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Original Article

CALCIUM CARBONATE ISOLATION FROM EGGSHELL TO MEET PHARMACOPOEIAL STANDARDS AND ITS EFFECTIVENESS AS AN ANTACIDS

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ABSTRACT

Objective: Calcium carbonate is widely used in the pharmaceutical field as excipients and therapeutic agents. Calcium carbonate can be obtained from limestone, chalk, marble and dolomite. Other alternative is from eggshell. Calcium carbonate source from eggshell has several advantages including higher calcium carbonate content, fewer contaminants metal limit, and more brittle. Therefore, in this study, calcium carbonate had been isolated from eggshells which was expected to meet the requirements of Indonesian Pharmacopoeia (sixth edition) and having activity as antacid.

Methods: Calcium carbonate were isolated from eggshells by mechanically and physically organic separation. The quality of calcium carbonate was examined according to the Indonesian Pharmacopoeia parameters including loss on drying; acid-insoluble substance, magnesium and alkali salt; limit of arsenic, lead, iron, mercury, heavy metal, and barium. Additional physicochemical characterization of calcium carbonate including particle size analyzer, FTIR and XRD were compared with those of commercial calcium carbonate.

Results: The results showed that the isolation produced 98.5±0.5 % of calcium carbonate. The calcium carbonate powder had an average size of 21±1.0 µm, while that of commercial was 8±1.3 µm. The resulted calcium carbonate revealed similar XRD patterns compared with that from commercial Calcium carbonate from the market. Based on database from FTIR instrument, the calcium carbonate sample had 99% similarity level compared with that from the reference. The sample of Calcium carbonate isolated from the eggshell (>mesh 100) had lower antacid activity (23.83 mEq) than that of commercial (24.56 mEq).

Conclusion: Calcium carbonate from eggshell fulfilled the requirements of Indonesian Pharmacopoeia.

Keywords: Eggshell, Calcium carbonate, Pharmaceutical grade, Antacid

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INTRODUCTION

According to FAO data, global egg production increased by 49% from 2000 (55 million metric tons) to 82 million metric tons in 2019 [1]. This significant increase in production is almost certainly accompanied by an increase in egg waste, specifically eggshells. Eggshell waste, on the other hand, can be used to make calcium-fortified foods and in other industries [2]. Eggshells have been used as animal feed [3], soil amendment [4], calcium bioavailability and bio-accessibility [5], renewable catalyst [6] and human consumption in a variety of food applications [2] in several studies.

The main component of eggshell is calcium carbonate (94%). Other reported components include magnesium carbonate (1%), calcium phosphate (1%), and organic compounds (4%), which include collagen, sulfated polysaccharides, and other proteins [7, 8]. Calcium carbonate is widely used in the pharmaceutical industry as excipients such as buffering agents [9], tablet diluent as a co-process [10, 11], cross-linking agent [12] and therapeutic agents such as calcium supplements [13], antacid [14-16] as well as treating high phosphate levels [17]. However, the use of calcium carbonate derived from eggshells in the pharmaceutical industry has received little attention.

Gaonkar *et al.* conducted research on the use of eggshells in the pharmaceutical field, among other things. In the study, eggshells were used as calcium supplement tablets in women. Eggshells were powdered and formed into tablets before being tested for dissolution in 0.1 N. HCl medium [18]. However, no attempt was made in this study to obtain calcium carbonate powder with pharmaceutical-grade specifications from eggshells. Another study found that chicken eggshell powder was more effective than inorganic calcium carbonate in reducing bone loss. The experiment was carried out on ovariectomized mice [19].

The physicochemical properties of calcium carbonate from eggshells have been extensively studied, including eggshell morphology using SEM and TEM, x-ray diffraction, FTIR, thermogravimetric analysis, specific surface area [7, 20-22]. However, the characterization of calcium carbonate from eggshells has yet to be completed, particularly in accordance with pharmacopoeia requirements in order to obtain pharmaceutical-grade calcium carbonate. The purpose of this research is to obtain calcium carbonate from egg shells, characterize it to meet pharmacopoeia requirements, and test its activity as an antacid.

MATERIALS AND METHODS

Materials

Eggshells (*Gallus gallus* domestic us) were obtained from a food processing plant (Amanda Brownies, Jawa Barat Indonesia). The calcium carbonate Frac Depot LLC was used as a reference.

