Application of crude natural enzymes for extraction of Wali seed [Brucea javanica (L) Merr]

H Muliasari*, A D Ananto, R F Deccati, D Almira and Solahuddin
Department of Pharmacy, Faculty of Medicine, University of Mataram, Mataram, West Nusa Tenggara, Indonesia

Corresponding author: handamuliasari@unram.ac.id

Abstract. Brucea javanica (L) Merr is a medicinally important plant, commonly known as Wali. The extract of its seed has various secondary metabolites responsible for its pharmacological properties. Unfortunately, the recent method of extraction lacks in the percentage of the yield and has disadvantages to the environment. Enzymatic extraction of bioactive compounds is a potential alternative. In this research, we use some crude natural enzymes that are amylase, protease (bromelain), and cellulase isolated from corn kernels, pineapple fruit, and beef rumen fluid, respectively. The aim of this research was to determine the effectiveness of crude natural enzymes for the extraction of metabolite compounds in wali seed comparing with the solvent extraction method. For each of one gram of wali seed powder was extracted by using ultrasonic-assisted extraction (UAE) method with solvent of (1) methanol; (2) ethanol 96%; (3) bromelain; (4) amylase; (5) cellulase; (6) bromelain+amylase; (7) bromelain+cellulase; (8) amylase+cellulase; (9) amylase+bromelain+cellulase. The extracts of (1) and (2) were then evaporated; while extracts of (3) to (9) were freeze-dried. The appearance of extracts (1) and (2) were bright-yellow viscous liquid; while extracts (3) to (9) were solid-milky powder. Cellulase increases the extraction yield up to 40% and 22% in comparison with methanol and ethanol, respectively. The semiquantitative analysis showed that methanol extracts the metabolites with the highest concentration but has the lowest amount of yield extract. However, cellulase showed the highest ability to extract metabolites tested. The flavonoid content extracted using cellulase enzymes resulted in an increase of 273% and 170% in comparison with methanol and ethanol, respectively. Thus, the use of crude cellulase enzyme isolated from beef rumen fluid is the best choice to extract wali seed among other enzymes and solvents used.

1. Introduction
Wali seed [Brucea javanica (L) Merr] has been widely used in Sesaot District, Indonesia to treat diabetes mellitus [1]. It is also used in Chinese medicine and is known to have cold character and bitter taste so that it has various pharmacological activities such as antipyretic and detoxifying properties. In addition, wali seed is used to treat lung, prostate and intestinal cancer, and has the potential as an antimalarial, anti-inflammatory, and antiviral agent with low toxicity [2]. The ethanol extract of wali seed is reported to have antidiabetic activity in vivo and is able to repair the pancreas damage [3,4]. Thus, wali seed extract is very valuable to be developed as medicine.

The challenges in drug development of wali seed extract including the extraction of bioactive components and bioactivity screening processes [5,6]. The current extraction process still uses conventional methods such as maceration and Soxhletation which are less effective and use large
amounts of organic solvents which are also harmful to the environment. Therefore, it is necessary to develop a more effective and safer extraction method. The extraction process with high yields to be carried out in this research is the enzyme-assisted extraction technique [7,8]. Enzymatic extraction in this study used some hydrolase enzymes such as cellulase, amylase, and protease (bromelain) enzymes isolated from natural ingredients, which are beef rumen fluid, corn kernels, and pineapple fruit, respectively.

Enzymes have various advantages over conventional extraction with organic solvents, such as they are cheaper, not toxic, and able to increase the yield extract. Extraction with enzymes can increase the yield extract because enzymes work to hydrolyze and damage cell walls of plant material so that all secondary metabolite components are extracted out of the cell [8]. Here we reported the effectivity of crude enzymes isolated from natural sources that were used in the extraction of bioactive compounds in wali seed comparing with organic solvents.

2. Materials and Method

2.1. Plant materials
Wali fruits were collected from Sesaot Forest, Sesaot District, West Lombok, West Nusa Tenggara Province, Indonesia. The seeds of Wali fruits were separated and then air-dried and powdered.

2.2. Preparation of crude natural enzymes
The crude of cellulase enzyme was isolated from beef rumen fluid following the method of [9] with modification. Isolation and simple purification of amylase enzyme from corn kernels were conducted following the method of [10] and [11]. Protease (bromelain) was isolated and purified from pineapple fruit by using the procedure described by [12] and [13].

