Optimal conditions for extracting bioactive compounds from leaves of *Cleistocalyx operculatus roxb* using an enzyme cocktail

Vu Dinh Giap[1], Do Thi Cam Van[1], Vu Dinh Duy[2], Vu Kim Dung[3], Nguyen Thi Hai Ha[4]

[1]*Hanoi University of Industry (HaUI)*

[2]*Join Vietnam - Russia Tropical Science and Technology Research Center*

[3]*Vietnam National University of Forestry*

[4]*Vinschool Vinhomes Ocean Park*

Tối ưu điều kiện chiết xuất hoạt chất sinh học từ lá vối (*Cleistocalyx operculatus* roxb) sử dụng enzyme cocktail

Vũ Đình Giáp[1], Đỗ Thị Cẩm Vân[1], Vũ Đình Duy[2], Vũ Kim Dung[3], Nguyễn Thị Hải Hà[4]

[1]*Trường Đại học Công nghiệp Hà Nội (HaUI)*

[2]*Viện Sinh thái nhiệt đới - Trung tâm nhiệt đới Việt - Nga*

[3]*Trường Đại học Lâm nghiệp*

[4]*Trung học Vinschool Ocean Park*

https://doi.org/10.55250/jo.vnuf.8.2.2023.010-018

Article info:
Received: 23/08/2023
Revised: 12/10/2023
Accepted: 02/11/2023

Keywords:
Cleistocalyx operculatus Roxb, flavonoid, hydrolytic enzymes, oxidative enzymes, Polyphenol.
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ABSTRACT

In recent years, both scientists and food producers have shown great interest in natural compounds with high biological activity. Leaves of Cleisto (Cleistocalyx operculatus Roxb) contain polyphenolic compounds with high biological activity, including flavonoids such as quercetin, kaempferol, myricetin, tannins, and other phenolic compounds. These compounds are highly beneficial for human health due to their potent antioxidant activity and various other biological activities. These polyphenols play a crucial role in protecting the body against various diseases caused by free radicals. They have the ability to prevent cellular and DNA damage. Polyphenols derived from mulberry leaves have significant potential for the production of high-value functional foods. In this study, the total polyphenol content, and antioxidant activity of leaves of Cleisto were studied. These bioactive compounds were shown to be more abundant in the dried extract of Cleisto than in the control sample (enzymes inactivated) following 21 hour of incubation with laccase and celluclast® 1.5L (each enzyme at least 30 U/gram dry biomass) at 45°C, pH 5.0. The highest total polyphenol content and antioxidant activity achieved was 23.95 mg GAE/gds and 80.32%, respectively. Therefore, enzyme-assisted extraction technique has various benefits and is a potential method to obtain bioactive chemicals from leaves of Cleisto.

Từ khóa:
Cleistocalyx operculatus Roxb, enzyme oxy hóa, enzyme thủy phân, flavonoid, Polyphenol.

TÓM TẮT

Trong những năm gần đây, các nhà khoa học cũng như nhà sản xuất thực phẩm rất quan tâm đến các hợp chất tự nhiên có hoạt tính sinh học cao. Lá vối chứa các hợp chất polyphenol giàu hoạt tính sinh học, bao gồm các flavonoid như quercetin, kaempferol, myricetin, tannin và các hợp chất phenolic. Các hợp chất này rất tốt cho sức khỏe con người do chúng có hoạt tính chống oxy hóa cao và nhiều hoạt tính sinh học khác. Những polyphenol này đóng vai trò quan trọng trong việc bảo vệ cơ thể chống lại nhiều loại bệnh do gốc tự do gây ra. Chúng có khả năng ngăn chặn sự tổn thương tế bào và DNA. Các polyphenol có nguồn gốc từ lá vối có tiềm năng lớn để sản xuất thực phẩm chức năng có giá trị cao. Trong nghiên cứu này, hàm lượng polyphenol tổng số và hoạt tính chống oxy hóa của cao chiết lá vối được nghiên cứu. Các hoạt chất sinh học này được chứng minh có nhiều trong dịch chiết mẫu lá vối khi ủ với hỗn hợp laccase và Celluclast® 1,5 L (30 U/g sinh khối khô) ở 45°C, pH 5,0 sau 21 giờ ủ. Hàm lượng polyphenol tổng số và hoạt tính chống oxy hóa lớn nhất đạt được lần lượt là 23,95 mg GAE/gds và 80,32%. Do đó, kỹ thuật chiết xuất có sự hỗ trợ của enzyme có nhiều lợi ích khác nhau và là phương pháp tiềm năng để thu các hoạt chất có hoạt tính sinh học từ lá vối.

