Impact of commercial cellulase enzyme on the quality of *Clinacanthus nutans* extracts

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Abstract. The use of enzymes such as cellulase and pectinase to aid in the fruit and vegetable juice extraction is a common practice. However, this practice is very limited in the production of juice or extract from green leaves. This study was carried out to evaluate the impact of commercial cellulase enzyme on the volume yield recovery, total chlorophyll and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of the *Clinacanthus nutans* leaves and stem. Leaves extract showed higher volume yield recovery, total chlorophyll content and DPPH scavenging activities (0.72±0.02 mL, 50.10±3.96 mg/L and 70% respectively) compared to the stem extract (0.60±0.1 mL, 39.70±4.73 mg/L and 25% respectively). It was also found that the cellulase enzyme concentrations were proportional to the volume yield recovery, and DPPH scavenging activity of the *C. nutans* extract over time. However, there was no significant effect of enzyme concentration on the total chlorophyll content. The results obtained can be considered very satisfactory and cellulase enzyme can be considered to be used in the aqueous extraction of *C. nutans* to aid the extraction process in order to increase the volume yield recovery, total chlorophyll content and DPPH scavenging activities.

1. Introduction

Fruit and vegetable extracts are produced using combinations of physical maceration and/or enzyme-assisted reaction of the fruit or vegetable to expel the extract, in some cases leaving large amount of insoluble waste pomace. Enzymes actually help in the extraction process to increase the volume as well as the quality (in terms of taste, colour, viscosity, and composition) of the juice.

The most common enzymes used in commercial extraction process are pectinase and cellulase. Pectinase is an enzyme that breaks down the cell walls and pectin. Pectin is the compounds found in plant cell walls that located in the plate of middle lamella. The benefits of using pectinase are to increase the volume of extract produced, lower the viscosity of the extract and reduce the cloudiness of the extract caused by suspended pieces of cell wall [1]. Primarily, pectinase is responsible for the degradation of the long and complex molecules called pectin that occur as structural polysaccharides in the middle
lamella and the primary call walls of young plant cells [2]. Cellulase enzyme likewise has the same function as pectinase to hydrolyze cellulose, pectin, and hemicellulose in certain natural product or plants [3].

Clinacanthus nutans Lindau (family Acanthaceae) or known as 'Sabah snake grass' or 'Belalai Gajah' has been traditionally used as important herbal medicine in Asia. In Thailand, dried leaves are accepted to cure creepy crawly, snake chomp and skin rashes [4]. C. nutans products are commonly being used as the replacement of topical acyclovir for the treatment of herpes simplex infection (HSV) and varicella-zoster infection (VZV) in many hospitals [5]. In Malaysia, this plant had been utilized customarily for its capability to cure several types of disease such as cancer and snake bites [6]. People in Indonesia use it to treat diabetes and looseness of the bowels [7]. Due to its benefits, this plant has gained a lot of interest and has been commercially exploited to produce a number of products, such as extract, juice, herbal drinks, tea and many more.

However, most of C. nutans products that are available in the market are normally extracted with solvent such as ethanol. Production of leaves extract by using enzyme is not common and it is a challenge to produce extract from green leaves because the water content in the leaves is low. There is limited literature available for the extraction of green leaves using water and enzyme. Therefore, this study focused to produce extract from C. nutans leaves and stems to increase the volume yield, total chlorophyll content and antioxidant activity by manipulating the use of commercial enzyme which is cellulase enzyme.

2. Methodology

2.1. Sample Preparation
The plant of C. nutans was bought from a local farmer at Kampung Wang Tepus, Jitra, Kedah. The fresh C. nutans was washed thoroughly under running tap water and separated into two parts; leaves and stems.

2.2. Cellulase Enzyme Treatment
The fresh plant material (150 g) was weighted and finely ground with 100 ml of distilled water using handheld stick mixer (Kenwood HB714M Tri Blade Hand Blender, UK) for approximately 5 min. The finely ground mashed sample (10 g) was mixed with different concentrations of Cellulclast® (cellulase enzyme) (Novozyme, Denmarks) [0.2, 0.4, 0.9, 4.3, and 8.5 filter paper unit (FPU)] in a universal bottle and then incubated in a waterbath at 50 °C for 180 min. At different time intervals, the sample was collected and immediately cooled in ice slurry to reduce the temperature and then the sample was mechanically extracted by using garlic press to yield the extract. The volume of the extract was measured and stored maximum up to 3 days at -7 °C until further analysis.

2.3. Determination of Total Chlorophyll Content
Accurately 1 mL of the samples were extracted with 4 mL of 80 % (v/v) acetone under dark condition for 15 minutes. Then, the mixture was centrifuged at 2500 rpm for 10 min. The absorbance of supernatant was measured at 655 and 649 nm using UV-VIS spectrophotometer (Helios- Zeta Thermo Scientific, United State). The total chlorophyll content was calculated (Eq. 1).

\[ \text{Total chlorophyll content (mg/L)} = 6.45(A_{655}) + 17.72(A_{649}) \]  
(Eq. 1)

where 6.45 and 17.72 are the factor for chlorophyll a and b respectively.

