



Formulation of Instant Granule Containing Nano Calcium from the Shell of Freshwater Mussels (*Anodonta woodiana*) for Autism Children

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Abstract

The small particle size of nano calcium from freshwater mussels shell will tend to increase the absorption of calcium by human's body and will be very well consumed by children with special needs to suppress hyperactivity disorders that caused by mercury. This study aimed to create and determine the best instant granule formulation containing nano calcium from freshwater mussels shell. The research method began with the manufacture of freshwater mussels shell powder and isolated nano calcium. Nano calcium was tested for its solubility in acid. The granule formula was made with stevia variations. Instant granule evaluation included granule flow test, rest angle, compressibility, water content, solubility, hedonic test, and calcium bioavailability. The results showed that nano calcium was soluble in 1:1 ratio of citric and tartrate acids, so that each formula needs 1.2% citric and 1.2% tartrate acids. Based on the flavor, aroma, and texture, the most preferred granule formula contained 600 mg nano calcium, 60 mg citric acid, 60 mg tartaric acid, 400 mg stevia each sachet weighing 5 g. The best instant granule evaluation showed that the flow rate was 4.253 g/sec, 33.90 rest angle, 4.65% compressibility, 2.21% water content, soluble time 58 second and calcium bioavailability was 100%.

Key words: Calcium bioavailability, detoxification, freshwater mussels, instant granule, nano calcium.

Formulasi Granul Instan Mengandung Nano Kalsium dari Cangkang Kerang Air Tawar (*Anodonta woodiana*) untuk Anak Autisme

Abstrak

Partikel berukuran kecil nano kalsium dari cangkang kerang air tawar akan cenderung meningkatkan penyerapan kalsium oleh tubuh dan sangat baik dikonsumsi oleh anak berkebutuhan khusus (autisme) untuk menekan gangguan hiperaktivitas yang disebabkan oleh merkuri. Penelitian ini bertujuan untuk membuat dan menentukan formula granul instan terbaik yang mengandung nano kalsium dari cangkang kerang air tawar (*Anodonta woodiana*). Metode penelitian diawali dengan pembuatan tepung cangkang kerang air tawar dan isolasi nano kalsium. Nano kalsium diuji kelarutannya dalam asam. Formula granul dibuat dengan variasi stevia. Evaluasi granul instan meliputi uji alir granul, kompresibilitas, kadar air, kelarutan, uji hedonik dan bioavailabilitas kalsium. Hasil penelitian menunjukkan bahwa nano kalsium larut dalam asam sitrat : asam tartrat 1 : 1, sehingga setiap formula membutuhkan 1,2% asam sitrat dan 1,2% asam tartrat. Berdasarkan rasa, aroma dan tektur, formula granul yang paling disukai adalah formula mengandung 600 mg nano kalsium, 60 mg asam sitrat, 60 mg asam tartrat, 400 mg stevia pada setiap kemasan 5 g. Evaluasi granul instan terbaik menunjukkan laju alir 4,253 g/detik, sudut istirahat 33,90, kompresibilitas 4,65%, kadar air 2,21%, waktu larut 58 detik dan bioavailabilitas kalsium 100%.

Kata kunci: Bioavailabilitas kalsium, detoksifikasi, granul instan, kerang air tawar, nano kalsium.

1. Introduction

Currently, autism is a disease with a high indication (1: 250 children). Various factors may be the cause of autism, including metallothionein dysfunction and/or heavy metal poisoning. The detoxification of heavy metals and nutritional therapy with supplements containing zinc, cysteine, and glutathione is recommended for the treatment of metallothionein dysfunction in autism¹. Approximately 88 percent of autistic children have a damaged intestinal condition (autistic colitis). There is their suspicion of heavy metal poisoning. The process of removing toxins (detox) can be done by supplementation is divided into several categories, namely, improve, provide essential nutrients, toxins, and improve neurotransmitters. One of the freshwater commodities that have the potential as a source of calcium is the shell of freshwater mussels. Freshwater mussels are one of the preferred freshwater commodities of the community. Utilization of shell from freshwater mussels as a source of Ca, Mg, P, Na, K, and Zn minerals help to overcome one of the problems in people with autism that is hyperactivity due to heavy metal contamination especially Hg. Nanocalcium is the calcium produced by utilizing nanotechnology to form calcium with a very small size (nanometer). Nanocalcium is a highly efficient predigestive mineral entering the body cells because the very small size causes easy entry of the receptor so that it can be absorbed rapidly and perfectly into the body². Nanocalcium has a very small size of 9 to 10 nm which causes the receptor to quickly enter the body perfectly, so that nano calcium can be absorbed by the body almost 100%³. The word nano refers to particle size of 1 to 100 nm⁴. According to Muller and Keck⁵ the nano size ranges from 200 to 400 nm, whereas according to Mohanraj and Chen⁶ nano are particles measuring 10 to 1000 nm. The nanocalcium produced from the extraction using a base is better than using an acid based on high rendement value and high white degree⁷. Calcium with nano size obtained from the shells of freshwater mussels is one of the applicable uses in the manufacture of min-