1. INTRODUCTION

Polyphenols are a widely prevalent group of natural compounds found in plants. They are present in the leaves, stems, and fruits of many plant species in high concentrations, such as in tea leaves, cocoa beans, berries, grapes, pomegranates, and persimmons. It is known for its potential health benefits due to their antioxidant and anti-inflammatory properties [1].

The active polyphenols found in the leaves of *Cleistocalyx operculatus Roxb* are likely to be a mixture of various compounds, including flavonoids such as quercetin, kaempferol, and myricetin, tannins, and other phenolic compounds. These polyphenols have been studied for their potential health-promoting effects, including antioxidant activity, anti-inflammatory properties, and potential benefits for cardiovascular health. It protects the body against many different diseases caused by free radicals and is found in many leaves of syzygium nervosum [2, 3].

Vietnam is one of the places in the world with the most different kinds of plants and animals. It is home to more than 72,000 kinds of higher plants, including about 2.250 kinds of fungi [4]. Fungi can make hydrolytic (cellulase, xylanase, etc.) and oxidizing (laccase, etc.) enzymes outside of their cells to attack lignocellulose structures in plant cell walls. Cellulase is an enzyme that breaks down cellulose, a complex carbohydrate found in plant cell walls. While cellulase's primary role is to break down cellulose, it can also indirectly impact the oxidation of polyphenols [5]. Cellulase enzymes can disrupt the plant cell structure, releasing polyphenols and other compounds trapped within the cells. These released polyphenols are then more accessible to other enzymes, including laccase, which can oxidize them [6]. Meanwhile, laccase is an enzyme that catalyzes the oxidation of various compounds, including polyphenols. It does this by transferring electrons to molecular oxygen, leading to the formation of reactive oxygen species. In the context of polyphenols, laccase can oxidize them to form more complex and stable compounds. This oxidation process can lead to changes in color, flavor, and other properties of the polyphenols and the plant tissues containing them [7]. So, cellulase indirectly affects polyphenol oxidation by breaking down plant cell walls and releasing polyphenols, making them more accessible to oxidation by enzymes like laccase. Laccase, on the other hand, directly catalyzes the oxidation of polyphenols through the generation of reactive oxygen species. These processes are part of the natural biochemical pathways in plants and are important for various physiological and ecological functions. It's worth noting that the oxidation of polyphenols can have both positive and negative effects [8]. On the other hand, it can lead to the formation of stable compounds with altered properties, which can be beneficial for plant defense mechanisms or the production of certain flavors and aromas. On the other hand, excessive oxidation can lead to the degradation of desirable compounds and contribute to spoilage in foods and beverages. The purpose of this study is to enzymatically hydrolyze persimmon leaves with the assistance of cellulase and laccase oxidase enzymes, in order to release bioactive polyphenol compounds without the need for prior chemical pre-treatment.

2. RESEARCH METHODOLOGY

2.1. Materials

The fungal strain *Clitopilus prunulus* BV 18 was isolated from Ba Vi National Park (Hanoi, Vietnam), and kept in the laboratory, HaUI Institute of Technology, Hanoi University of Industry.

Leaves of *Cleistocalyx operculatus Roxb* (*Cleisto*) were obtained from Hai Duong province (Vietnam). Samples were dried to constant weight at 60°C, ground into powder and stored in desiccant bags, and kept at a cool and dry place.

Commercial celluclast® 1.5L enzyme from *Trichoderma reesei* (specific activity 700 U/g, Novozymes, Denmark), optimum temperature and pH at 40-45^{o}C and pH 5.5.

