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EXTRACTING CONDITIONS OPTIMIZATION AND BIOACTIVITY OF POLYSACCHARIDES FROM THE PODS OF HARICOT VERT

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Abstract. Polysaccharides from the pods of haricot vert (*Phaseolus vulgaris* L.) were extracted using a simple heating method, by varying extracting temperature, heating time, solid-to-liquid ratio, and solvent composition. The obtained results were processed using statistical analysis that helped to identify the optimal conditions for the polysaccharides' extraction process. Moreover, the results of the bioassays were compared and statistically analysed to look for the correlation among extracting conditions, compositions, and bioactivities. The extracts of optimal conditions showed significant antioxidant and α-amylase inhibition. Correlations among extracting conditions, compositions, and bioactivities were evaluated based on partial least square (PLS) regression model. Therefore, this study represents a promising production method of bioactive polysaccharides extract in the food and pharmaceutical industry.

Keywords: polysaccharide extraction, optimization, haricot vert, bioactivity.

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Introduction

Haricot vert (*Phaseolus vulgaris* L.), is a common plant that is widely grown and consumed worldwide [1]. *P. vulgaris* is rich in polysaccharides, such as starch, non-starch polysaccharides, dietary fiber, and oligosaccharides, besides proteins, lipids, and secondary metabolites [1,2]. The starch and non-starch polysaccharides from *P. vulgaris* have been reported as antioxidants and immunoenhancing agents [1-4], whereas, dietary fibers have been shown to improve human health, reduce cholesterol, modulate blood glucose levels, and prevent constipation [5-9]. Additionally, an antioxidant water-soluble pectic polysaccharide in *P. vulgaris* has been identified, the structure has been elucidated by hydrolysis along with NMR analysis [4]. While many studies on *P. vulgaris* focused on chemical properties and bioactivities of carbohydrates from the seeds [5-9], few investigations on the polysaccharides from the pods have been reported [3,4].

Previous studies reported several methods to obtain polysaccharides from natural materials, for example, using enzyme, hot, or microwave-assisted extraction. Though using the microwave assisted method can effectively increase the extracting yields and save the processing time [10,11], it demands expensive and complicated instruments that could not apply on a large scale. Whereas, enzyme extraction is commonly utilized to extract polysaccharides from medicinal materials, which was energy-saving and environmental friendly [12,13]. However, this method is quite expensive and enzymes are easily inactivated [14]. Among these, hot extraction was the simplest method for the extraction of polysaccharides, that could be applied in the industry [15]. Neutral or alkaline solvents could be used for extracting the acidic polysaccharides [15], but the yield and quality of the product could decline at a too high temperature for a long time exposure. Thus, efficient conditions, including extracting temperature, time, and solvents should be assessed for the optimal extraction.

The goal of this study was the optimization of the polysaccharides extracting process and evaluation of the antioxidant and α-amylase inhibiting activities of the obtained extracts. Statistical methods have been used to identify the correlation between extracting conditions and bioactivities. The optimized process could be applied on a larger scale for the preparation of polysaccharide products in food or pharmaceutical industries.

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Experimental

Generalities

Haricot vert pods purchased from a local market, Nam Tu Liem district, Ha Noi, Vietnam were cleaned using water, further, the seeds were removed, and dried until constant weight, and powdered. The lipids of the materials were removed by soaking in ethanol 90% for two hours. The powder was then dried under vacuum and prepared for extracting polysaccharides.

2,2-Diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, deoxyribose, 2-thiobarbituric acid, trichloroacetic acid, nitro blue tetrazolium chloride (NBT), enzyme xanthine oxidase, *α*-amylase, starch azure (Brilliant blue R) were purchased from Sigma (St. Louis, MO, USA). Other chemicals were analytical grade reagents.

The absorbance of experimental solutions was measured on a BioTek Synergy HTX microplate reader (BioTek Instruments, Inc., USA).