2.3. Extraction process
The three crude enzymes were diluted to concentration of 27.7% (w/v) for extraction of wali seed. Then, for each of 1 gram of wali seed powder was added by 10 ml of different solvents as follows: (i) methanol; (ii) ethanol 96%; (iii) bromelain; (iv) amylase; (v) cellulase; (vi) bromelain + amylase; (vii) bromelain + cellulase; (viii) amylase + cellulase; (ix) bromelain + cellulase + amylase. The extraction of each was conducted with ultrasonic extraction for 20 minutes and then filtered to obtain the extract. Extract (i) and (ii) were then evaporated, while extract (iii)-(ix) were freeze dried.

2.4. Identification of secondary metabolites extracted
Secondary metabolites extracted from wali seed were identified by using Thin Layer Chromatography (TLC). An amount of 0.1 g of each extract was diluted with 10 ml of methanol and then 20 µL of them was spotted into the TLC plate. Identification of flavonoid, alkaloid, and tannin were conducted to all extracts by using appropriate solvents.

2.5. Semiquantitative analysis of secondary metabolites extracted
Secondary metabolites extracted from Wali seed based on TLC were semiquantitatively analyzed by using ImageJ software [14].

3. Results and Discussion

3.1. Crude natural enzymes
Crude enzymes that have been isolated from natural ingredients are listed in Table 1. Three types of hydrolase enzymes, namely amylase, protease (bromelain), and cellulase were extracted from corn kernels, pineapple fruit, and beef rumen fluid, respectively. The crude of cellulase and bromelain enzymes were obtained with higher yields than amylase, which are 7.97% (w/v) and 7.30% (w/w). The color of the crude enzymes produced still retains the color of the raw materials.
3.2. Extraction of wali seed by using organic solvents and crude natural enzymes

The differences in the characteristics of wali seed extracts extracted with organic solvents and crude natural enzymes are shown in Table 2. Wali seed extracts produced by using organic solvents i.e. methanol and ethanol, were a bright yellow viscous liquid; while extraction by using crude enzymes produces wali seed extract in the form of milky white powder, which has a certain color mixture depends on the type of enzyme used to extract. What is interesting here is that the use of crude natural enzymes, either single or mixed enzymes, can produce a higher yield percentage of extract than organic solvents. Cellulase increased the extraction yield up to 39.51% and 21.56% in comparison with methanol and ethanol, respectively. The mixture of bromelain and cellulase enzymes produced the highest yield with an increase of 57.03% and 39.08% compared with methanol and ethanol, respectively.

These results indicated that not only the pure commercial enzymes [15], but also the crude natural enzymes isolated by a simple method can be used to extract wali seed with a higher percentage of yield extract compared to extraction by using organic solvents. Metabolites in plants are generally protected by lignin, cellulose, pectin, and the other cell wall components. The enzymes used in extraction act on hydrolyze the cell wall components and increase the permeability of the cell wall, causes metabolite compounds to leave the cell, thus resulting in a higher yield of the metabolites. This action is in contrast to the extraction mechanism with organic solvents which work by penetrating into the cell and then pulling metabolite compounds out of the cell. Apart from producing a higher yield percentage, extraction with crude natural enzymes are also nontoxic, cheaper, and less harmful to the environment [16].

| Crude Enzymes | Source          | % Yield (w/w) | Color         |
|---------------|----------------|--------------|---------------|
| Amylase       | Corn kernels   | 4.75%        | Light orange  |
| Protease      | Pineapple fruit| 7.30%        | Light yellow  |
| Cellulase     | Beef rumen fluid| 7.97%       | Dark green    |

Table 2. Characteristics of wali seed extracts extracted with organic solvents and crude natural enzymes.

| Solvent used          | % Yield extract (g dry extract/1 g dry simplicia) | Form of extract | Color of extract | Odor of extract         |
|-----------------------|-----------------------------------------------|-----------------|------------------|-------------------------|
| Methanol (MeOH)       | 10.65%                                       | Liquid          | Bright yellow    | Wet wood odor          |
| Ethanol 96% (EtOH)    | 28.60%                                       | Liquid          | Bright yellow    | Wet wood odor          |
| Bromelain             | 50.45%                                       | Powder          | Milky white      | Soy milk odor          |
| Amylase               | 42.77%                                       | Powder          | Milky white      | Corn powder odor       |
| Cellulase             | 50.16%                                       | Powder          | Cream-Dark green | Pungent acid odor      |
| Bromelain+Amylase     | 42.98%                                       | Powder          | Cream-white      | Milk odor              |
| Bromelain+Cellulase   | 67.68%                                       | Powder          | Cream-Dark green | Acid odor              |
| Amylase + Cellulase   | 58.72%                                       | Powder          | Cream-Dark green | Acid odor              |
| Bromelain+Cellulase + Amylase | 58.06% | Powder | Light brown | Milk-acid odor |

