THE INFLUENCE OF EXTRACTION TEMPERATURES ON THE ANTIOXIDANT ACTIVITY OF POLYSACCHARIDES FROM *MYXOPYRUM SMILACIFOLIUM* LEAVES

ẢNH HƯỞNG CỦA NHIỆT ĐỘ CHIẾT XUẤT ĐẾN HOẠT TÍNH CHỐNG OXY HÓA CỦA POLYSACHARIDES TỪ LÁ SÂM ĐÁ (*MYXOPYRUM SMILACIFOLIUM*)

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(Received: July 17, 2023; Revised: August 25, 2023; Accepted: August 29, 2023)

Abstract - The aim of this study is to assess how different extraction temperatures affect the *in vitro* antioxidant potential of polysaccharides derived from the leaves of *Myxopyrum smilacifolium*. The findings indicated that, the polysaccharides exhibit the highest antioxidant activity when extracted at a temperature of 90°C. Fourier transform infrared spectroscopy analysis confirmed the presence of characteristic absorption groups typically found in polysaccharide structures in the PS-T90 sample. The average molecular weight of the extracted polysaccharides was approximately 9.30×10^5 Da. The total antioxidant capacity of PS-T90 was determined to be 0.2646 ± 0.0007 mg GA/g or 0.1725 ± 0.0007 mg AS/g. Additionally, the IC_{50} values for the polysaccharides against 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radicals were measured to be 1.04 mg/mL and 3.37 mg/mL, respectively. These exceptional bioactive properties suggest the potential of PS-T90 as a valuable resource for antioxidant development.

Key words - ABTS; antioxidant activity; DPPH; Myxopyrum smilacifolium; polysaccharide

Tóm tắt - Mục tiêu của bài báo này là nghiên cứu ảnh hưởng của nhiệt độ chiết đến tiềm năng chống oxy hóa *in vitro* của polysaccharide thu được từ lá Sâm đá (*Myxopyrum smilacifolium*). Kết quả cho thấy, ở nhiệt độ chiết là 90°C thì hoạt tính chống oxy hóa của polysaccharide là cao nhất. Phân tích quang phổ hồng ngoại biến đổi Fourier cho thấy, PS-T90 thể hiện các nhóm hấp thụ đặc trưng thường thấy trong các cấu trúc polysaccharide. Trọng lượng phân tử trung bình của polysaccharide được chiết xuất là khoảng $9{,}30\times10^5$ Da. Tổng hàm lượng chống oxy hóa quy tương đương của PS-T90 được xác định là 0,2646 ± 0,0007 mg GA/g hoặc 0,1725 ± 0,0007 mg AS/g. Hơn nữa, các giá trị IC_{50} trong các mô hình bắt gốc tự do DPPH và ABTS của PS-T90 lần lượt là 1,04 mg/mL và 3,37 mg/mL. Hoạt tính sinh học nổi bật như vậy có thể thúc đẩy việc sử dụng PS-T90 như một nguồn đầy hứa hẹn để phát triển chất chống oxy hóa.

Từ khóa - ABTS; khả năng chống oxy hoá; DPPH; Myxopyrum smilacifolium; polysaccharide

1. Introduction

Polysaccharides (PS) have garnered significant attention in recent decades [1-3]. They play diverse and crucial roles in nature, serving as valuable sources of food and cosmetic ingredients, as well as in nutraceutical and pharmacological applications [4, 5]. The extraction process of polysaccharides unveils their structural diversity and potential biological functions, granting them unique properties [6]. Consequently, the extraction of polysaccharides has become a prominent research topic in the field of biomedicine, owing to their physiological and pharmacological properties that are vital for their application and further research and development [7, 8].