Polysaccharide extraction procedure

After removing lipids, the haricot vert powder was subjected to extraction with designed extraction time, solid-to-liquid ratio, temperature, and solvent. The mixtures were filtered, then, the filtrates were neutralized to pH 7 if necessary and removed protein by using Sevag reagent (chloroform: *n*-butanol, 4:1, v:v). The water layers were collected, evaporated under vacuum to the one-quarter volume of the initial solutions. Four times the volume of ethanol was then mixed with the concentrated extracting solutions and kept at 4°C for 12 h. At this stage, the polysaccharides would be precipitated and subsequently obtained by centrifugation at 4000 rpm for 20 min and washed again in ethanol. The polysaccharides were obtained by freeze-drying. The effects of the extraction time were evaluated by comparing the yields, total polysaccharides content, and total phenolic content of the extracts from 30 to 240 min. Also, the effects of the solvent composition and temperature conditions were studied through those when increasing temperature from 60°C to 90°C in several solvents, including 0-30% NaOH and 0-30% ethanol. The polysaccharides yield was calculated by the Eq.(1).

Polysaccharides yield (%)=

$$\frac{Weight\ of\ the\ polysaccharides}{Weight\ of\ the\ materials} \times 100 \qquad (1)$$

In addition, the total polysaccharide content was determined by the phenol sulphuric assay [16], whereas, total phenolic content was evaluated by the Folin-Ciocalteu method [17].

α-Amylase inhibiting and antioxidant assays

The α-amylase enzyme inhibitory activity was evaluated by the previously reported method [18] with some modifications. The substrate was prepared by boiling 80 mg of potato starch in 8 mL of phosphate buffer (pH 7.0) for 5 min, then it was left to cool down at room temperature. Next, 50 µL of the substrate and 100 µL of the sample solution were mixed with 30 mL of 100 mM phosphate buffer (pH 7.0). After 5 min, 5 µL of *α*-amylase 50 µg/mL solution was added, and the solution was incubated at 37°C for 15 min. Added 50 µL of glacial acetic acid, then 50 mL iodine solution in the solution and measured the absorbance at 650 nm by using the microplate reader. Acarbose was used as a reference reagent.

The DPPH radical-scavenging activity was conducted by modifying a previous method [19]. Each sample (20 µL) was mixed with 380 µL of DPPH in methanol then incubated at 37°C for 20 min. The absorbance was measured at 517 nm. The scavenging capacity (SC) was calculated using Eq.(2).

$$\%SC = \frac{A_0 - A_s}{A_0} \times 100 \qquad (2)$$

where, A_o- absorbance of the reference reagent;
A_s- absorbance of the tested sample.

Each sample was analysed in 3 replicates. The results were expressed as the IC_{50} values in µg/mL, which corresponded to the sample concentration required to scavenge 50% of the DPPH free radicals. Ascorbic acid was used as a reference sample.

Superoxide radical scavenging activity was measured by a reported method with some modification [20]. A volume of 100 µL of the sample in DMSO was mixed with 300 µL of the phosphate buffer 50 mM pH 7.8, 200 µL xanthine 0.5 mM, 100 µL NBT 0.2 mM, and 100 µL of xanthine oxidase. The mixture was incubated at 37°C for 60 min then measured at the wavelength of 550 nm. Superoxide radical-scavenging activity was expressed by the degree of NBT reduction of a test group in comparison to that of the control (without test compound).

Hydroxyl radicals inhibition was evaluated by a modified method of the previously reported assay [20]. The mixture containing 50 µL of the test sample, 100 µL of the phosphate buffer 50 mM pH 7.8, 100 µL of deoxyribose 2.8 mM, and 100 µL of $Fe(NH_4)_2(SO_4)_2$ 500 µM was incubated for 1 h at 37°C. After adding 250 µL of trichloroacetic acid (10%, w:v) and 250 µL of thiobarbituric acid (1%, w:v), the reaction mixture

was boiled for 15 min in a water bath. The colour development was measured at 532 nm.

Statistical analysis

The data and charts were performed and processed on Microsoft Excel (Microsoft, USA), while surface plots were conducted on STATISTICA 12 software (Dell Inc., USA Round Rock, Texas). A PLS regression model was developed, using SIMCA-P software vs. 14 (Sartorius, Germany), to determine the correlation among extracting conditions, compositions, and bioactivities of the polysaccharide extracts.