2.2. Cellulase assay

Cellulase will break down the CMC (carboxymethyl cellulose) chain into monosaccharides. The content of reducing sugars formed is determined using the 3.5-dinitrosalicylic acid (DNS) reagent and measured using a spectrophotometer at a

wavelength of 540 nm. The DNS reagent turns yellow in an alkaline solution and is reduced to 3-amino-5-nitrosalicylic acid, which exhibits an orange-red color. Extract 10 mL of the solution, centrifuge it at 5.000 rpm for 20 minutes to remove biomass, and collect the supernatant for use as the crude enzyme solution. Carry out the reduction reaction by taking 1.0 mL of the crude enzyme solution and placing it into a 16 × 100 mm test tube. Add 1.0 mL of 1% CMC solution, allow the reaction to occur at 30°C, pH 5.5 for 60 minutes [9].

Laccase activity was determined by the ABTS oxidation method [2.2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid]. The reaction solution consisted of 50 µl enzyme solution, 100 µl sodium acetate buffer (100 mM, pH 5), 50µl ABTS (10 mM). Samples were incubated at 45 C for 30 minutes, spectrophotometrically measured at 420 nm [10].

2.3. Growth conditions of C. prunulus BV 18 on solid cultivation

For the production of enzymes on a larger scale, 1-2 kg of lignocellulose (rice straw) material was soaked overnight. Subsequently, the material is dampened and introduced into plastic bags with high heat resistance, a trace element solution was added of 20 mL Kg^{-1} (80 mg L^{-1} of $FeSO_4.7H_2O$, 200 mg L^{-1} of $ZnSO_4.7H_2O$, 3.0 g L^{-1} of $MgSO_4.7H_2O$, 0.5 g L^{-1} of $MnSO_4.H_2O$, 70 mg of L^{-1} of $CaCl_2$, 8.0 mg L^{-1} of $CuSO_4.5H_2O$, 5.0 mg L^{-1} of H_3BO_3, 1.0 g L^{-1} of NaCl), then sterilized at 121ºC for 30 minutes. Two petri dishes containing agar-malt medium are inoculated with homogenous fungal spores in 160 ml of distilled water containing 0.9% NaCl. The entire fungal inoculum is transferred into the plastic bags along with the straw or wood chips substrate. The entire culturing process is conducted under aseptic conditions. After culturing at 25°C at pH 6.0. During incubation of 15 days, aliquots were taken from fungal liquid cultures after intervals of 3 days for the measurement of laccase activity.

2.4. Characterization of enzymes laccase

The crude enzyme solution obtained after liquid fermentation was further precipitated with the inorganic salt ammonium sulfate with concentrations from 50%. Concentrate the enzyme at 11 C, then centrifuge (10 min, 4.000 rpm) to collect protein residue. The fractions obtained after centrifugation, protein residues will be washed and re-mixed with 100mM sodium acetate buffer (pH 5.0), continue centrifugation to collect protein residues, repeat 2-3 times. The effect of pH on laccase activity was tested with a pH of 3.0 to 8.0 in 100 mM sodium acetate buffer (pH 4.0 - 5.5) and 100 mM sodium phosphate (pH 6.0 - 8.0). The optimum temperature was determined at 30 to 50°C on an ABTS substrate in sodium acetate buffer (100 mM) at pH 5.0.

2.5. Determination of suitable conditions for extraction of polyphenols from leaves of Cleisto by enzyme mixture

Leaves of *Cleisto* (5.0 gram per reaction) were milled for 5 minutes to powder, added water (1:5, w/v) and sonicated at 50ºC for 30 min. And then, the samples were incubated with enzyme mixture (10 U/gds celluclast® from Novozymes and 10 U/gds laccase from *C. prunulus* BV 18) at 50°C, pH 5.0 and monitored at 2, 18, 21, 24, 42, and 45h. Moreover, the effects of different enzyme activity (U/gds, U of each enzyme laccase and celluclast® 1.5L per gram dry biomass) were investigated, including 10, 20, 30, 40, and 50 U/dgs. The reaction was carried out in 100 mM MOPS buffer (pH 5.0) at 45°C, pH 5.0 during 45 h. The control sample with heat-inactivated enzyme (95ºC for 30 min) was used. The hydrolyzate solution was filtered through whatman filter paper No.41. The extract was centrifuged at 10.000 rpm for 30 min until dry. The obtained samples were analyzed for their total polyphenol content and antioxidant activity.