Myxopyrum smilacifolium, a renowned herbal medicine, has been traditionally employed in Viet Nam and other Southeast Asian countries like Laos, Cambodia, Indonesia, Thailand, and India for the treatment of various ailments including cough, rheumatism, cephalalgia, nostalgia, and otopathy [9, 10]. Extensive research has demonstrated the antimicrobial, cytotoxic, anti-inflammatory, and antioxidant properties of extracts derived from *M. smilacifolium* [10, 11]. However, the specific mechanisms underlying these bioactivities have yet to be fully understood, and polysaccharides may play a crucial role as one of the main components responsible for these biological effects [12, 13]. Previous studies have shown that extraction temperature affects the composition and antioxidant activity of the extracts [14-16]. Numerous studies have investigated the impact of extraction temperature on polysaccharide (PS) extraction efficiency and antioxidant activity in various plant sources [17-19], there is a lack of research specifically exploring the influence of extraction temperatures on the antioxidant activities of polysaccharides extracted from *Myxopyrum smilacifolium* leaves.

This paper aims to conduct a series of experiments to investigate the impact of extraction temperatures on the in vitro antioxidant activities of polysaccharides extracted from *Myxopyrum smilacifolium* leaves. The evaluation will be performed using assays for total antioxidant activity, DPPH radical scavenging activity, and ABTS scavenging activity. Additionally, the polysaccharide will be characterized through Fourier transforms infrared spectroscopy and high-performance gel-permeation chromatography to gain further insights into their structural properties.

2. Experimental

2.1. Plant and chemicals

The leaves of *Myxopyrum smilacifolium* were obtained on June 10th, 2021 from Bach Ma National Park located in Thua Thien Hue, Vietnam. The identification and deposition of the plant material were carried out by the Department of Biology, University of Sciences, Hue University. Gallic acid, ascorbic acid, quercetin, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were procured from Sigma-Aldrich Co. (USA). H_2SO_4, phenol, $(NH_4)_2MoO_4$ were obtained from Guangdong Co. All reagents and chemicals used in this study were of analytical grade.

2.2. Extraction of polysaccharides (PS)

The dried *Myxopyrum smilacifolium* leaves was crushed into a fine powder and sieved through a 200-mesh sieve. The powdered samples were dispersed in a 250 mL flask with a sample-to-water volume ratio of 1:50. The extraction temperature was varied between 60, 70, 80, 90, and 100 °C to investigate its impact on the polysaccharide yield and antioxidant activity. Other extraction conditions included an extraction time of 3 hours and 3 replications. The mixture was then cooled to room temperature using cold water and was then filtered. The resulting solution was concentrated using a rotary evaporator under reduced pressure to obtain the extract solution (50 mL). Ethanol 96 was added to the concentrated extract solution to precipitate the polysaccharides completely with an ethanol 96° to-extract volume ratio of 5:1. The obtained precipitation was collected by centrifugation and sequentially washed with cold ethanol and acetone. Finally, the product was vacuum-dried at 40°C to obtain the crude water-soluble polysaccharide powder.

Qualitative and quantitative analysis of polysaccharides were examined by phenol-sulphuric acid – colorimetric method using D-glucose as standard at a wavelength of 490 nm [20].

2.3. Antioxidant activities

2.3.1. TAC

The total antioxidant activity of the samples was assessed using the method described by Nair et al. [21]. An aliquot of the sample (0.3 mL) was meticulously combined with an equal volume of a reagent solution (3 mL). This reagent solution was meticulously composed of 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate. The resultant mixture was subjected to an incubation period at 95°C for a duration of 90 minutes, followed by a controlled cooling process to attain a temperature of 25°C, ensuring optimal conditions for subsequent absorbance measurements. The measurements were conducted at a specific wavelength of 695 nm. The total antioxidant capacity was quantified in terms of the equivalents of gallic acid (GA) and ascorbic acid (AS).

2.3.2. DPPH radical-scavenging activity

The DPPH free radical scavenging activity of the samples was determined following the method outlined by Le et al. [22]. Briefly, 2 mL of samples dissolved in DMSO at various concentrations (25 – 125 µg/mL) were added to 1 mL of 100 µM DPPH solution dissolved in methanol. The mixture was incubated at room temperature for 30 minutes, and the absorbance was measured at a wavelength of 517 nm using a UV-Vis spectrophotometer. Quercetin and ascorbic acid were used as reference compounds. The radical scavenging activity was evaluated by determining the IC50 value.