Results and discussion

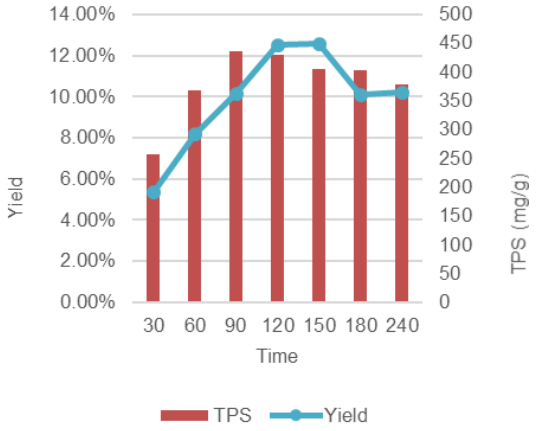
Effect of extracting time on the extraction yields and the total polysaccharide content

Extracting time may affect considerably the yield and quality of the extracts. In this paper, the effect of time was evaluated on the polysaccharides extraction using solvents, such as 10% ethanol, water, and 10% NaOH. As it is highlighted in Figure 1, the polysaccharides yield rose abruptly in the range of 120 min, hit the highest point in the next 30 min, then slightly declined in the further lengthening extracting time. In addition, the total polysaccharide content values peaked at 90 min. The extraction in the ethanol solvent has the highest efficiency between 120 and 150 min.

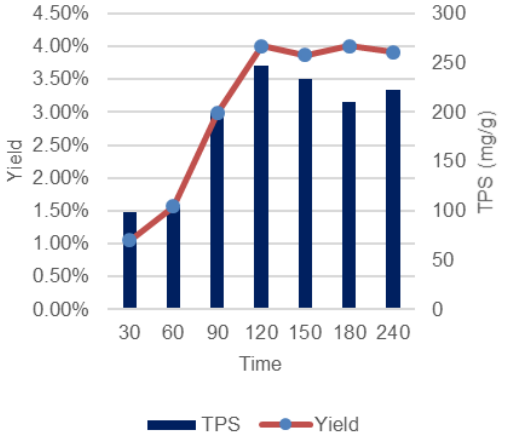
Also, the optimal extracting time for using water and 10% NaOH could be from 120 to 150 min, as shown in Figures 2 and 3. Accordingly, the yields and total polysaccharide contents of both two processes peaked after 2 h and reached a plateau in the next periods. Therefore, the extraction time of 120 min was selected for the subsequent experiments.

Effect of solid-to-liquid ratio on the extraction yields and the total content of polysaccharide

The ratio between the weight of material and solvent volume, or solid-to-liquid ratio, may affect deeply the extraction rate and contents of the products. In this paper, the differences when the solid-to-liquid ratio changed from 1:10 to 1:50 were evaluated. Figures 3 and 4 illustrated the effects that the polysaccharide yield reached the highest points when the ratios were between 1:20 and 1:30. The extraction yields and total polysaccharide contents decreased gradually when increasing the volume of solvents for the extraction. Too low solid-to-liquid ratio possibly declined the viscosity and density of the extracting solutions [21], thus, negatively affected the later polysaccharides precipitation. Furthermore, it may cause a higher cost for the process. Therefore, the ratio of solid-to-liquid should be set at 1:20 for the highest efficiency.



(a)



(b)

Figure 1. Effect of extracting time on the yield and total polysaccharide contents of 10% ethanol *(a)* and water extracts *(b)*.