eral supplements that can be used as a heavy metal detoxification supplement. With an increasing number of autistic people, such as a 56% increase in 1998 - 2013, supplement products containing nano calcium powder have the potential to be developed as an alternative to a CFGF (casein free gluten free) diet and its calcium content can help replace calcium from cow's milk containing casein. Supplements containing nano calcium from freshwater shells can be consumed by autism children who are safe to eat and can suppress hyperactivity and can be consumed by people who have osteoporosis. The right nutritional interventions can have a huge impact on autism brain function, memory, learning, attention, focus, mood, behavior, growth, and overall health⁸. This study aimed to create and determine the best supplement formulation containing nano calcium from freshwater shells that can detoxify mercury based on hedonic test and quality test.

2. Materials and Methods

2.1. Tools

Equipment used include Hammer mill, hot plate (IKA, Aachen, Germany), furnace (CV. Dinar Multi Gemilang, Bogor, Indonesia), tray and basin, Filter 16, 20 and 30 mesh, Oven (CV. Dinar Multi Gemilang, Bogor, Indonesia), analytical balance (Ohaus, New Jersey, USA), glassware, granul flow tester apparatus (Intralab, Bandung, Indonesia), Tap density tester (Intralab, Bandung, Indonesia), Moisture balance brand (AND, California, USA).

2.2. Materials

The materials used we re shell of freshwater mussels (*Anodonta woodiana*), HCl, NaOH (Merck, Darmstadt Germany), Citric Acid, Tartaric Acid (Sigma, Tokyo, Japan), Stevia, PVP (Polyvinyl Pyrrolidone), Lactose, Essence, Dyes (Brataco, Bogor, Indonesia).

2.3. Methods

The research stages included preparation of the sample from freshwater mussels, making flour from the shells of freshwater mussels, isolation of nano calcium from

shell flour of freshwater mussels, nanocalcium solubility test, instant granule formulation making then hedonic test and quality analysis.

2.3.1. Making Flour from the shells of freshwater mussels

Samples of freshwater mussels (*Anodonta woodiana*) were obtained from the inundated waters of the Salabintana Experimental Pond Sukabumi, West Java, Indonesia. Freshwater mussels used with a weight between 200-300 g was cleaned and dumped. Flour from the shell was made by modification of the Sada method, referred to in Wahyuni⁹ modified in the sieve stage. The shell of fresh water mussels that has been separated previously from its dirt was cleaned and dried in the sun for approximately 6-8 hours, then boiled in 1 L NaOH 1N solution at 50°C for 3 hours. The shell that has been boiled then neutralized by washing, and then dried by oven at 121°C for 15 minutes. The dried shells from freshwater mussels were then crushed using a hammer mill and filtered with a coarse filter and nylon mesh to become flour. Nano calcium was obtained and isolated from the resulting flour.

2.3.2. Isolation of nano calcium from Shell flour of freshwater mussels

The flour was then extracted with HCl solvent at 90°C for 1.5 hours. The extraction results were further filtered by filter paper to obtain liquid/filtrate. The obtained filtrate was further precipitated by addition of NaOH 3N and stirring and sterilized until precipitation no longer formed. The precipitate obtained was then separated by decantation. The precipitate was then carried out a neutralization process using aquadest until reach pH 7. The next stage was the drying stage of sediment with oven and continued with combustion in the furnace at a temperature of 600°C so as to form nano calcium powder. Particle measurement results using the SEM with 20.000x and 10.000x magnification showed that nano-sized particles of nano calcium powder produced range 121-430 nm. It tends to increase the calcium absorption by the body¹⁰.

2.3.3. Calcium bioavailability analysis

Calcium bioavailability analysis of nano calcium powder was performed in vitro using dialysis method. Calcium in the sample was hydrolyzed from its bond with proteins using digestive enzymes (pepsin) found in the stomach and small intestine. The free calcium present in the sample solution diffuses through the semipermeable membrane into a dialysis sac containing the NaHCO₃ buffer. Calcium in dialysate shows the amount of calcium absorbed by the body. Calcium analysis uses atomic absorption spectroscopy at a wavelength of 422.7 nm.