2.3.3. $ABTS^{\cdot+}$ radical-scavenging activity

The free radical scavenging capacity of the samples was assessed using the ABTS radical decolorization assay, following the method described by Re et al. [23]. Briefly, 0.1 mL of samples at various concentrations (ranging from 25 µg/mL to 125 µg/mL) was mixed with 3.9 mL of $ABTS^{+\cdot}$ solution, and the absorbance of the resulting mixture was measured at 734 nm. Ascorbic acid was used as a reference compound. The ABTS scavenging activity was determined by calculating the IC_{50} value.

2.4. Determination of Molecular Mass

The average molecular weight of the polysaccharide was determined using gel permeation chromatography (GPC) with an Agilent 1100 Series system coupled to an MS detector, specifically the microTOF-QII Bruker (USA), following the method described by Fukuda et al. with slight modifications [24]. The purified polysaccharides were dissolved in 0.1 M $NaNO_3$ solution and injected into the system, maintaining a consistent flow rate and column temperature. Pullulan, a polysaccharide with known molecular masses of 50, 100, 200, 400, and 800 kDa, was used as a standard. The separation was performed on an Ultrahydrogen 500 column (7.8 mm x 300 mm, 10 µm) using 0.1 M $NaNO_3$ as the mobile phase. The chromatography was carried out at 40°C with a flow rate of 1 mL/min.

2.5. Infrared Spectroscopy Analysis

The mixture of the dried PS powder (2 mg) and KBr powder was ground and pressed into 1 mm-thick pellets. The as-prepared sample was conducted by an infrared spectrophotometer (IRPrestige-21). The scan range was from 4000 to 400 cm^{-1} with a resolution of 8 cm^{-1}.

2.6. Statistical analysis

Data were calculated as percentage in dry weight of sample. Data analyses were performed using the Statistical Analysis System on Excel software and Origin 8.0. Assessing the repeatability of experiments based on the relative standard deviation and Horwitz equation. Confidence intervals of experimental data were calculated and means were statistically considered significantly different when $P \leq 0.05$.

3. Results and discussion

3.1. Effect of extraction temperature on the yield of the polysaccharides from Myxopyrum smilacifolium leaves

Experiments were conducted to evaluate the influence of extraction temperature on the yield of polysaccharides from *Myxopyrum smilacifolium*. The extraction temperatures tested were 60°C, 70°C, 80°C, 90°C, and 100°C while keeping other extraction conditions constant:

a sample-to-water volume ratio of 1:50, an extraction time of 3 hours, three replicates, and a ratio of 96^0 ethanol to extract volume of 5:1. Table 1 presents the extraction yields of polysaccharides obtained at different temperatures ranging from 60°C to 100°C. The results showed that the extraction yields ranged from 3.38 % to 5.20%, with an increasing trend as the temperature rose. The highest yield of polysaccharides (5.20%) was obtained at 100°C.

Table 1. *Percentage of pure PS and Total antioxidant capacity (TAC) of polysaccharides from Myxopyrum smilacifolium leaves*

Sample	PS (%)	TAC		DPPH	ABTS
		mg GA/g	mg AS/g	IC_{50} (mg/mL)	IC_{50} (mg/mL)
PS-T60	3.38 ± 0.03	0.2385 ± 0.0006	0.1590 ± 0.0004	1.22	3.94
PS-T70	4.26 ± 0.05	0.2479 ± 0.0004	0.1639 ± 0.0007	1.19	3.82
PS-T80	4.68 ± 0.03	0.2541 ± 0.0003	0.1671 ± 0.0002	1.10	3.62
PS-T90	5.09 ± 0.04	0.2646 ± 0.0007	0.1725 ± 0.0007	1.04	3.37
PS-T100	5.20 ± 0.02	0.2524 ± 0.0006	0.1662 ± 0.0003	1.10	3.63