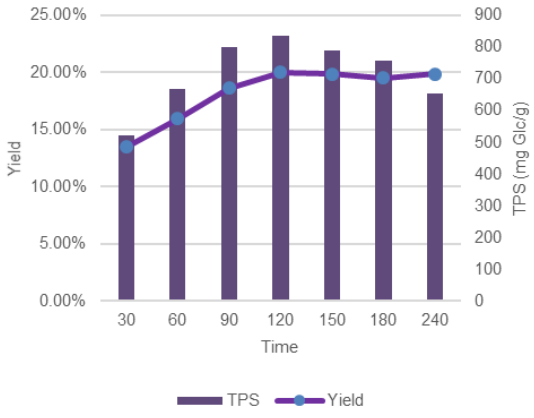
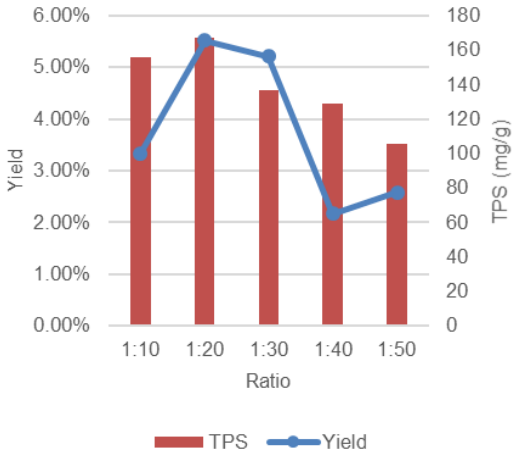
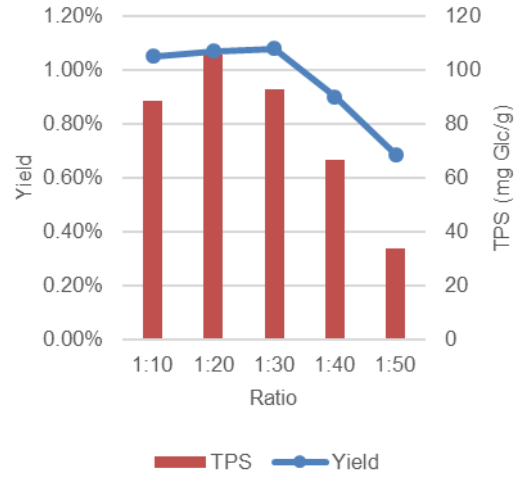


Figure 2. Effect of extracting time on the yield and total polysaccharide contents of 10% NaOH extracts.



(a)



(b)

Figure 3. Effect of solid-to-liquid ratio on the yield and total polysaccharide contents of 10% ethanol *(a)* and water extracts *(b)*.

Figure 4. Effect of solid-to-liquid ratio on the yield solid-to-liquid ratios and total polysaccharide contents of 10% NaOH extracts.

Effect of solvents and temperature conditions on the extraction yields, total polysaccharides, and total phenolic contents

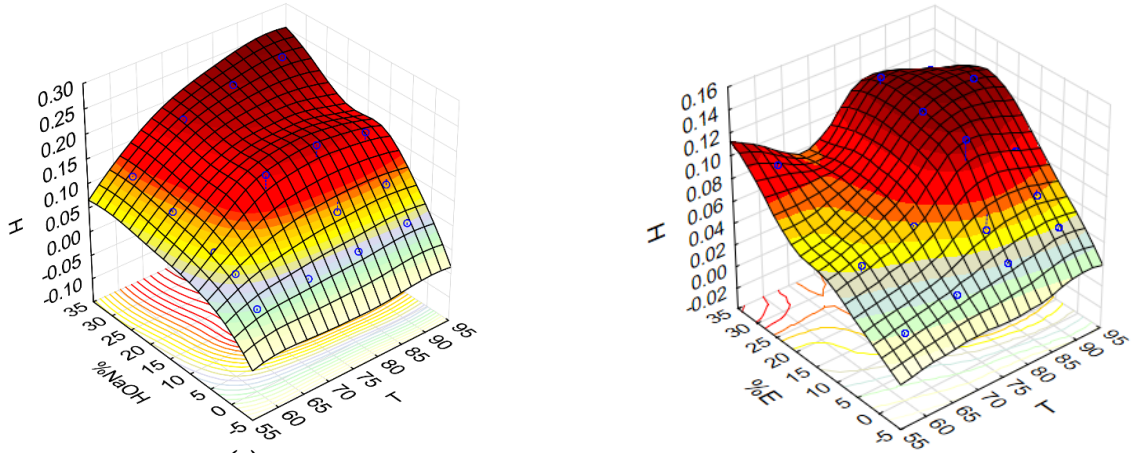
Temperature and solvent composition are the factors that may impact significantly the extraction yields of polysaccharides. In this report, the extraction yields by simultaneous effects of NaOH or ethanol solutions at different concentrations and temperatures with the optimized extracting time (2 h) and solid-to-liquid ratio (1:20, w:v) were evaluated. As can be seen in Figure 5*(a)*, when the NaOH concentration increased from 5% to 30%, the extracting yield rose sharply between 60°C to 70°C. However, there was no significant difference when the temperature raised gradually from 70°C to 90°C. Higher content of alkali may increase hydrolysis of glycosidic linkages that shorten the carbohydrate chains, while higher temperatures stimulated dissolution polysaccharides in the solvent [21]. Meanwhile, the extracting yields also showed an upward trend by the increase of ethanol concentration. However, it had declined slightly between 60°C and 70°C before rising along with the increase of temperatures from 70°C to 90°C. The highest yield was 13.65% when using ethanol 20% at 90°C. Overall, extracting in alkaline solution showed higher polysaccharide yields than using ethanol at the same temperatures. The polysaccharides could dissolve well in alkaline solutions, while ethanol may inhibit extracting large carbohydrates from haricot vert. The increase of polysaccharide yield by the ethanol concentrations may be due to the dissolution of several phenolics in the extracts.