3.3. Secondary metabolites content in the extracts based on semiquantitative analysis

Observation result of secondary metabolites content in wali seed extracts by using TLC followed by imageJ analysis includes the content of tannin, alkaloid, and flavonoid as shown in Figure 1 and Table 3. These three metabolites observed is based on the content of compounds from phytochemical screening results in methanol and ethanol extracts of wali seed [17,3]. The tannin compounds were shown with lavender spots on TLC at visible light after eluted with eluent of methanol-ethylacetate (7:3) and sprayed with 2% ferric chloride solution and 2% sodium carbonate solution. The alkaloid compounds were shown with a green color at visible light after eluted with eluent of methanol-ethylacetate (7:3) and sprayed with one percent ninhydrin solution. The flavonoid compounds were shown with a blue color at visible light after eluted with eluent of methanol-ethylacetate (7:3) and sprayed with one percent aluminum chloride solution.
with FeCl$_3$ 5% (Figure 1a) [18]. The spots visualized under UV light of 254 nm (Figure 1c) are presumed to be alkaloid compounds that appear after eluted by eluent of ethyl acetate-methanol-water (16:1:2) and sprayed with Dragendorff reagent [19]. Flavonoid compounds are the blue-light spots in Figure 1e visualized under UV light of 366 nm. Identification of flavonoid used TLC eluted by eluent of chloroform-ethyl acetate-buthanol (5:4:1) and then sprayed with AlCl$_3$ 10%. The plots analysis in Figure 1b, 1d, and 1f showed the same pattern that peak number 1 (methanol extract) was the peak with the largest area, so that all data were normalized to peak 1 which means that the quantification of the compounds based on spot visualization on TLC is relative to methanol extract.

![TLC and ImageJ plot analysis of wali seed extracts.](image)

**Figure 1.** TLC and ImageJ plot analysis of wali seed extracts. (a),(c),(e): TLC visualized of tannin, alkaloid, and flavonoid, respectively. (b),(d),(f): ImageJ plots of tannin, alkaloid, and flavonoid, respectively. Spot number (0) Quercetin as standard; spots and peaks number (1)-(9): wali seed extracts using solvent of (1) methanol; (2) ethanol; (3) Bromelain; (4) Amylase; (5) Cellulase; (6) Bromelain+Amylase; (7) Bromelain+Cellulase; (8) Amylase + Cellulase; (9) Bromelain+Cellulase+ Amylase.

The result of ImageJ semiquantitative analysis (Table 3) showed that methanol can extract the compounds (tannin, alkaloid, flavonoid) with the highest concentration, although the amount of extract yield is the least. Ethanol extracts lower metabolites than methanol and some enzymes. Among the
various enzymes used, cellulase showed the highest ability to extract metabolites. In the quantification of flavonoid content (mg/gram extract) compared to quercetin standard, methanol produced the highest levels of flavonoids, followed by cellulase and ethanol. However, flavonoid content in the total extract after being multiplied by the amount of extract, cellulase produces the highest flavonoid content followed by bromelain+cellulase, amylase+cellulase, and bromelain+amylase+cellulase, as shown in Figure 2. Thus, cellulase is the best choice for extraction among other enzymes and organic solvent used.

Table 3. Semiquantitative analysis results of imageJ software.

| Spot number | Solvent used         | Tannin  | *Percent area ±STD | Flavonoid (mg/0.1 gram of extract) |
|-------------|----------------------|---------|--------------------|-------------------------------------|
| 1           | Methanol (MeOH)      | 100 ± 0 | 100 ± 0            | 100 ± 0                             | 9.793557                            |
| 2           | Ethanol 96% (EtOH)   | 31.79106 ± 1.1775 | 51.33546 ± 2.200367 | 59.80497 ± 0.475672 | 5.855 |
| 3           | Bromelain            | 69.90435 ± 1.6619 | 37.28383 ± 1.480884 | 26.5933 ± 1.980079 | 2.608308 |
| 4           | Amylase              | 42.78569 ± 1.3635 | 52.78299 ± 2.360555 | 29.7422 ± 1.703881 | 2.922198 |
| 5           | Cellulase            | 84.97608 ± 2.2022 | 63.18103 ± 2.671867 | 79.7242 ± 4.791589 | 7.761983 |
| 6           | Bromelain+Amylase    | 71.11754 ± 2.2022 | 38.11174 ± 2.671867 | 31.0629 ± 4.791589 | 3.090995 |
| 7           | Bromelain+Cellulase  | 77.19757 ± 0.8379 | 68.85071 ± 1.481638 | 40.6906 ± 5.4851 | 4.113613 |
| 8           | Amylase+Cellulase    | 49.80249 ± 0.9279 | 37.10488 ± 3.012052 | 39.1164 ± 15.9272 | 3.78998 |
| 9           | Bromelain+Cellulase  | 58.39326 ± 1.4953 | 60.38267 ± 1.38834 | 32.78623 ± 4.231368 | 3.214064 |
|             | + Amylase            | 1.9373   | 1.329471           | 0.386571                            | 0.386571                            |