2.6. Determination of total polyphenol content

The polyphenol content of the extract was determined using colorimetry and the Folin-Ciocalteu reagent. This reagent contains a phospho-tungstenic acid oxidizing agent; phenol hydroxy groups are readily oxidized during reduction; and this oxidizing agent produces a blue color with maximal absorbance at 765 nm. The sample's polyphenol content is proportional to the sample's concentration and is

calculated as gallic acid. The reaction mixture is comprised of 1.0 ml of the sample to be quantified and 6 ml of distilled water that has been vigorously agitated. Then, add 0.5 milliliter of 10% Folin-Ciocalteu reagent, agitate vigorously, and allow to stand. After 5 minutes, add 1.5ml of 20% Na_2HCO_3 and agitate vigorously. Add 10 milliliter of water distillate to a volume of 10 ml. Two hours later, measure the absorbance of all samples at 765 nm using a spectrophotometer that has been calibrated [11].

2.7. Determination of antioxidant activity

The technique that was used to assess the 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of an extract sample was reported [12]. The sample of dried extract was dissolved in DMSO at a concentration of one hundred percent. DPPH was mixed with ethanol that was 96% strong. An UV spectrophotometer was used in order to determine a sample's absorbance at a wavelength of 515 nm. The free radical scavenging capacity (SC, %) of the extract was calculated as follows:

$$\text{SC}\ (\%) = \left[100 \quad \frac{\text{Abs(test)} - \text{Abs (blank)}}{\text{Abs (negative)}} \times 100\right] \pm \sigma$$

The standard deviation (σ) is calculated by Duncan's formula as follows:

$$\sigma = \sqrt{\frac{(\sum x_i \quad \bar{x})^2}{n \quad 1}}$$

3. RESULTS AND DISCUSSION

3.1. Induction of carbohydrate sources on the laccase production

Laccase synthesis in *C. prunulus* is efficient after a 15 days cultivation period as Figure 1. Throughout the solid-state cultivation process employing rice straw as the substrate, laccase activity exhibited a rise until the 12th day of culture (increasing from 87.0 to 2.316 mU g^{-1}), followed by a subsequent decline. Despite the decline, the enzyme activity remained notably elevated (1.980 mU g^{-1}) until the conclusion of the cultivation period. This pattern can be attributed to the enzyme activity's continuous growth up until the 12th day of culture. The original pH of the culture medium was 6.0, but it dropped to a somewhat more acidic level of 5.5 after some time. The *C. prunulus* strain's ability to produce laccase on lignocellulosic substrates was examined. This fungus was grown in solid cultures that also included lignocellulosic materials (rice straw) as a source of carbon. The biosynthetic output of the fungal strain's laccase is increased by using rice as a carbon source. It is stated that simple sugars joined by glycosides create cellulose in carbon sources. The laccase activity of the *C. prunulus* culture filtrate was often equivalent to or lower than that reported for these fungi, but utilizing accessible and affordable raw carbon sources as rice straw.

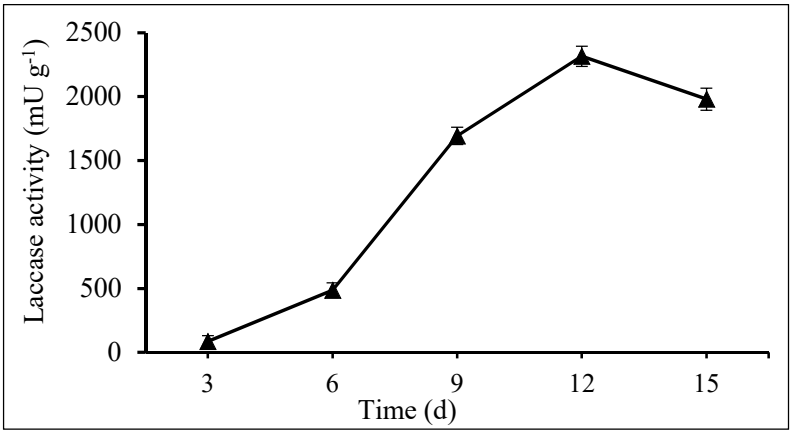

Figure 1. Time courses of laccase production by *C. prunulus* BV18 in solid-state cultures using rice straw as the growth medium. Each experiment was carried out three times and error bars show the standard deviation