Total polysaccharide and total phenolic contents were evaluated to compare the qualities of the products due to extracting conditions. Figures 6 presents the correlations among temperatures, NaOH or ethanol concentration, and total polysaccharide contents. On the one hand, the total polysaccharides content of the extract was peaked at 835.3 mgGlcE/g when using 20% NaOH at 80°C. In addition, the total polysaccharide contents grew stably when the temperature rose from 60°C to 80°C, and the NaOH percentage increased from 0% to 20%. The higher NaOH concentration and higher temperature may lead to the decomposition of glycogen structure and declining of the total polysaccharide content [22]. On the other hand, the polysaccharide content of the extracts grew sharply from 60°C to 80°C when increasing the ethanol percentage to 20%. The extract had the

highest content of polysaccharides in 20% ethanol at 80°C. The figures showed a sudden decrease at higher ethanol concentration and temperature since ethanol might inhibit the solubility of polysaccharides.

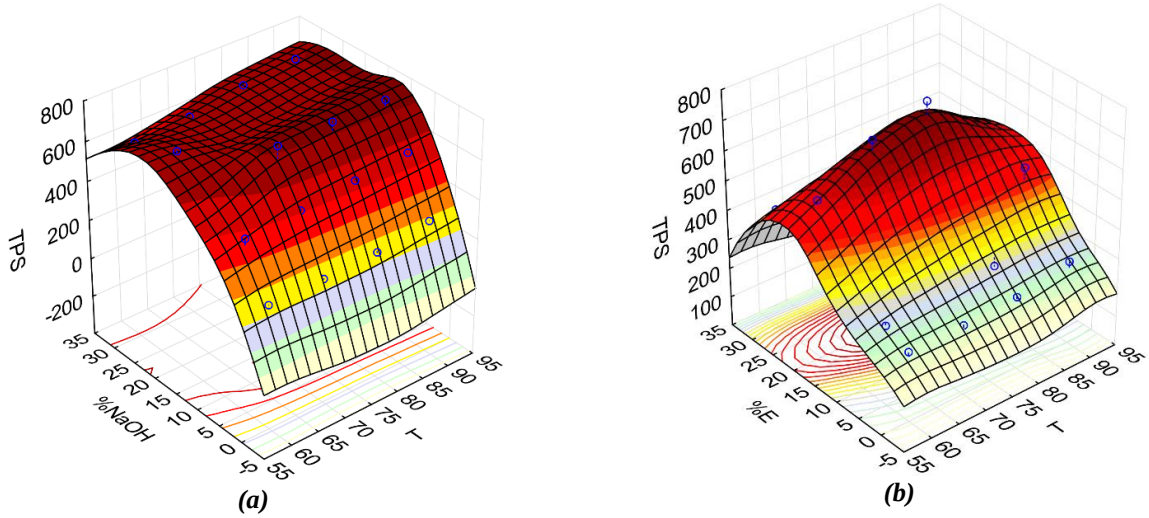
Besides polysaccharides, the content of phenolic compounds was also evaluated because of their impact on the antioxidant activities of the extracts. Figure 6*(a)* illustrates the correlations between total phenolic contents and extracting conditions when the NaOH solution was used as solvent. As can be seen, the product had the highest phenolic content when extracting in 10-15% NaOH at 65-75°C. There was a downward trend when both temperatures and alkaline concentrations increased. In general, the phenolic compounds may dissolve in a slightly alkaline solution at a certain temperature, thus, 10-15% NaOH at about 70°C may be an optimized condition for extracting both polysaccharides and phenolics. However, at a higher temperature and alkaline content of the solvent, phenolic compounds could be destroyed. On the other hand, the total phenolic contents of the extracts rose significantly by the increase of temperatures and ethanol contents in the solvent. As shown in Figure 6*(b)*, the best condition for extracting phenolics was in 25-30% ethanol at 75-80°C. The phenolic content could decline considerably if increasing the temperature to 90°C because of the decomposition of secondary metabolites at high temperatures. Thus, the extraction should not be carried out at a too high temperature.