The observed increase in polysaccharide yield at higher extraction temperatures can be attributed to several factors. Firstly, it is speculated that high temperatures may disrupt the cell membrane structure, making it easier for polysaccharides to be released into the solution. Additionally, the viscosity of the extraction solution decreases at higher temperatures, leading to improved solubility of polysaccharides. This increased solubility facilitates the release and dissolution of polysaccharides from the plant material [25]. Overall, the combination of membrane damage and enhanced solubility at higher temperatures contributes to the higher yield of polysaccharides observed in this study.

3.2. Antioxidant activity assay of polysaccharides

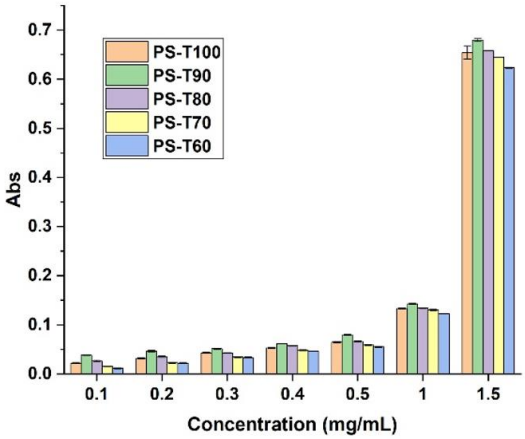

Figure 1. *Antioxidant activity of polysaccharides of Myxopyrum smilacifolium leaves in total antioxidant capacity model*

Figure 1 illustrates the impact of extraction temperature on the antioxidant activities of the polysaccharides. The findings reveal that the antioxidant activities of the polysaccharides exhibit a significant increase with increasing concentration from 0.1 to 1.5 mg/mL. Notably, the antioxidant activities of the polysaccharides demonstrate a proportional relationship with the extraction temperature within the range of 60°C to 90°C. However, beyond this temperature range, a decline in antioxidant activities is observed. We propose that the properties and activities of polysaccharides are linked to their molecular characteristics, such as spatial structure and molecular weight. The elevation of extraction temperature may enhance both the yield and molecular weight of the extracted polysaccharides [25, 26]. It is possible that high-molecular-weight polysaccharides may exhibit reduced antioxidant activity, thus contributing to the observed decrease in antioxidant activities beyond a certain extraction temperature.

The antioxidant capacity of the polysaccharides is quantified by the number of equivalents of gallic acid or ascorbic acid. The standard curve equations used are as follows: for gallic acid: A (Abs) = 0.2018 C_{GA} + 0.2295 (R = 0.9998); and for ascorbic acid: A (Abs) = 4.5974 C_{AS} – 0.3231 (R = 0.9952). The total antioxidant capacity of the polysaccharides, at a concentration of 1.5 mg/mL, ranges from 0.2385 ± 0.0006 to 0.2646 ± 0.0007 mg GA/g or from 0.1590 ± 0.0004 to 0.1725 ± 0.0007 mg AS/g (Table 1). These results affirm that the polysaccharides possess antioxidant capacity.