3.2. Characterization of laccase from C. prunulus BV18

The crude enzyme solution generated from liquid fermentation was then precipitated using ethanol and the inorganic salt ammonium sulfate (40, 50, 55, 60, 65, and 70%). Precipitation was performed at 11°C, followed by centrifugation (10 min, 4.000 rpm) to obtain protein residue. To assess enzyme activity, the fractions produced after centrifugation and solvent evaporation were reconstituted with 100 mM sodium acetate buffer (pH 5.0) as the results shown in Table 1.

Table 1. Precipitation of crude laccase by different methods

Precipitation method	Concentration (%; v/v)						
	40%	**50%**	**55%**	**60%**	**65%**	**70%**	Laccase activity (U/ml)
Ethanol	32	68	67	**79**	43	27	
Amoni sulfate	196	**215**	190	143	109	93	

As the Table 1, the laccase enzyme activity within the ethanol fractions was notably lower compared to that in the ammonium sulfate fractions. Within the ethanol precipitation fractions, the highest enzyme activity reached 79 U/ml at a solvent concentration of 60%. However, when the concentration exceeded 60%, the enzyme activity decreased to 27 U/ml at a solvent concentration of 70%. In contrast, among the ammonium sulfate fractions, a solvent concentration of 50% yielded the highest laccase activity at 215 U/ml. As the solvent concentration increased progressively, there was a slight decline in enzyme activity, reaching a minimum of 93 U/ml at a solvent concentration of 70%. The enzymatic activity resulting from ammonium sulfate precipitation was 2.7 times greater than that of ethanol precipitation. Consequently, the method of enzyme recovery utilizing the inorganic salt ammonium sulfate is preferred due to its comparatively lesser impact on laccase activity when contrasted with the polar solvent ethanol.

The laccase pH range was determined to be between 3.0 and 8.0. The results demonstrated that the laccase enzyme activity derived from the strain *C. prunulus* attained a relatively high value in the pH range of 5.0 to 5.5, with a maximum value between 96 and 100%. Compared to the enzyme activity at pH 5, the enzyme activity decreased marginally by 85% when the pH increased to 6.0. After the pH continued to rise, enzyme activity began to decline abruptly, reaching a minimum of 35% at pH 8 (Figure 2A).

The optimal temperature has a significant impact on the activity and rate of the reaction, and each enzyme only functions within a specific temperature range. The optimal temperature range for the reaction between laccase obtained from *C. prunulus* BV18 and ABTS substrate was between 30 and 50ºC. Figure 2B demonstrates that the enzyme activity increased steadily from 40% at 30ºC to its maximum at 45ºC, and then began to decrease gradually after 45ºC. Thus, the results of the study demonstrated that laccase biosynthesized from the fungus *C. prunulus* BV18 has an optimal temperature of 45ºC and pH 5.