Cleaning and separation of eggshell organic compound

Eggshells were cleaned of mucus and sterilized with steam before being mechanically separated from the membrane. The eggshell was cleaned with running water, then dried in the oven (40 °C, 24 h) after three rinses with distilled water. Dry eggshells (5 kg) were sterilized in an autoclave (121 °C, 15 min) [23] and then dried in an oven (40 °C, 24 h) to form sample 1 (S_1). To separate the membrane from the eggshell, the eggshell was pounded inside the mortar and sieved (mesh 10) (the membrane retained in the sieve while the eggshell were pass through the sieve). The powdered eggshells that had been separated from its membrane was named as Sample 2 (S_2) [7].

Eggshell size reduction

S_2 was ground into eggshell powders using a grinding machine (Universal Crusher Kodi Machinery, WF-30B Type). Recovery and particle size distribution of the powders were studied. The ground eggshell powders which has been sieved with a mesh size of 43 µm was called as Sample 3 (S_3). Sample 4 (S_4) was the ground eggshell powders that had been passed through a 43 µm mesh sieve [7].

Particle size distribution

The particle size distribution of Sample 3 (S_3), Sample 4 (S_4), and commercial calcium carbonate (CCC) were studied using a laser diffraction particle size analyzer (Beckman Coulter LS I3 320 type) in a dry powder system. Calculations ranging from 0.375 m to 2000 m [24].

Eggshell powder purification from organic compounds

Sample S_4 (500 g) was boiled in water (3 L) for one hour. Substances that float to the top during the boiling process were discarded. The precipitated eggshell powder was dried in the oven (160 °C, 2 h) and named as Sample 5 (S_5) to which the purified eggshell powders [7].

Characterization purified powders

The purified powders were investigated for microbial and proximate content. TPC of powders before (S_4) and after purification (S_5) was determined using Indonesia National Standard method number SNI-2981:2009. Using Indonesia National Standard method number SNI-2897:2008, the proximate content of powders before (S_4) and after purification (S_5) were determined.

Calcium carbonate assay

Volumetric analysis were used to determine the calcium carbonate content in eggshell powders before (S_4) and after purification (S_5). About 200 mg of samples were transferred to a 250 ml beaker after being dried at 200 °C for 4 h and accurately weighed. After thoroughly moistening the powder with water, hydrochloric acid 3 N was added dropwise. The sample were titrated with 0.05 M disodium edetate to give a distinct blue color. Next, 100 ml of water was added, then 15 ml of 1 N sodium hydroxide, and 300 mg of hydroxy naphthol blue. Each ml of 0.05 M edetate disodium contains 5.004 mg of CaCO3 [23].

Loss on drying

Sample 5 (S_5) loses no more than 2.0% of its weight after drying at 200 °C for 4 h [23].

Acid insoluble substances

Sample 5 (S5) (5.0 g) was mixed with 10 ml of water, then hydrochloric acid were added dropwise with agitation until the bubling stopped, then added water to make the mixture 200 ml, and filtered. The insoluble residue was washed with water until no chloride was found, then ignited. The residue's weight should not exceed 10 mg (0.2%) [23].

Limit of heavy metal, magnesium and alkali salts

The ICP-OES (inductively coupled plasma optical emission spectrometry) method was used to determine the metal content limit in eggshell. Sample 5 (S_5) was weighed in a porcelain dish (approximately 1 gram) and dried over the stove. The sample was placed in the furnace at a heating rate of 100 °C every 30 min until it reached 600 °C and was kept for 3 h. The sample was taken out of the furnace and allowed to cool to room temperature. After cooling, samples were treated with 2 ml of 65% HNO_3 to dry and 2 ml of HCl 6 N to dry before being evaporated on the stove. The solution was cooled to room temperature before being transferred to a 50 ml flask and bound with aquabidestilata. The metal content limit in eggshell was determined using the ICP-OES method (Agilent Technologies 700 series ICP-OES) with the following process parameters: power (0.95 kW), plasma flow (15 L/min), auxiliary flow (1.5 L/min), nebulizer flow (55 L/min), viewing height (6 mm), and instrument stabilization (15 s) [25].

Eggshell powder physicochemical characterization

XRD (Shimadzu) was used to compare the crystallinity of purified powders (S_5) to commercial calcium carbonate (CCC). Fourier Transform Infrared Spectroscopy (Agilent Technologies Carry 630 FTIR) was used to compare whole eggshell powders (S_1), ground eggshell powders that passed through 43 m mesh (S4), and purified eggshell powders (S5). Scanning Electron Microscope was used to determine the morphology of eggshell (JEOL JSM 6360 LA) [20].