2.3.4. Nano calcium Solubility Test and Instant Granule Formulation

The nano calcium powder was further tested for solubility in an acid solvent which were citric acid, tartaric acid and in 1:1 ratio of citric and tartrate acid. Use of amounts of citric acid and tartaric acid for pharmaceutical preparations 0.3-2.0%. Instant granule formulation of nano calcium was based on the best previous solubility test result. The result of a child dose conversion for the use of nano calcium powder was 600 mg per day. The ingredients for the manufacture of instant granules such as nano calcium powder, stevia, lactose, PVP are weighed at a certain amount, then sieved using a mesh screen 30. The nano calcium, lactose, acid, stevia powder was put into a basin and then stirred until homogeneous for approximately 5 minutes, added PVP that had been liquefied and left overnight and then stirred until a compact mass was formed. Instant granule formulation was made with the variation of wet stevia. Mass addition was sieved using mesh 16 sieves to form the wet granules. Granules were dried in a drying cupboard which had been covered at the temperature of 40-50°C overnight to form dried granules. Dried granules were sieved using mesh screen 20. In the manufacture process of empty granules (placebo), nano calcium powder was replaced with lactose in equal amounts and in the same manner. Placebo was made as a control to see its effect on characteristics and hedonic granules.

2.3.5. Instant Granules Evaluation

Instant granules that have been prepared according to formula with different use of stevia sweeteners were then evaluated. Evaluation of instant granules includes granule flow test, break angle test, compressibility test, moisture test, solubility test and hedonic test.

f. Flow Tower Test

The granular flow test was carried out with as much as 25 g of granules passed into the granule flow tester until the granule period passes through the funnel, then timed. Measurements were made 3 times.

g. Rest Angle Test

Determination of the rest angle was done by inserting a mass of granules into the funnel. The falling mass will form a cone, then measured in height and diameter of the cone. This experiment was done 3 times.

h. Compressibility Test

The test was performed to determine the compressibility of granules and the attachment of binder with other granule materials. If the value of the compression increment before and after the tap is small, the more homogeneously mixed with the binder and the other granule. This test was done by weighing 50 g granules, inserted into the tube on the powder taping density tester. The granules volume in the tube were recorded before and after the stamping. This test was performed three times and calculated the price of compressibility (%) by the Carr equation.

i. Water content test

Examination of granule water content was done by using Moisture Balance. Each formula was inserted 1 g of instant granules into the prepared tool, at 105°C for 10 minutes and then the levels listed on the Moisture Balance are recorded.

j. Solubility Test

A total of 8 g of instant granules was introduced into 250 mL water, then calculated by stopwatch, until the entire instant granules

dissolved and record the time listed in the stopwatch.

k. Organoleptic Testing

Organoleptic tests include a hedonic quality test of the color, aroma and texture and taste of the dissolved granules. Tests were conducted on panelists in the age range 18-30 years as many as 20 people. Testing is done to find out the most preferred formula by panelists.

l. Calcium bioavailability analysis

Calcium bioavailability analysis was performed on selected instant granules according to the results of the hedonic test using dialysis method.

3. Results

3.1. Solubility Result of Nano calcium and Instant Granule Formulation

Nano calcium powder obtained from freshwater mussel flour has a white color. The yield of the obtained nano calcium powder was 8.53%. The results of the solubility test showed that the nano calcium soluble in a mixture of citric acid and tartaric acid Based on the results of the solubility test subsequently prepared an instant granule formula of nanocalcium. Instant granules formulation were made containing 1:1 ratio of citric acid and tartaric acid taking into account that the use of citric acid and tartrate for pharmaceutical preparations in the range of 0.3-2.0%. Instant granule formulation of nano calcium is shown in Table 1. The prepared nano calcium granules have a light yellow, granular, weak odor and slightly sweet to sweet taste (Figure 1). Instant granules of nano calcium dissolved in water can be seen in Figure 2. Evaluation done on physical properties of instant granules aims to ensure the quality of granules produced. The evaluation includes the nature of flowability, rest angle, compressibility, water content, and solubility. The results of the evaluation of the instant nano calcium granules are shown in Table 2. Results of assessment of quality of hedonic formula of granule instant nano calcium are shown in Table 3.