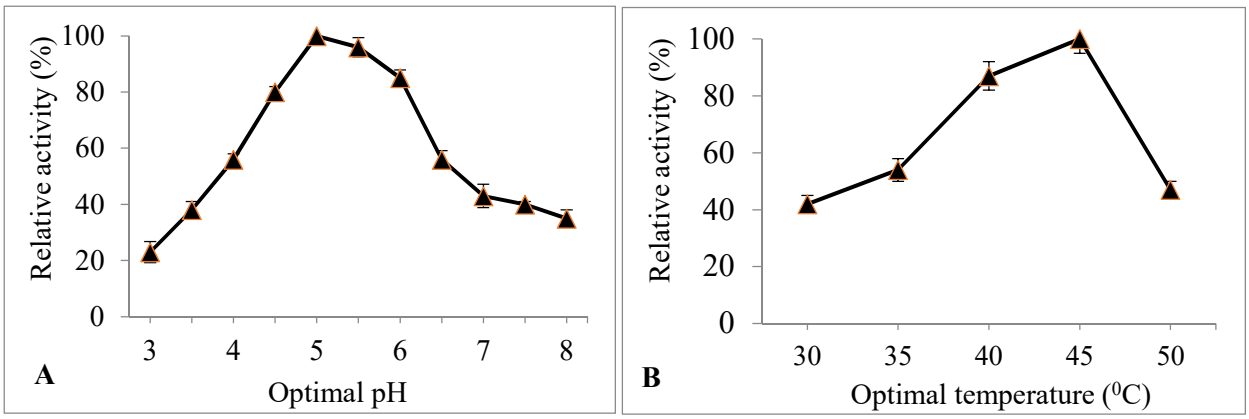

Figure 2. Optimal pH (A) and (B) temperature of laccase from *C. prunulus* BV18

3.3. Effect of enzyme-assisted extraction time on total polyphenol content

Hydrolysis time has a significant effect on the extraction of compounds from the leaves of *Cleisto*. The efficiency of the process has a positive correlation with the time of extraction. The efficacy of extending the extraction time is limited and subject to specific constraints.

Figure 3 presents the results obtained from the investigation on the impact of the duration of combined enzyme and ultrasonic hydrolysis on both the extraction yield and the principal constituents found in the extract.

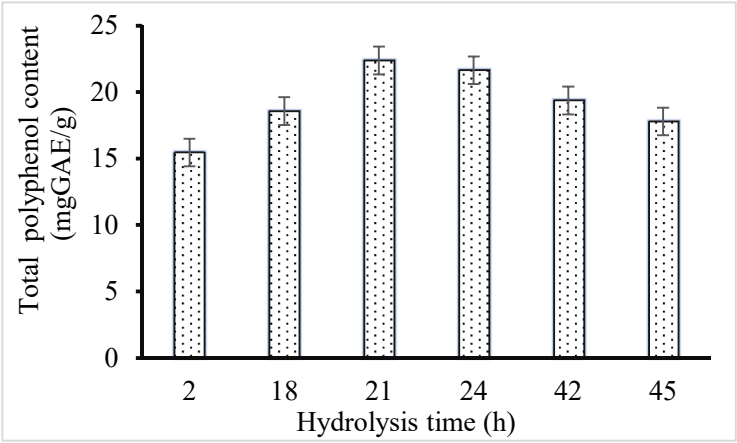

Figure 3. Effect of hydrolysis time on total polyphenol content from *Cleisto* extract

The polyphenol content exhibited a rapid increase, rising from 15.98 to 17.32 mg GAE/g, as the hydrolysis time was extended from 2 to 18 hours. Notably, the optimal extraction period for achieving the highest concentration was found to be 18 hours. Then, when the extraction time was further extended to a total of 21 hours, the polyphenol content gradually decreased and finally reached 23.43 mg GAE/g. After a 24 hours period, the polyphenol content reached 21.38 mg GAE/g. With extended durations of 42 and 45 hours, the polyphenol content significantly decreased to 19.17 and 18.43 mg GAE/g, respectively. The lowest value of polyphenol content was at 45 hours, showing a significant difference compared to the content observed at 21 hours. This can be explained by the fact that, when the extraction period is prolonged, the polyphenol compounds within and outside the raw materials are near to equilibrium, resulting in a gradual increase in polyphenol content and a decreasing tendency in the extracted material. The study conducted by Khoi & Duyen (2017) [13] on water chestnut husks revealed that with increasing time, the polyphenol content initially increased and then gradually decreased. In the time range of 1 to 9 hours, the extraction efficiency significantly increased, and the polyphenol content reached 89.13 mg GAE/g db (increased 3.3-fold). From 9 to 24 hours, the extraction efficiency decreased gradually, and the polyphenol content decreased by 33.38 mg GAE/g (decreased 1.35-fold). In another study by Quyen *et al.* (2016) [14] on soil gourd leaves. When increasing the extraction time from 10 to 40 minutes, the content increased rapidly from 17.16 to 34.80 mgGAE/g extract and 40 minutes was the time to obtain the highest polyphenol content. However, if the extraction time was increased to 50 and 60 minutes, the polyphenol content increased only slightly and there was almost no difference.