Acid neutralizing capacity

Sample: Eggshell powders (S_3) in various mesh sizes (<20; 20-40;40-60; 60-80; and>80) and commercial calcium carbonate samples. The samples were weighed and placed in a 250 ml beaker, to which 70 ml of water was added and stirred for 1 minute with a magnetic stirrer. A magnetic stirrer was used to continuously stir 30 ml of 1.0 N VS hydrochloric acid into the test solution. Following the addition of the acid, the mixture was stirred for exactly 15 min before being titrated. The excess hydrochloric acid was titrated with 0.5 N sodium hydroxide to achieve a stable pH of 3.5 (for 10 sec. to 15 sec.). The equation is used to calculate the amount of mEq of acid used. All tests shall be conducted at a temperature of 37±3° [23].

$$\text{Total mEq} = (30 \times N_{HCl}) - (V_{NaOH} x N_{NaOH})$$

N_{HCl} = normality of hydrocloric acid VS

V_{NaOH} = volume of sodium hydroxide used for titration

N_{NaOH} = normality of sodium hydroxide VS

RESULTS AND DISCUSSION

Organic compound separation and eggshell size reduction

Many microorganisms have been found in eggshells [26]. As a result, after being cleaned of mucus, eggshells were sterilized in an autoclave [23] to reduce the number of microorganisms. Another reason for autoclave sterilization was to make the separation process of the membrane from eggshells easier. The eggshell's cross-sectional morphology (fig. 1) revealed that the eggshell membrane was attached to the outer layer of the membrane. Membranes in eggshells are made up of organic compounds (4%) such as collagen, sulfated polysaccharides, and other proteins [7]. To isolate calcium carbonate from eggshells, the membrane must be separated. In this study, the membrane was mechanically separated using a sieve (mesh 10). The separation and particle size reduction process were carried out in three batches. The results (table 1) revealed that the process efficiency was 82.7%.

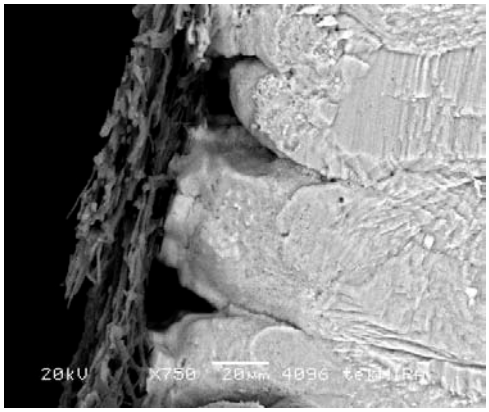

Fig. 1: SEM micrograph of eggshell (S_1) in cross-section

Table 1: Process efficiency

Batch no.	Yields	
	S_2 (%)	**S_3 (%)**
1	88.4	79.3
2	86.6	85.0
3	85.3	83.7
	86.8±1.5	82.7±3.0

S_2: separated eggshell, Yield = (weight of S_2/initial weight (5 Kg))x100, S_3: ground eggshell powders, yield = (weight of S_2/initial weight (5 Kg))x100

Particle size distribution

The particle size distribution of ground eggshell powders (S_3) and eggshell powders that passed through a mesh of 43 m (S_4) were determined using a laser diffraction size analyzer [24] and

compared to that of commercial calcium carbonate (CCC). The particle size distribution of S_4 was also determined to compare with commercial calcium carbonate, which requires 99.9% passes through the mesh 43 sieve according to The Frac Depot LLC's Certificate of Analysis. The results (table 2) revealed that the mean particle size of S_4 (21±1.0 m) was nearly three times larger than that of CCC (8±1.3 m). All experiments were conducted in triplicate. S_4 had one peak in the differential volume (fig. 2), whereas CCC had two peaks. Because the CCC requirement was 99.9% pass through 43 µm mesh, S_4 was selected for further processing.