Though the highest total phenolic contents of the NaOH extracts were gently lower than in the ethanol extracts, the NaOH addition gave higher extracting yields and higher total polysaccharide contents at the same temperature condition. The optimal temperature ranges for extracting the highest yield, polysaccharides, and phenolics contents using NaOH solvents were at 65-70°C, which was lower than the temperature of 75-80°C required when using ethanol solutions.



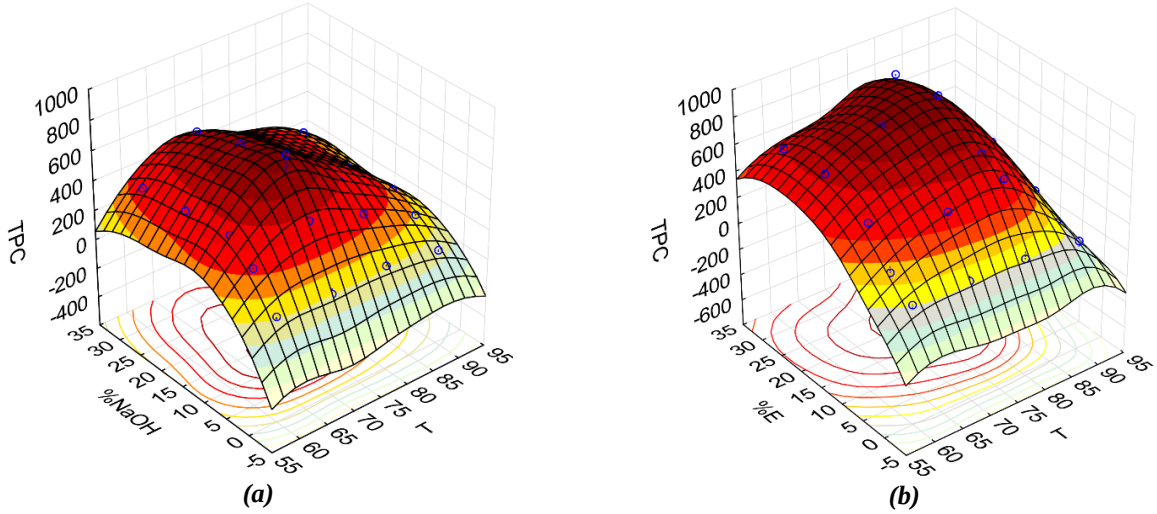
(a) *(b)*

Figure 5. Response surface plots (3D) for the effect of temperature, NaOH concentration *(a)*, or ethanol concentration *(b)* on extraction yields.