Table 1. Instant granule formulation of nano calcium

Instant Granule Materials	The Amount Added to the Formula			
	F1	F2	F3	F4 (Placebo)
Nano Calcium	600 mg	600 mg	600 mg	600 mg
Citric Acid	6 mg	6 mg	6 mg	6 mg
Tartaric Acid	6 mg	6 mg	6 mg	6 mg
Stevia	100 mg	300 mg	500 mg	300 mg
PVP	50 mg	50 mg	50 mg	50 mg
Lactose	4.2375 g	4.0375 g	3.2375 g	4.6375 g
Aroma	qs	qs	qs	qs
Dye	qs	qs	qs	qs
Total	5 g	5 g	5 g	5 g

qs : quantumstatic

4. Discussion

4.1. Nano Calcium from Freshwater mussel shell flour

The precipitation method is a mixture of acids and bases that produce crystalline solids and water¹¹. In this study, precipitation was done by dissolving calcium from the shell into an acid solvent then added sodium hydroxide. The mixing of acids and bases produces a fine nano-sized calcium deposit. The freshwater mussels shell that have been crushed with a hammer mill into a light brown shell powder. Isolation of calcium from freshwater shells was done by demineralization using HCl. Calcium carbonate (CaCO_3) in shell flour reacts with HCl will form CaCl_2 . Calcium chloride (CaCl_2) was then precipitated with NaOH resulting in a precipitate of calcium hydroxide and sodium chloride. Calcium hydroxide (Ca(OH)_2) will turn into calcium oxide (CaO) during the foaming process. The yield of the nano calcium powder was obtained from freshwater mussels shell (*Anodonta woodiana*) at the treatment time of 1.5 hours extraction was 8.53%. The results of the calcium bioavailability test of nano

calcium powder were 32.67 to 35.58%. The calcium bioavailability rate of nano calcium is high as more than 20%.

4.2. Instant Granule Evaluation

The results of instant granule evaluation of flow rate, rest angle and compressibility are presented in Table 2.

4.2.1. The flow rate

The flow rate represents the amount of time required by some granules to flow through the funnel on the flowmeter. Examination of granule flow time is done on dried granules. This is done with the aim to find out whether the instant granules of nano calcium contain citric acid and tartrate made to meet the requirements so it was expected to produce good granules. Flow rate evaluation showed that F1 and F2 had rates with cohesive characteristics whereas F3 and placebo flowed easily. The cohesive properties of granules of formula 1 and 2 are possible because the particle size of the granules is too small. Fine particles with large surface area are more cohesive than particles of larger size

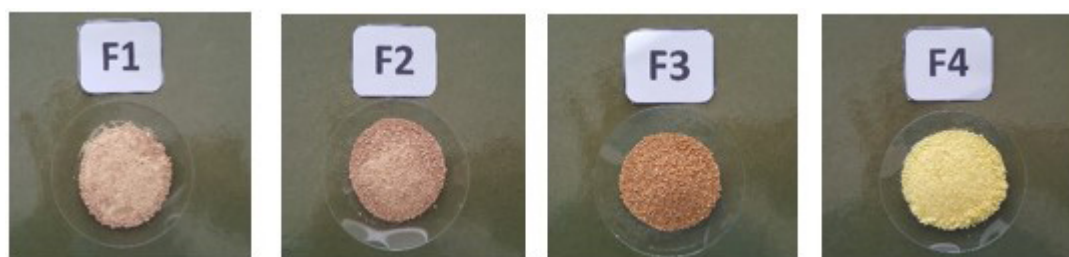


Figure 1. Instant granules contain nano calcium from freshwater mussel shell flour

Table 2. Results of evaluation of instant granule

Instant Granule Materials	The Amount Added to the Formula			
	F1	F2	F3	F4 (Placebo)
Flowability (g/sec)	3.417	3.787	4.253	5.297
Rest Angle (°)	34.76	32.37	33.90	29.99
Compressibility (%)	1.90	2.52	4.65	4.42
Water Content (%)	2.057	2.183	2.210	0.760
Solubility (sec)	56.5	56.6	58.0	50.0

due to the influence of gravity. F1 flow rate is 3.417 g/s, F2 3.787 g/s, F3 4.253 g/s and placebo 5.297 g/s. Based on the results of the flow tests, formula 1, 2, and 3 met the conditions that have been determined where good granule conditions have a flow time of 4-10 g/s.

4.2.2. The rest angle

The rest angle is an important granule test to determine the flow properties of granules. The powder will form a cone, a more flat the cone is produced the smaller the silent angle. The height and diameter of the cone formed on the measured power test and calculated the rest angle for each instant granule formula. The value of the acceptable rest angle between 20-40°. The rest angle 40° indicates a good flow potential. Evaluation of the rest angle indicates that all formulas have a good flow type that is easy to flow because the instant granules of nano calcium are more hygroscopic which gives rise to the adhesion-cohesion style, the surface area increases in the granules.