*These data were normalized to methanol extract

**These data are based on plot quantifications comparing with quercetin standard

Many other studies have demonstrated that the use of carbohydrate enzymes increases the amount of phenolic compounds extracted as well as antioxidant activity in comparison with the solvent extraction method. Yazdi et al [20] reported that pure commercial cellulase increases the phenolic compounds extracted up to 60%. The combination of cellulase, pectinase, and tannase enzymes under their optimal conditions increased the extraction yield up to 112% and the antioxidant property of the enzymatic extracted compounds was 71% more than the control extract. Compared with those commercial enzymes, the crude cellulase enzyme in this research increased the extraction yield up to 40% and 22% in comparison with methanol and ethanol, respectively. Semiquantitative analysis of flavonoid content extracted using cellulase enzymes resulted in an increase of flavonoids reaching 273% and 170% in comparison with methanol and ethanol, respectively. The increase in yield extract and the amount of extracted metabolites because beef rumen fluid contains not only cellulase enzymes, but also several other carbohydrate enzymes such as -1,4-D-endoglucanase, -1,4-D-exoglucanase, protease, amylase, xylanase, pectinase, and some polysaccharide-degrading enzymes [21,22]. The enzymes contained in the beef rumen fluid work to hydrolyze the ether and/or ester bonds in polysaccharides that compose plant cell walls so that it increases the extraction efficiency of metabolite compounds [16].
4. Conclusion
The use of crude cellulase enzyme isolated from beef rumen fluid is the best choice to extract wali seed among other enzymes and solvents used. Cellulase increases the extraction yield up to 39.51% and 21.56% in comparison with methanol and ethanol, respectively. Methanol extracts the metabolites with the highest concentration but the lowest amount of extract. The semiquantitative analysis showed that cellulase showed the highest ability to extract metabolites tested. The flavonoid content extracted using cellulase enzymes resulted in an increase of 273% and 170% in comparison with methanol and ethanol, respectively.

5. Acknowledgments
We would like to thank University of Mataram for funding this research.

References
[1] Hamdin, C.D., Muliasari, H., dan Ananto, A., 2016. Clinical Condition Changes on Patients of Diabetes Mellitus at Sesao Village After Treatment Using Makasar Fruit Seeds (Brucea Javanica L. Merr) Parameters: Blood Glucose, Blood Pressure, Heart Rate and Respiratory rate. ICST 2016 : The 1st International Conference on Science and Technology. 97.
[2] Chen, M., R. Chen, S. Wang, W. Tan, Y. Hu, X. Peng, Y. Wang. 2013. Chemical components, pharmacological properties, and nanoparticulate delivery systems of Brucea javanica. International journal of nanomedicine. 2013:8, 85-92.
[3] Muliasari, H., C.D.Hamdin, A.D. Ananto, M. Ihsan. 2017a. Hypoglycemic Effect of Brucea javanica (L) Merr Leaves and Seed Extract in Alloxan-induced Diabetic Rats. Prosiding The 2nd International Conference on Science and Technology 2017, 62-67. ISBN 978-602-61265-1.
[4] Muliasari, H., C.D.Hamdin, A.D. Ananto, M. Ihsan. 2017b. Histologi Pankreas Tikus Diabetes Setelah Pemberian Suspensi Biji Buah Makasar (Brucea javanica (L.) Merr). Jurnal BioWallacea Vol 3 No 3 Sept 2017, hal. 115-118. ISSN 2442-2622.
[5] Ma, H., Bai, Y., Li, J., Chang, Y. 2018. Review: Screening bioactive compounds from natural product and its preparations using capillary electrophoresis. Electrophoresis, 39, 260–274.
[6] Khan, R.A. 2018. Natural products chemistry: The emerging trends and prospective goals. Saudi Pharmaceutical Journal 26 (2018) 739–753. https://doi.org/10.1016/j.jsp.2018.02.015.
[7] Shannon, E. and Abu-Ghannam, N. 2018. Enzymatic Extraction of Fucoxanthin from Brown Seaweeds. International Journal of Food Science & Technology. doi:10.1111/ijfs.13808.
[8] Nadar, S.S., Rao, P., Rathod, V. K. 2018. Enzyme assisted extraction of biomolecules as an approach to novel extraction technology: A review. Food Research International 108 (2018) 309–
330. https://doi.org/10.1016/j.foodres.2018.03.006.