3.4. Effect of enzyme-assisted extraction time on antioxidant activity

The findings of the research demonstrate a link between the amount of total polyphenols and the *Cleisto* leaf extract's antioxidant activity. The DPPH free radical assay is often used for the rapid evaluation of antioxidant efficacy, offering a notable advantage in terms of time efficiency compared to other methodologies. The powerful scavenging capacity of dried *Cleisto* leaf extracts has been shown via the investigation of many chemical approaches. However, these technologies are costly and generate a lot of pollution. In this study, the investigation focused on evaluating the free radical scavenging activity of the

hydrolysis product of *Cleisto* leaf extract obtained using enzyme-assisted extraction. The DPPH free radical was used as a means to assess this activity.

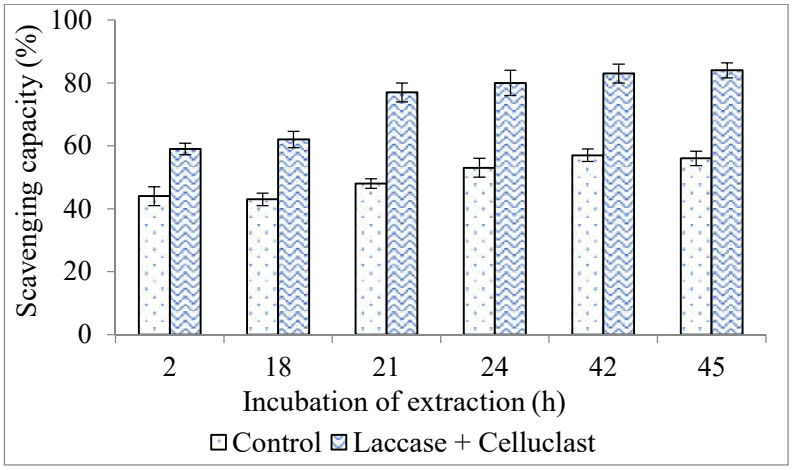

Figure 4. Effect of incubation of extraction on scavenging capacity: ( ) Enzyme-assisted extraction (10 U/gds celluclast® and 10 U/gds laccase) and ( ) inactivated enzyme (control)

The free radical scavenging capacity of the dried *Cleisto* leaf extract is shown in Figure 4. The sample was treated with enzyme mixture, the product had the maximum free radical scavenging capacity after 21 hours of incubation (79.01%). Scavenging capacity ranged from 79.65 to 81.29% for hydrolysis products produced after 24 to 45 hours, which was significantly greater than the control (inactivated enzyme), which ranged from 42.40 to 47.21%.

3.5. Effect of enzyme activity on total polyphenol content

Table 2. Effect of different enzyme mixture on the total polyphenol content

Enzyme activity (laccase and celluclast® 1.5L, ratio 1:1, U/gds)	The total polyphenol content (mg GAE/gds) obtained by enzyme-assisted extraction (45°C, pH 5.0, 21h)	
	Enzyme mixture (test sample)	Inactivated enzyme (control sample)
0	-	6.32 ± 0.2
10	9.04 ± 0.3	-
20	13.35 ± 0.5	-
30	21.49 ± 1.0	-
40	22.32 ± 0.4	-
50	23.43 ± 0.3	-
60	23.95 ± 1.2	-

The results obtained in Table 2 demonstrate a significant impact of enzyme activity on polyphenol content. When the total enzyme activity was 30.0 U/gds, the maximum polyphenol content was found to be 23.95 mg GAE/gds, and when the total enzyme activity was 10 U/gds, the lowest polyphenol content was found to be 9.04. When the enzyme activity was increased from 10 to 30 U/gds, the polyphenols increased (9.04 up to 21.49 mg GAE/gds). However, if the enzyme increases above 30 U/gds, the total polyphenol increase is not significant (21.49 to 23.95 mg GAE/gds). Meanwhile, the control sample only achieved 6.32 mg GAE/gds. The observed phenomenon can be attributed to the correlation between elevated substrate and enzyme quantities and a subsequent rise in reaction rate. As enzyme content increased, the reaction rate remained stable or even escalated, especially upon reaching a threshold where enzyme concentration equaled or surpassed substrate concentration. Consequently, the utilization of a more active laccase enzyme led