2.4. Determination of Antioxidant Activity
The antioxidant activity of C. nutans extract was assessed by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical assay as described by Barros et al. [8] with modification. The samples were reacted with the stable DPPH radical in an ethanol solution. Accurately, 0.1 mL of sample was added with 3.9
mL of 6 x 10^5 mol/L ethanol DPPH solution. The mixture was mixed thoroughly and incubated for 30 min in dark condition. Then, the absorbance was measured at 517 nm by using spectrophotometer (Helios- Zeta Thermo Scientific, United State). Scavenging activity (% SA) on DPPH radical was calculated using Eq. 2.

\[
% \text{SA} = \left( \frac{\text{Abs}_{\text{control}} - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{\text{Abs}_{\text{control}}} \right) \times 100
\]  \hspace{1cm} (Eq. 2)

2.5. Proximate Analysis

Proximate analysis was carried out only on the best extract of C. nutans. The purpose of proximate analysis was to determine the quality of the extract by analyzing the content of moisture, protein and ash according to the Official Methods of Analysis of the Association of the Analytical Chemistry [9].

2.5.1. Moisture Content. Moisture content of the samples were measured using moisture analyzer (MA 35M, Sartorius, United States). A material test chamber was used to dry the samples till constant weight. The percentage of moisture content was calculated as per Eq. 3:

\[
\% \text{Moisture} = \left( 1 - \frac{\text{Weight}_{\text{dry sample}}}{\text{Weight}_{\text{wet sample}}} \right) \times 100
\]  \hspace{1cm} (Eq.3)

2.5.2. pH. pH of the extracts was determined by using pH meter (Hanna Instruments, United States).

2.5.3. Ash Content. Ash content was measured following official methods of analysis of the association of the analytical chemistry [9]. The samples were incinerated in a furnace at 550 °C overnight. The remaining inorganic material was cooled, weighed and calculated (Eq. 4).

\[
\% \text{Ash Content} = \left( \frac{A-B}{C} \right) \times 100
\]  \hspace{1cm} (Eq.4)

where, A = weight of crucible with sample (g); B = weight of crucible with ash (g); C = weight of sample (g)

3. Results and Discussion

3.1. Effect of enzymatic treatment on volume yield of the extracts

Cellulase enzyme treatment shows a significant impact on the volume yield of C. nutans extracts (Figure 1). The volume yield was increased proportionally to the enzyme concentration over time in both leaves and stem. The volume yield was higher in leaves compared to the stem. The use of enzyme is a common practice in fruit and vegetable juice extraction. The enzyme’s role is to break down the cell wall and pectin and eventually increased the volume of the extract. This result was in agreement with the study conducted on the impact of commercial enzyme on white dragon fruit and goldenberry which showed that the use of enzyme was not only beneficial to increase the volume yield, but it also increased the quality of the extract by decreasing the viscosity, reducing the cloudiness and preventing gelatinization of the extract [10-11].

3.2. Effect of enzymatic treatment on total chlorophyll content of the extract

In contrast to volume yield, total chlorophyll content was decreased significantly over time (Figure 2). However, no significant effect of the enzyme concentration on the total chlorophyll content in both leaves and stem. Total chlorophyll content was higher in the leaves (50.10 ± 3.96 mg/L of FW) compared to the stem (39.7 ± 4.73 mg/L of FW). The results show that chlorophyll was very sensitive toward heat treatment. About 70% of the chlorophyll was degraded after 180 min of heat treatment at 50 °C. Study conducted on the kinetics degradation of chlorophyll in pandanus juice during pasteurization show the similar results. The chlorophyll was decreased with temperature at any given time [12].
Figure 1. Effect of cellulase enzyme treatment on volume yield of *C. nutans* extract in A) Leaves B) Stem at 50 °C, where □= control, ● = 0.22 FPU, ○=0.4 FPU, ▼=0.9 FPU, Δ=4.3 FPU, and ■=8.5 FPU. Data represent means of three replicates. Error bars indicate confidence interval at 95%.

Figure 2. Effect of cellulase enzyme treatment on total chlorophyll content of *C. nutans* extract in A) Leaves B) Stem, where □= control, ● = 0.22 FPU, ○=0.4 FPU, ▼=0.9 FPU, Δ=4.3 FPU, and ■=8.5 FPU. Data represent means of three replicates. Error bars indicate confidence interval at 95%.

3.3. *Effect of enzymatic treatment on antioxidant activity*
Cellulase enzyme treatment shows a significant impact on the antioxidant activity. Antioxidant activity was increased when enzyme concentration was increased over time (Figure 3). Interestingly, antioxidant activity of both extracts was inversely proportion to the total chlorophyll content. This phenomenon might be due to the accumulation of acylated phenolic compound resulted from degradation of compound during heat treatment [13].

The antioxidant activity in the leaves was significantly higher compared to the stem. The highest antioxidant activity in the leaves reached up to 70% whereas the maximum antioxidant activity in the stem was only about 25%. The use of cellulase enzyme helped in liberating more antioxidant from the leaves compared to the stem. Most of literature showed a positive correlation between total chlorophyll contents and the DPPH antioxidant activity which suggested that chlorophyll has the ability to inhibit DPPH [14] and exhibit high free radical-scavenging activity in most of the plant extract [15].