Table 2: Particle size distribution by laser diffraction particle size analyzer

Parameter	Particle size (µm) of		
	S3	S4	CCC
Mean	212±10.6	21±1.0	8±1.3
d_{10}	<21±3.3	<2±0.4	<0.7±0.1
d_{25}	<73±11.2	<8±1.1	<1.2±0.1
d_{50}	<180±14.8	<18±1.4	<5.5±0.3
d_{75}	<317±13.5	<32±1.2	<11.2±1.2
d_{90}	<448±9.1	<47±1.1	<19.1±1.3

S3: ground eggshell powders (S_3), S4: ground eggshell powders that passed through 43 µm mesh (S_4), CCC: commercial calcium carbonate (CCC), *All experiments were conducted in triplicate

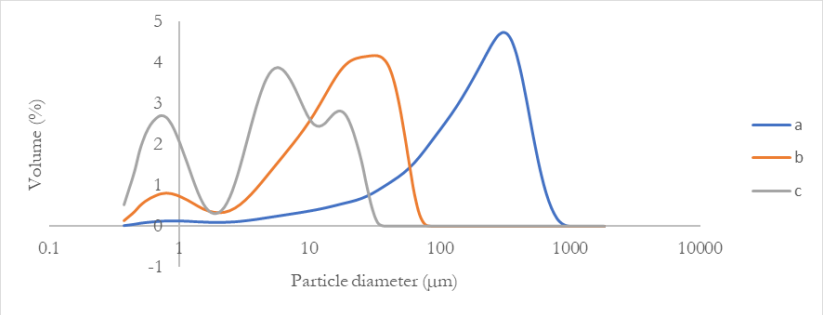

Fig. 2: **Differential volume of (a) ground eggshell powders, S3; (b) ground eggshell powders that passed through 43 µm mesh, S4 (c) commercial calcium carbonate, CCC**

Eggshell powder purification

The eggshell contains about 4% organic compounds such as collagen, sulfated polysaccharides, and other proteins [7]. Initially, the organic compounds in the membrane were mechanically separated from eggshells. The proximate levels of mechanically separated powders (S_4) were determined using the SNI method, and their similarity to calcium carbonate was determined using FTIR.

The results (table 3) revealed that the proximate levels of S_4 were approximately 1.78±0.2%, and the degree of similarity of S_4 with calcium carbonate from the database in FTIR equipment was 98% (table 4), whereas whole eggshell powders, S_1 (without membrane separation) was 96%. (table 4). S_4's microbial content has a Total Plate Count (TPC) of 2.5 x 10^5 cfu/g (table 3). There was no requirement for microbial content on calcium carbonate. TPC of S_4 was still relatively large when compared to the requirements of other excipients obtained from natural sources, such as acacia (TPC 10^4 cfu/g) and agar (TPC 10^3 cfu/g) [13]. As a result, purifying eggshell powders was necessary to reduce the amount of organic compounds which can cause microorganism contamination.

Table 3: Proximate and microbial content

No	Parameter	Result*	
		S_4	S_5
1.	Protein (%w/w)	1.70±0.19	1.50±0.34
2.	Fat (% w/w)	0.08±0.01	0.06±0.01
3.	Total Plate Count (cfu/g)	2.5 x 10^5	5,5 x 10^3

S_4: ground eggshell powders that passed through 43 µm mesh (before purification), S_5: purified eggshell powders (after purification), *: all experiment were conducted in triplicate

Physical purification was accomplished by boiling in water. During boiling process, organic compounds coagulated [27] and floated to the water's surface, whereas eggshell powders remain at the bottom. Organic compounds that floated were manually discarded. The precipitated powder was dried in a 2 hour oven at 160 °C. Another goal of this drying was to reduce the number of microorganisms [23, 28]. The proximate levels (table 3) of eggshell powders after purification (S_5) decreased by 12% from before purification (S_4). These findings suggested that organic compounds, particularly proteins, have coagulated and can be physically separated from eggshell powders. The total plate count (table 3) of eggshell powder after purification (S_5) was 25% lower than before purification (S_4). These findings indicated that the physical separation method of boiling followed by heating at 160 °C for 2 h can reduce microorganisms in eggshells and increase the degree of similarity of eggshell powders after purification (S_5) with calcium carbonate data base to 99% (table 4).

Table 4: Similarity of eggshell with $CaCO_3$ in library from FTIR spectrum

No	Sample	Similarity with library of $CaCO_3$ (%)
1.	S_1	96
2.	S_4	98
3.	S_5	99

S_1: whole eggshell powders, S_4: ground eggshell powders that passed through 43 µm mesh, S_5: purified eggshell powders