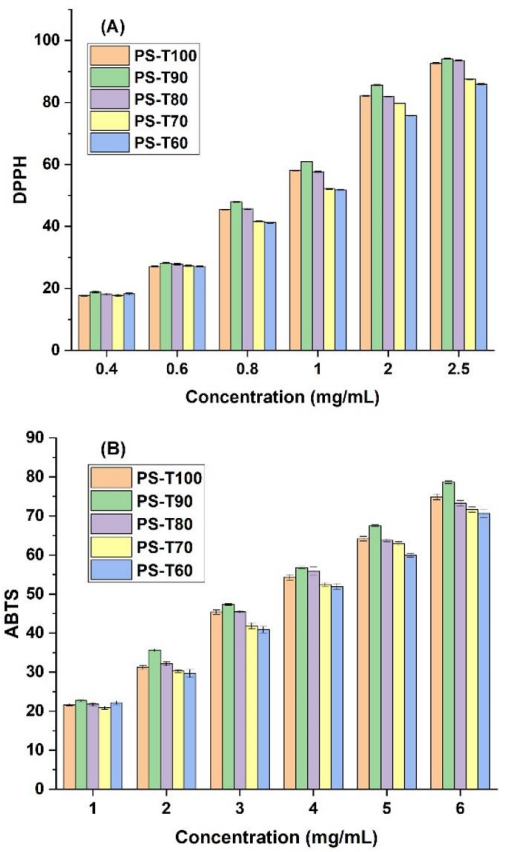

Figure 2. *Antioxidation activities of the polysaccharide samples; (A) DPPH method; (B) ABTS method*

The subsequent phase involves examining the antioxidant activity of the samples by evaluating their ability to scavenge free radicals. To accomplish this, we utilized DPPH and ABTS as model radicals, which donate

hydrogen atoms or electrons. The results of these evaluations are presented in Table 1 and Figure 2.

Overall, it is clear that the DPPH radical scavenging activities and ABTS radical scavenging activities of polysaccharides of *Myxopyrum smilacifolium* leaves increase with the polysaccharide concentration. In terms of the DPPH radical scavenging activity at the concentration of 2.5 mg/mL is over 85%. The same ABTS radical scavenging activities could be seen at the concentration of 6 mg/mL is over 70%. The antioxidant capacity of the PS-T90 was relatively good, with low IC_{50} values (1.04 mg/mL for DPPH radical and 3.37 mg/mL for ABTS radical). The free radical scavenging activity of the polysaccharides can be arranged as follows: PS-T90> PS-T80 ≈ PS-T100 > PS-T70 > PS-T60.

In this regard, the radical scavenging capability of the PS-MSL is notably comparable to that of various medicinal fungi and plants reported previously [27-29]. Notably, the DPPH radical scavenging activity of the PS-MSL surpasses that of polysaccharides obtained from different sources, including the rhizome of *Dryopteris crassirhizoma* Nakai (IC_{50} of 2.04 mg/mL) [27], *C. sinensis* polysaccharides (IC_{50} of 1.23 mg/mL) [28], and the polysaccharides from almonds and pistachio (IC_{50} = 2.87 mg/mL and IC_{50} = 1.61 mg/mL, respectively) [29]. Moreover, the polysaccharides of *Myxopyrum smilacifolium* demonstrates superior ABTS radical scavenging activity compared to both *O. sobolifera* polysaccharides (IC_{50}: 4.83 mg/mL) [22] and *C. militaris* polysaccharides (IC_{50}: 6.99 mg/mL) [30]. These findings highlight the promising antioxidant potential of the isolated polysaccharides and their potential applications in various therapeutic and nutraceutical products.

The above experiments (TAC, DPPH, and ABTS tests) demonstrated that PS-T90 has more antioxidant activity than other polysaccharides. Thus, the PS-T90 was used in further investigation.