Figure 3. Effect of cellulase enzyme treatment antioxidant activity of C. nutans extract in A) Leaves and B) Stem, □= control, ● = 0.22 FPU, ○=0.4 FPU, ▼=0.9 FPU, Δ=4.3 FPU, and ■=8.5 FPU. Data represent means of three replicates. Error bars indicate confidence interval at 95%.

3.4. Proximate analysis of the best extracts of C. nutans

Cellulase enzyme treatment showed a significant effect on the volume yield and antioxidant activity but there is no significant effect on enzyme concentration on total chlorophyll content. From the results, it can be concluded that cellulase enzyme at concentration of 8.5 FPU and incubated for 180 min is the best condition to extract C. nutans leaves. Therefore, this extract was further analyzed for the proximate analysis. Proximate analysis is one of the methods to evaluate the quantitative quality of food. The results show that there is no significant effect of the C. nutans extract treated with 8.5 FPU cellulase enzyme at 50 °C for 180 min compared to untreated C. nutans extract (Table 1). It can be concluded that although cellulase enzyme treatment increased the volume yield and antioxidant activity of the extract, it does not affect the quantitative quality of the extract measured by proximate analysis.

4. Conclusion

The use of enzyme is a common practice in fruit and vegetable extractions to enhance the quality of the extract by increasing the volume recovery, decreasing the cloudiness and lowering the viscosity. The enzyme works by hydrolyzing the cellulose, pectin and hemicellulose in the plant tissue samples. In this
Table 1. Proximate analysis of the best C. nutans extracts.

| Sample                                      | pH       | Moisture (%w/w) | Ash (%w/w) |
|---------------------------------------------|----------|-----------------|------------|
| Untreated                                   | 7.16 ± 0.05ª | 93.18 ± 0.60ª  | 28.63 ± 0.28ª |
| Treated with 8.5 FPU cellulase enzyme at 50 °C for 180 min | 7.30 ± 0.10ª | 93.00 ± 0.35ª  | 28.96 ± 0.40ª |

Data are means ± standard error (N=3). Values that are followed by different letters within each column are significantly different (P<0.05) using Student’s Paired T-Test.

study, it can be concluded that the cellulase enzyme has shown a significant effect on the volume yield and antioxidant activity. However, there is no significant effect on the enzyme concentration on total chlorophyll content. The volume yield and antioxidant activity were increased in proportion to the enzyme concentration over time in both leaves and stem and it were higher in leaves compared to the stem. In contrast to volume yield and antioxidant activity, total chlorophyll content was decreased significantly over time. Enzymatic treatment is one of the promising aids to be considered in fresh green leaves extraction to enhance the quality of the extract. Optimization study should be conducted to determine the enzyme concentration and other related parameters to maximize the extraction process of C. nutans.

References
[1] Pasha KM, Anuradha P and Subbarao 2013 J. Pure Appl. Sci. Technol. 16: 89-95.
[2] Kashyap DR, Vohra PK, Chopra S and Tewari R 2001 Bioresour. Technol. 77(3): 215-227.
[3] Wilkins MR, Widmer WW, Grohmann K and Cameron RG 2007 Bioresour. Technol. 98(8): 1596-1601.
[4] Sakdarat S, Shuprom A, Ayudhya TDN, Waterman PG and Karagianis G 2006 Thai J.
[5] Sangkitporn S, Polihan K, Thawatsupa P, Bunchok M, and Chawalithumrong P 1993 J. Med. Assoc. Thai 18: 226-231.
[6] Roosita K, Kusharto CM, Sekiyama M, Fachrurozi Y and Ohtsuka R 2008 J. Ethnopharmacol.
[7] Hariana HA 2005 Niaga Swadaya. 412pp
[8] Burros L, Carvalho AM and Ferreira ICFR 2010 Food Chem Toxicol. 48:1466–1472
[9] AOAC 1998 16th Edition, 4th Revision: AOAC International.
[10] Nur-Alia AR, Siti Mazlina MK, and Taip FS 2010 IEM Journal 71(4): 25-31.
[11] Ramadan MF and Moersel JT 2007 J. Sci. Food Agr. 87: 452-460.
[12] Punchira V, Montira N, Karan S, Phatcharanee T and Saowluck W 2010 AJOFAI 3(01): 44-51.
[13] Padayachee A, Netz G, Netzel M, Day L, Zabaras D, Mikkelsen D and Gidley MJ 2012 Food Chem. 134(1): 155-161.
[14] Ferruzzi MG, Böhm V, Courtney PD and Schwartz SJ 2002 J. Food Sci. 67(7): 2589–2595. 115(2): 72-81. Phytochemistry 13(2): 13-24.
[15] Silva-Beltran NP, Ruiz-Cruz S, Cira-Chavez LA, Estrada-Alvarado MI, Ornelas-Paz JJ, Lopez-Mata MA, Lizette D, Ayala-Zavala JF and Marquez-Rios E 2015 Int. J. Anal. Chem. Article ID 284071

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