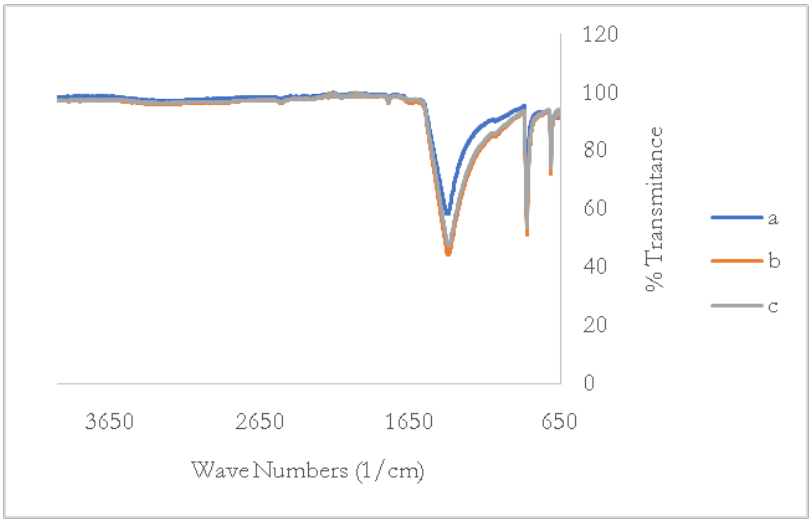

Fig. 3: FTIR spectrum of (a) whole eggshell powders, S_1; (b) ground eggshell powders that passed through 43 µm mesh, S_4 and (c) purified eggshell powders, S_5

Calcium carbonate assay

The calcium carbonate content was determined using complexometry titration methods according to the Indonesian Pharmacopoeia. $CaCO_3$ was determined using ethylenediamine tetraacetic acid (EDTA) as a complexing agent. The sample was dried at 200 °C for 4 h to remove any water content and crystalline water that may have been present. The sample was then dissolved in HCl and ionized into calcium and carbonate ions. When calcium ions react with EDTA, they form complex compounds. The results of the calcium carbonate determination (98.5±0.5%) (table 5) showed that the sample meets the requirements of the Indonesia Pharmacopoeia (98.0-100.5%).

Impurity

The impurity of calcium carbonate was determined using the Indonesian pharmacopoeia's loss on drying, acid-insoluble substance, and residual metal content limit parameters. The Indonesian Pharmacopoeia method was used to calculate the loss on drying and acid-insoluble substance. The results showed that the requirements for loss on drying and acid-insoluble substance were met (table 5). The ICP method was used to determine metal content residue because it is easier and faster than traditional methods such as Atomic Absorption Spectroscopy [25]. The results showed that the metal content limit in $CaCO_3$ from eggshell met the requirements (table 5).

Table 5: Parameter test results based on Indonesian pharmacopeia requirements

No	Parameter	Result*	Requirements
1.	Assay (%)	98.5±0.5	98.0–100.5
2.	Loss on drying (%)	0.8±0.1	≤2.0
3.	Acid-insoluble substance (%)	0.09±0.01	≤0.2
4.	Limit of arsenic (ppm)	0.001	≤ 3
5.	Barium	Negative	negative
6.	Limit of lead (ppm)	0.7	≤ 3
7.	Limit of iron (%)	0.004	≤ 0.1
8.	Limit of mercury (ppm)	0.001	≤ 0.5
9.	Limit of heavy metal (ppm)	≤ 20	≤ 20
10.	Magnesium and alkali salt (%)	0.3	≤ 1.0
11.	Total plate colony (cfu/g)	5.5 x 10^3	NA
12.	Organic residue (protein and fat) (%)	1.56	NA

*:all experiments were conducted in triplicate

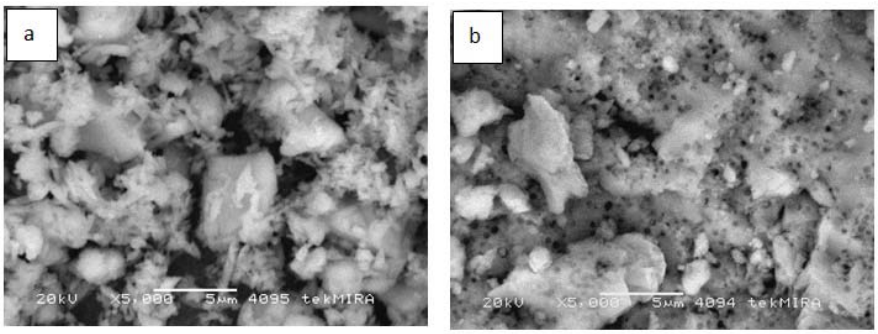

Fig. 4: SEM micrographs of (a) commercial calcium carbonate at 5000x magnification and (b) grinded eggshell powders at 43 m mesh (S5) at 5000x magnification

Eggshell powder morphology

The morphology of eggshell powders was studied using a scanning electron microscope (SEM). The results showed that eggshell powders (fig. 4b) were more porous than commercial calcium carbonate (fig. 4a). The porosity of eggshell powders could be due to voids caused by dissolved organic compounds that were removed and discarded during purification.

