL SECTION

Materials and methods

Icariside II of analytical grade purity was acquired from Xi'an Day Natural Inc., located in China. The whey protein concentrate (80%) was purchased from

Foodcom S.A., a company based in Warsaw, Poland. Additionally, Tween 80 and lecithin were obtained from Sigma-Aldrich, Germany. All other reagents used in the experiments were of analytical grade.

Antioxidant activity measurements with Briggs-Rauscher oscillating system

The antioxidant activity of pure ICS and the complexes with different molar ratio ICS-WPC-Tween 80-Lecithin (2-1-0-0, 2-1-3-3, 2-1-3-0, 2-1-0-3) was observed. An equivalent quantity of ICS (4 mg) from the different samples was diluted in 50 mL distilled water. To maintain consistency and reliability in the results for the BR antioxidant measurements the protocol described by Szabó et al. was closely followed [18]. The temperature was regulated at a constant level by employing a double-walled vessel with a 50 mL capacity connected to a thermostat. Continuous water circulation within the temperature jacket ensured a stable temperature of 37°C to simulate the physiological body conditions. The oscillations were electrochemically observed using an Ag/AgI indicator electrode and a Pt-wire counter electrode. The setup was linked to a computer via a PCI 6036 E data acquisition interface.

Using double distilled water, stock solutions of the following concentration were prepared for the oscillation reaction: $[H_2SO_4]_0$=0.27 M, $[KIO_3]_0$=0.125 M, $[MA]_0$=0.25 M, $[MnSO_4]_0$=0.032 M, and $[H_2O_2]_0$=6.6 M. From each solution 5 mL was added, the mixing order was as follows: malonic acid, $MnSO_4$, H_2SO_4 and KIO_3 and finally using H_2O_2 the oscillation reactions were initiated.

Antioxidant activity measurements with DPPH• free radical scavenging

Similarly, as above, the antioxidant activity of pure ICS and the complexes with different molar ratio ICS-WPC-Tween 80-Lecithin (2-1-0-0, 2-1-3-3, 2-1-3-0, 2-1-0-3) was analyzed. As standard the antioxidant capacity of ascorbic acid was used. From each complex, according to their molecular ratio, an equal amount of ICS (4 mg) was diluted in 10 mL of methanol. A methanol stock solution of 1.01×10^{-4} M DPPH· was prepared and stored in an amber volumetric flask as a prevention from decomposition. Various concentrations of antioxidant solution were prepared with the addition of different volumes (ranging from 0 to 800 µL) of stock solution (4000 µg/mL) to 3 mL of the DPPH· stock solution, followed by the dilution with methanol to achieve a final volume of 5 mL. This process resulted in the preparation of a range of solutions. The acquired solutions were placed in the dark for 20 minutes for the reaction to take place. The addition of antioxidants to the DPPH· solution resulted in a color change that was observed with a UV-VIS spectroscope at 517 nm wavelength [24].

Statistical analysis

All the measurements were conducted in triplicate and the results are reported as the mean value ± the standard error of the mean. A one-factor ANOVA test was performed and statistical significance was established for $p<0.05$.

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