4.2.3. Compressibility

Compressibility testing of all formulas of both nano calcium granules and placebo

granules has a compressibility index with an excellent flow type that is below 20%. Compressibility is a trait to form a stable and compact mass when pressed. Compressibility was measured using a bulk density tester. The results showed that the value of instant nano calcium granule compressibility yielded formula 1, 2, 3 and placebo were all below 5%. This was due to the bonding solution (PVP) which has the properties to maintain the stability and cohesiveness of granules. The same binder solution in the four different formulation gives almost the same compressibility index.

4.2.4. Moisture content

Evaluation of granular moisture content showed that the nano calcium instant granule formula met the requirements, the water content of formula 1 was 2.057%, the formula 2 was 2.183% and the formula 3 was 2.21%. The average instant granule water content for formula 1 was 2.057%, F2 was 2.183% and F3 was 2.21% indicating that all the formula fulfilled the requirements based on the standard set by SNI. The value of water content for traditional drinks is not more than 3%. Testing of moisture content in granule aims to determine the water content in granules be-

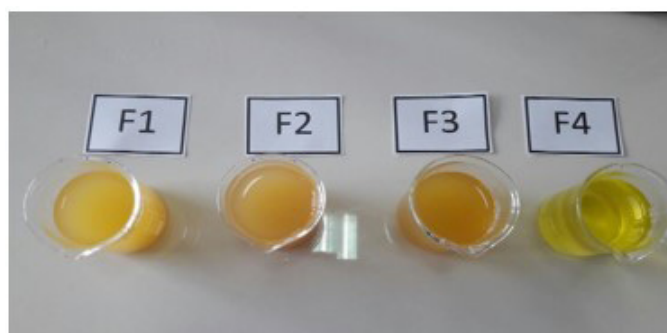


Figure 2. Instant granules of nanocalcium are diluted in water

Table 3. Results of assessment of quality of hedonic formula of granule instant nano calcium

Test Parameters	Nano Calcium Instant Granules			
	F1	F2	F3	F4 (Placebo)
Colour	3.60 ^a	3.05 ^a	3.10 ^a	3.60 ^a
Texture	2.90 ^a	2.85 ^a	2.75 ^a	3.55 ^b
Sense	2.15 ^b	1.65 ^a	2.20 ^b	3.90 ^c
Aroma	3.30 ^{ab}	3.20 ^a	3.75 ^b	3.35 ^{ab}

cause water can affect the length of storage granules. The higher the moisture content the easier the microbial preparation during storage.

4.2.5. The solubility test

The granule's soluble time indicates the amount of time required by the granules in a serving size to be completely soluble in a given volume of water.

The solubility test results showed faster soluble formulas 1 and 2 than formula 3. The solubility of the formula 1 was 56.5 seconds, the formula 2 was 56.6 seconds, and the formula 3 is 58 seconds. The results of the granule soluble time calculation showed no different values ranging from 56 to 58 seconds, while the placebo faster soluble is 50 seconds. Based on the results of the break-point test, the compressibility and moisture content, all granules of formula 1, 2 and 3 have met the requirements, and based on the flow of formula 3 was the best formula.

4.2.6. Organoleptic Granule Instant Test

Organoleptic tests were performed on instant granules after dissolving, and the tests were performed on aroma, color, taste, and texture. The hedonic test was conducted by 20 panelists. The result of the hedonic test in the panel is concluded by using statistical analysis. The statistical results showed that on the color parameter, all instant granule formulas showed no difference. For taste, aroma and texture parameters of formula 3 were preferred granules compared to F1 and F2.

4.2.7. Calcium bioavailability

The results of the calcium bioavailability test of the instant granule was 100%. The

calcium bioavailability rate of the F3 instant granule was high as more than 20%.

5. Conclusion

Nano calcium from Freshwater Mussels shell (*Anodonta woodiana*) was soluble in 1:1 ratio of citric and tartrate acid, so that each formula needed 1.2% citric and 1.2% tartrate acid. Based on the statistical results of the hedonic test on the instant granules showed that the color parameters were not significantly different, whereas the most preferred flavor, aroma and texture of the instant nano calcium granule was formula 3. Instant granule formula 3 contained 600 mg nano calcium, 60 mg citric acid, 60 mg tartaric acid, 400 mg stevia, 50 mg PVP and 3.83 g lactose. The best instant granule evaluation results showed that the flow rate was 4.253 g / sec, 33.90 rest angle, 4.65% compressibility, 2.21% water content, soluble time 58 second and calcium bioavailability 100%.

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