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Saponin Content of *Sapindus rarak* Pericarp Affected by Particle Size and Type of Solvent, its Biological Activity on *Eimeria tenella* Oocysts

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Abstract: *Sapindus rarak* is a species of soapberry and in Indonesia the trunks commonly used as board cast, match sticks and wooden crafts and the saponin containing fruits are used as a detergent for traditional clothes (called batik). Saponin has biological activity to damage protozoa. The study has been carried out to investigate the effects of particle size and type solvent on the concentration of saponin extracted from the *Sapindus rarak* pericarp powder (SRP) and to evaluate the activity of SRP on oocysts of *Eimeria tenella in vitro*. Completely randomized design with 2 factors was arranged to analyze saponin content. First factor was type of solvent, i.e., 70% methanol and water. Second factor was particle sizes of SRP, i.e., 75, 300 and 600 μm and the saponin analyses of each treatment was done in triplicates. Determination of total saponin as described by Hiai and Nakajima and total sapogenin according to Hiai method with some modification. *In vitro* assay was performed at several concentrations of SRP in water (100-0.01 mg/mL). Results showed SRP with 75 μm particle size showed the highest foam height and the highest total saponin content compared to those having 300 and 600 μm of particle size. Solvent and particle size significantly affected saponin content. Saponin content of SRP was higher when it was extracted with 70% methanol (35.98%) than extracted using water (32.53%). SRP with 75 μm particle size had higher saponin content (43.52%) than those with 300 and 600 μm particle size (37.54 and 21.71%). Sapogenin content extracted with 70% methanol and water was not significantly different (30.55 and 30.36%), but particle size affected the sapogenin content. At 75 μm particle size, the sapogenin content was significantly higher (35.51%) than 300 and 600 μm particle size (33.46 and 22.40%). The SRP with concentration of 100-1 mg/mL water inhibited sporulation of *E. tenella* oocysts more than 90%. It is proved SRP has the ability to destroy the development of *Eimeria tenella*.

Key words: Saponin, *Sapindus rarak*, particle size, solvent, *Eimeria tenella*

INTRODUCTION

Sapindus rarak is a species of soapberry and in Indonesia the trunks commonly used as board cast, match sticks and wooden crafts and the fruits are used as a detergent for traditional clothes (called batik). The major secondary compound in *S. rarak* fruits are saponins and sesquiterpenes (Chung *et al*., 1997; Wina *et al*., 2005). Saponin were reported to show several biological properties such as anticarcinogenic (Rao, 1995), stimulate immunity (Cheeke, 2001), haemolytic (Astuti *et al*., 2009), anti-inflammatory (Jian-Ping *et al*., 2007), reduced cholesterol (Afrose *et al*., 2009, 2010; Astuti *et al*., 2009). Additionally, saponins showed antimicrobial properties, especially against fungi and protozoa, including protozoa in the rumen (Cheeke, 2001; Wina *et al*., 2003).

Eimeria tenella is one of the eleven different types of Eimeria, a group of intracellular protozoan parasites, that causes avian coccidiosis and can lead to death in chicken (Cook, 1988). This disease has a significant economic loss in the poultry industry (Dalloul and Lillehoj, 2006).

Usually, chemoprophylaxis (antibiotic growth promoters and anticoccidial drugs) have controlled the disease but have caused drug resistance (Tipu *et al*., 2002; Abbas *et al*., 2011) and toxicity in animal (Nogueira *et al*., 2009). Therefore, it is necessary to look for alternatives from plants as anticoccidial agent that are safer and better for the animal and the meat consumer. Molan *et al*. (2009) reported Pine bark extract have ability to decrease the sporulation of the oocysts of *E. tenella*, *E. maxima* and *E. acervulina*. *S. rarak* may become one of the alternatives because its saponin may suppress the development of *E. tenella* oocysts.