[9] Setyoko, H., & Utami, B. (2016). Seminar Nasional XIII Pendidikan Biologi FKIP UNS 863 Isolasi dan Karakterisasi Enzim Selulase Cairan Rumen Sapi untuk Hidrolisis Biomassa Isolasi and Characterization of Cellulase Enzymes Cow’s Liquid Rumen for Biomass Hydrolysis. 13(1), 863–867.

[10] Wahjuni, S., Suarya, P., Saputra, I. M. A. (2017). Isolasi Enzim Amilase Dari Kecambah Biji Jagung Lokal Seraya (Zea mays L.) Untuk Hidrolisis Pati. Issn 1907-9850. JURNAL KIMIA, 11(2), 122–128.

[11] Bahri, S., Mirzan, M., & Hasan, M. (2012). Karakterisasi Enzim Amilase Dari Kecambah Biji Jagung Ketan (Zea mays ceratina L.). Jurnal Natural Science, 1(1), 132–143.

[12] Mohan, R., Sivakumar, V., Rangasamy, T. & Muralidharan, C., 2016. Optimisation of Bromelain Enzyme Extraction from Pineapple (Ananas comosus) and Application in Process Industry. American Journal of Biochemistry and Biotechnology, 12, pp.188-95.

[13] Nurhidayah, N., Masriany, M., & Masri, M. (2013). Isolasi dan Pengukuran Aktivitas Enzim Bromelin dari Ekstrak Kasar Batang Nanas (Ananas comosus) Berdasarkan Variasi pH. Biogenesis: Jurnal Ilmiah Biologi, 1(2), 116–122. https://doi.org/10.24252/bio.v1i2.457

[14] https://imagej.nih.gov/ij/download.html

[15] Puri, M., Sharma, D., & Barrow, C. J. (2012). Enzyme-assisted extraction of bioactives from plants. Trends in Biotechnology, 30(1), 37–44. https://doi.org/10.1016/j.tibtech.2011.06.014

[16] Cheng, X., Bi, L., Zhao, Z., & Chen, Y. (2015). Advances in Enzyme Assisted Extraction of Natural Products. IC3ME, 371–375. https://doi.org/10.2991/ic3me-15.2015.72

[17] Risnadewi, W. N., Muliasari, H., Hamdin, C. D., & Andayani, Y. (2019). Comparative antioxidant activity of Brucea javanica (L) Merr seed extract derived from maceration and soxhletation method. AIP Conference Proceedings, 2199(December). https://doi.org/10.1063/1.5141312

[18] Sriwahyuni, I. (2010). Uji fitokimia ekstrak tanaman anting-anting (Acalypha indica Linn) dengan variasi pelarut dan uji toksisitas menggunakan Brine Shrimp (Artemia salina Leach). Skripsi. Malang: Jurusan Kimia Fakultas Saintek Universitas Islam Maulana Malik Ibrahim Malang.

[19] Marlina, S. D., Suryanti, V. dan Suyono. (2005). Skrining fitokimia dan analisis kromatografi lapis tipis komponen kimia buah labu siam (Sechium edule Jacq. Swartz.) dalam ekstrak etanol. Biofarmasi. 3 (1), 26 – 31.

[20] Ghandahari Yazdi, A. P., Barzegar, M., Sahari, M. A., & Ahmadi Gavlighi, H. (2019). Optimization of the enzyme-assisted aqueous extraction of phenolic compounds from pistachio green hull. Food Science and Nutrition, 7(1), 356–366. https://doi.org/10.1002/fsn3.900

[21] Lee, S. S., C. H. Kim, J. K. Ha, Y. H. Moon, N. J. Choi, & K. J. Cheng. 2002. Distribution and activities of hydrolytic enzymes in the rumen compartemenes of Hereford bulls fed alfalfa based diet. Asian-Australas. J. Anim. Sci. 15:1725-1731.

[22] Morgavi, D. P., K. A. Bauchemin, V. L. Nsereko, L. M. Rode, A. D. Iwaasa, W. Z. Yang, T. A. McAlister, & Y. Wang. 2000. Synergy between ruminal fi brolytic enzymes and enzymes from Trichoderma longibrachiatum. J. Dairy Sci. 83:1310-1321.