3.3. Characterization of PS-T90 extracted from Myxopyrum smilacifolium leaves

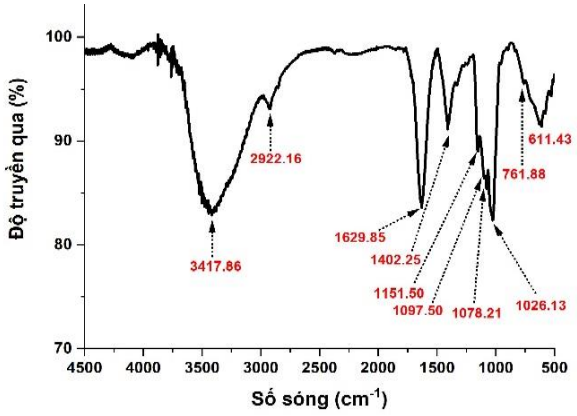

Figure 3. *The IR spectrum of PS- T90 from M. smilacifolium*

The FT-IR was conducted to investigate the characteristic bonding of the PS-T90 from *Myxopyrum smilacifolium* leaves, as shown in Figure 3. The intensity of bands around 3417 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide. The bands in the region of 2922 cm^{-1} were due to C-H stretching vibration, and the bands in the region of 1629 cm^{-1} were due to associated water. The strong absorption bands at 1402 cm^{-1} were due to C–O stretching vibrations [31]. The characteristic peak centering at 1151 cm^{-1} implies a glucopyranoside, whereas the peaks located at 1026 cm^{-1} could be indicated *α*-configurations and the presence of fructose residues, respectively [32]. The characteristic peak centering at 1078 cm^{-1} implies a mannopyranoside [32]. The peak located at 1097 cm^{-1} could be ascribed to the presence of galactose residues [33]. To this end, it can be said that the extracted PS-T90 from *M. smilacifolium* leaves possesses the typical absorption groups of polysaccharides in the structure.

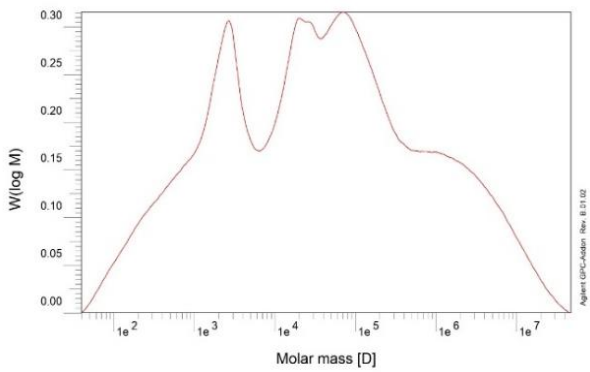

Figure 4. *Molecular mass chromatogram of PS-T90 from M. smilacifolium leaves*

Figure 4. shows a chromatogram of polysaccharide obtained by gel permeation high-performance liquid chromatography. The PS-T90 from *M. smilacifolium* leaves had a molecular weight of 9.30×10^5 Da. Three peaks centering at 3.12×10^3 Da, 3.42×10^4 Da and 1.02×10^5 could be distinguished. Besides peak width from about 10^2 Da to 10^7 Da, that value implied that the isolated polysaccharide contained a very large molecular weight distribution. Therefore, it can be stated that PS-T90 from *M. smilacifolium* leaves is a heterogeneous polysaccharide.

4. Conclusions

The experimental findings serve as a crucial foundation for further systematic research and development of *M. smilacifolium* polysaccharides. It is evident that the content and antioxidant activities of the polysaccharides are closely linked to the extraction temperature, with the highest antioxidant activity observed at 90°C. Fourier transform infrared spectroscopy analysis confirms that PS-T90 exhibits the characteristic absorption groups typically found in polysaccharide structures. The average molecular weight of the extracted polysaccharides is approximately 9.30×10^5 Da. At this specific temperature, the total antioxidant capacity of the PS-T90 is determined to be 0.2646 ± 0.0007 mg GA/g or 0.1725 ± 0.0007 mg AS/g. Furthermore, the IC_{50} values for the polysaccharides against DPPH and ABTS radicals are measured to be 1.04 mg/mL and 3.37 mg/mL, respectively. These results indicate that the *M. smilacifolium* polysaccharides exhibit notable antioxidant activity *in vitro*. These findings underscore the potential of these polysaccharides for

various applications in the fields of therapeutics and functional foods.

Funding: The authors acknowledge the support of Hue University under the Core Research Group Program, Grant No. NCM.DHH.2020.05. This research was funded by the Vietnamese Ministry of Education and Training under project number B2022-DHH-12 (L.T.H)

Conflicts of Interest: The authors declare no conflict of interest.

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