(a) *(b)*

Figure 6. Response surface plots (3D) for the effect of temperature, NaOH concentration *(a)*, or ethanol concentration *(b)* on the total polysaccharide content.



(a) *(b)*

Figure 7. Response surface plots (3D) for the effect of temperature, NaOH concentration *(a)*, or ethanol concentration *(b)*, on the total phenolic content.

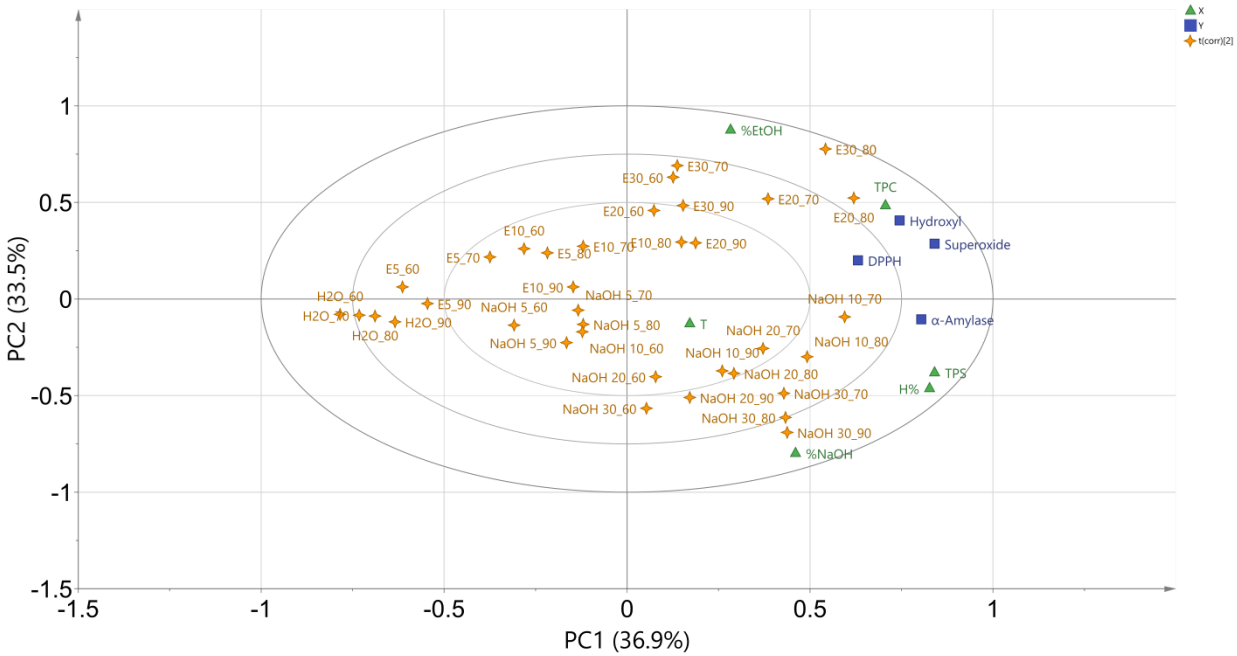


Figure 8. The PLS regression biplot. 4-Point stars, triangles, and boxes represented for samples, extracting conditions (*X*-variables), bioactivities (*Y*-replicas), respectively.

Correlation among extracting conditions, compositions, and bioactivities of the polysaccharide extracts

Partial least square (PLS) regression was conducted to identify the correlation between bioactivities and the compositions of the polysaccharide extracts (Figure 8). PC1 and PC2 performed 36.9% and 33.5% of the variations, respectively, indicated that most of the variations could be explained by the first two principal components. Previous reports presented successful results of this statistical approach on determining the links of bioactivities and sample compositions and reducing the complex dataset to interpret them more easily [23-25]. In this case, the extracting conditions, including temperature and percentage of solvents, total polysaccharide, and phenolic contents were selected as *X*-variables, meanwhile, bioactivities were set as *Y*-replicas. Close positions among variables and replicas on PLS biplots may illustrate considerable effects of a factor to the others. Besides, near positions of samples (cases) to a factor, which could be a variable (*X*) or replica (*Y*), may represent a higher value of these factors for these samples [23]. As can be seen in Figure 8, bioactivities of the extracts were highly affected by extracting yields, total polysaccharides content, and total phenolic contents.