Eggshell powder crystallinity

The XRD method was used to examine the crystallinity [29-31] of purified eggshell powders (S_5) and compare it to that of commercial calcium carbonate. Compared to commercial calcium carbonate, S_5 had a similar XRD pattern and intensity (fig. 5b) (fig. 5a).

Acid neutralizing capacity

The neutralizing capability of an antacid determines its efficacy [32] The acid neutralizing capacity of eggshell powder was determined using official methods from the Indonesian Pharmacopoeia [23] and compared to commercial calcium carbonate. As shown in the fig. 6, the particle size of the eggshell powder had a significant effect on the acid-neutralizing capacity. As the surface area of the eggshell powder particles increased, their acid-neutralizing capacity was also increased.

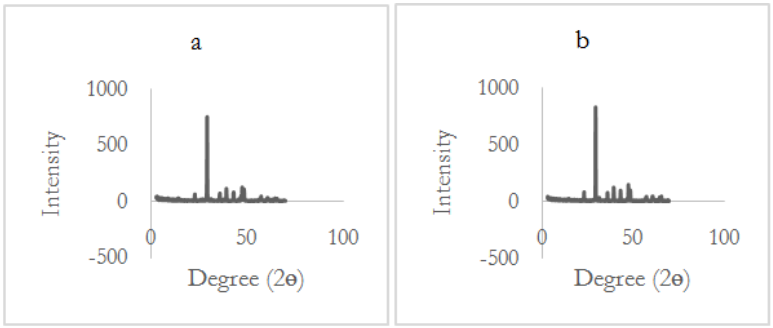

Fig. 5: XRD pattern of (a) commercial calcium carbonate, CCC and (b) purified eggshell powders, S5

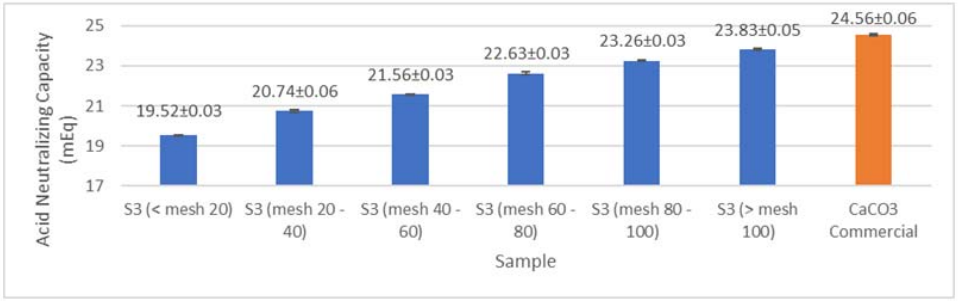

Fig. 6: Acid neutralizing capacity of eggshell powders and $CaCO_3$ commercial, all experiments were conducted in triplicate.

CONCLUSION

Eggshells can be used as a natural source to isolate calcium carbonate. Mechanical methods (separating membranes from eggshells) were used in this study, followed by physical purification methods (boiling eggshell powders to separate organic compounds from eggshells). Calcium carbonate from eggshells met the requirements of the Indonesian Pharmacopoeia (fifth edition) at 98.5% calcium carbonate content and impurity limit (loss on drying, acid-insoluble substance, limit of metal content, limit of magnesium and alkali salt.).

Furthermore, calcium carbonate from eggshells had physicochemical characteristics such as an average particle size of 21±1.0 µm, an XRD pattern that is similar to commercial calcium carbonate, a 99% similarity level to calcium carbonate from FTIR, and a more porous surface than commercial calcium carbonate from SEM.

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Nil

AUTHORS CONTRIBUTIONS

Conceptualization; D,S., M. A. and A. Y. C.; methodology, D. S., M. A., T. M. and A. Y. C.; formal analysis, A. Y. C, M. M., I. S.; investigation, D. S.; writing−original draft preparation, D. S. and A. Y. C.; writing−review and editing, M. M., I. S and A. Y. C.; visualization, D. S. and A. Y. C.; supervision, M. A., T. M. and A. Y. C. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTERESTS

The authors declare that we have conflicts of interest in this work.

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