to notable enhancements in product release. This underscores the role of laccase as a catalyst in glycosidic linkage hydrolysis within complex carbohydrates featuring two or more sugar units. Hence, the enzyme's activity proves indispensable in the degradation of lignocellulose, a constituent of plant cell walls. The enzyme's release from cellular components, including polyphenol-bound proteins, occurred upon cellular breakdown. Therefore, under the aforementioned circumstances, an enzyme mixture addition of approximately 30 U/gds proved optimal. In a study carried out by Dung & Huong (2019) [15] involving the utilization of the celluclast enzyme on custard-apple shells, the findings demonstrated that as the enzyme ratio was raised from 0.5% to 1.5%, the polyphenol content exhibited an increase from 35.89 to 47.66 mg GAE/gds. In a separate investigation conducted by Buu *et al.* (2018) [16] concerning the extraction of polyphenol compounds from black garlic, the polyphenol content exhibited a rise in tandem with an increase in cellulase enzyme concentration, progressing from 0 to 0.06%. This progression corresponded to an escalation in the measured polyphenol compound content, ascending from 6.52 to 11.05 mg GAE/gds. Despite continuing to increase the cellulase concentration to 0.1%, the polyphenol content still reached 11.11 mg GAE/gds.

3.6. Effect of enzyme activity on antioxidant activity

The study results show that, the correlation between polyphenol compounds for the antioxidant capacity of *Cleisto* leaf extract.

Table 3. Effect of enzyme activity on antioxidant activity

Enzyme activity (laccase and celluclast® 1.5L, ratio 1:1, U/gds)	Scavenging capacity (%) obtained by enzyme-assisted extraction (45°C, pH 5.0, 21h)	
	Enzyme mixture (test sample)	Inactivated enzyme (control sample)
0	-	48.54 ± 0.15
10	50.16 ± 0.10	-
20	69.52 ± 0.08	-
30	77.39 ± 0.13	-
40	78.09 ± 0.11	-
50	80.01 ± 0.10	-
60	80.32 ± 0.11	-

As shown in Table 3, effect of enzyme activity from 10 to 60 U/gds on antioxidant activity was investigated. The antioxidant capacity of the initial dried extract (control sample) was 48.54%. Enzyme treatment had a significant effect on the antioxidant capacity of the samples at 10 U/gds compared with the control samples (50.16 and 48.54%, respectively). However, as the results, in the rank of enzyme activity from 20 to 40 U/gds a significant change in the liberation of products was observed to be 69.52 to 78.09 % for scavenging capacity. At enzyme activity of 30 U/gds, the scavenging capacity is significantly greater compared to the control, measuring 77.39% and 48.54%, respectively. However, at higher enzyme mixture (e.g. up to 40 U/gds), the reaction products wouldn't be increased significantly. The highest scavenging capacity using enzyme mixture (60 U/gds) in the enzyme-assisted extraction process was up to 80.32% at 45°C, pH 5.0 during 21h incubation. The capacity to scavenge DPPH free radicals improved from 22.75 to 61.08% when the enzyme concentration grew from 0% to 0.1% in a researchs conducted by Buu et al. (2018) [16] on participants studying the extract of black garlic. The findings revealed that the DPPH free radical scavenging ability increased when the enzyme concentration climbed from 0 to 0.1%. However, there was no discernible difference in the capacity to scavenge DPPH free radicals between the 0.06, 0.08, and 0.1% enzyme concentrations that were achieved. Therefore, in comparison to the control extraction, the enzyme-assisted extraction technique yielded significantly higher levels of antioxidants.

4. CONCLUSION

This study aimed to investigate the total polyphenol content and antioxidant activity of *Cleisto*. The research revealed that these bioactive compounds were more abundant in the dried extract of *Cleisto* than in the control sample (with enzymes inactivated or without enzyme). This difference was observed following a 21 hour of incubation with laccase and Celluclast® 1.5L (each enzyme at least 30 U/gram dry biomass) at 45°C and pH 5.0. The highest total polyphenol content and antioxidant activity achieved was 23.95 mg GAE/gds and 80.32%, respectively. Therefore, enzyme-assisted extraction technique has various benefits and is a potential method to obtain bioactive chemicals from leaves of *Cleisto*.

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