Table 1

Antioxidant and α-amylase inhibition activities of some polysaccharide extracts.

Samples	*DPPH* (IC_{50}, µg/mL)	*Hydroxyl* (IC_{50}, µg/mL)	*Superoxide* (IC_{50}, µg/mL)	*α-Amylase* (IC_{50}, µg/mL)
10% NaOH	36.3±1.3	23.3±0.6	30.9±1.1	125.3±3.1
20% EtOH	34.2±0.8	26.2±0.3	38.2±1.4	146.9±2.8
Ascorbic acid	35.1±0.2	-	-	-
(+)-Catechin	-	20.1±0.3	32.5±1.0	-
Acarbose	-	-	-	84.2±1.8

Data were expressed by mean ± standard derivation, significant differences at p <5%

Antioxidant activity might depend deeply on the total phenolic content of the extract, whereas, the total polysaccharide content and polysaccharides extraction yields might have larger effects on α-amylase inhibition. Antioxidant activity of phenolic compounds was previously reported [23-26], meanwhile, other investigations presented considerable α-amylase inhibition of polysaccharides from haricot vert [27-28].

According to these results, the extraction should be carried out in a condition that could increase extracting both polysaccharides and phenolics from haricot vert pods. In addition, Figure 8 also indicates that the extract in ethanol 20% at 80°C might have the best antioxidant activity due to its phenolics compositions, while, the extract in 10% NaOH at 70°C might perform the greatest α-amylase inhibiting activity because of higher polysaccharides content. As mentioned above, the extracts in these conditions might have high contents of those two compositions. Comparing the results, while the extract in 10% NaOH showed higher DPPH, superoxide, and hydroxyl radical scavenging activities with higher polysaccharides yield and content, the extract in 20% ethanol contained more phenolic compounds and exhibited more significant α-amylase inhibition.

The antioxidant activity of polysaccharide extracts in 10% NaOH at 70°C and in 20% ethanol at 80°C was further evaluated in terms of IC_{50} values (Table 1). Both two extracts have certain contents of phenolic compounds that may show antioxidant activities. Interestingly, though having a lower phenolic content, at 806.7 µgGAE/g, than the 10% NaOH extract, at 868.4 µgGAE/g, the 10% NaOH extract showed higher hydroxyl and superoxide radical inhibitions. It should be noted that the antioxidant IC_{50} values of the alkaline extract approximated to the values of reference samples (ascorbic acid for DPPH assay, (+)-catechin for hydroxyl and superoxide assay), as shown in Table 1. Besides, the NaOH extract showed gently higher α-amylase inhibiting activity than the ethanol extract. However, these inhibitions were quite weak when compared to acarbose (reference sample), an oligosaccharide formulated into an anti-diabetic drug used to treat type 2 diabetes.

Conclusions

Polysaccharides extractions of haricot vert pods were carried out under different conditions, such as extracting time, temperature, solid-to-liquid ratios, and solvent compositions to optimize the processing method. While extracting time and solid-to-liquid ratio could be simply selected at 120 min and 1:20, respectively, the optimal temperature might vary due to the solvent compositions.

Correlations between bioactivity and compositions of the extracts were investigated from the PLS regression model, that demonstrated that polysaccharides might affect sharply the α-amylase inhibition, whereas, the phenolic contents exhibited strong effects on the antioxidant activity. Additionally were evaluated the comparative bioactivity of the products under different conditions, IC_{50} values of antioxidant and α-amylase inhibiting activities of the extract in 10% NaOH at 70°C and in 20% ethanol at 80°C. Accordingly to the obtained results, extracting in 10% NaOH with solid-to-liquid 1:20 (w:v) at 70°C for 2 h proved to be the optimal conditions that showed the highest extraction rate, polysaccharides contents, and also bioactivities.

The method could be used at a larger scale for the preparation of a natural antioxidant product in the food or pharmaceutical industry.

Acknowledgments

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