Smaller particles dissolve faster than larger ones because the surface of particle is wider, so that the plant

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material in powder form with micro particle size is expected to release more bioactive substances. The effect of particle size were studied mainly on grain feed ingredient such as corn (Amerah *et al*., 2008) but the information about effect of particle size of plants containing secondary compounds as feed additives is very limited. Zhang *et al*. (2009) used different particle size of *Zingiber officinale* root as antioxidant and hypocholesterol agent in broiler chickens. They found that reducing particle size of ginger powder linearly reduced cholesterol and increased glutathione peroxidase (Zhang *et al*., 2009). Hu *et al*. (2006) used *Astragalus membranaceus* root powder size comparing two different particle sizes and indicated that smaller particle was more effective than bigger size in improving growth performance and enhancing immune function of pigs.

Extraction is the process of separating the active substances of a material by using selective solvents in standard extraction procedures. The finer the size of the substance, it would be more optimal extraction process. The type of solvent used in the extraction related to the polarity of the solvent. The compounds that have the same polarity with solvent will be more easily dissolved. Polar solvent (water, methanol, ethanol, acetic acid) is suitable to extract polar compounds from plants.

The aim of research were to study the effect of solvent and particle size on saponin and sapogenin content of *Sapindus rarak* powder (SRP) and to evaluate the activity of SRP on oocysts of *Eimeria tenella in vitro*.

MATERIALS AND METHODS

Preparation of different particle *Sapindus rarak* powder (SRP): *S. rarak* fruits was obtained from Madiun, East Java, Indonesia. The fruits were dried in the oven at the temperature 60°C for 3 to 4 days. Pericarp and seed were separated and then pericarp was ground with Laboratory blender. The powder was screened through 30, 50 and 200 US mesh to obtain SRP with particle size of 600, 300 and 75 µm.

Foam test: A total of ±0.5 g of powder sample was introduced into 50 mL volumetric tube, then 9.5 mL of distilled water was added. The tube was strongly shaken for 30 times, then was allowed to stand for 30 min for stability foaming. The foam height was measured. Foam test was performed in three replications.

Extraction of saponin from *Sapindus rarak*: Two hundred milligrams of milled SRP with different particle size was weighed and extracted using 10 mL of 70% methanol or water in a test tube with a lid, then stirred using a vortex. The solution was kept in an ultrasonic bath for 30 min and then centrifuged for 10 min at 3000 rpm, then the supernatant was separated from the residue as saponin containing extract (methanol/water).

Determination of total saponin as described by Hiai *et al.* (1976).

Preparation of reagents: Sulfuric vanillin reagent was prepared as followed: 1.6 g of vanillin was weighed and added to 20 mL of absolute ethanol, then was mixed. Preparation of 72% sulfuric acid: 72 mL of H_2SO_4 was added slowly to 28 ml of water. Preparation of standard solution of diosgenin 0.5 mg/mL: 10 mg of diosgenin was dissolved in 20 mL of absolute methanol.

Saponin containing extract (0.25 mL) was pipette and put into a test tube, then 0.25 mL of vanillin reagent (fresh) and 2.5 mL of 72% H_2SO_4 were added (performed in an ice bath). The mixture was heated in waterbath at a temperature of 60°C for 10 min, then was cooled. The absorbance of mixture was measured by spectrophotometer at a wavelength of 544 nm. Diosgenin was used as a reference standard. The test was performed in duplicate.

Determination of total sapogenin was carried out according to the method of Hiai *et al*. (1976) with some modifications.

SRP was extracted with 70% methanol (SRM) and water (SRW). Each sample (SRM and SRW) was pipetted 4 mL and hydrolyzed with 2N HCl. SRW was added directly with 6 mL of 2N HCl, meanwhile SRM was evaporated first then added 10 mL of HCl 2N. Furthermore, sample was heated at 100°C for 60 min, then were added 10 mL of ethyl acetate was added and the tube was shaken for 30 times. The ethyl acetate layer was separated and was concentrated until ethyl acetate was exhausted. After that, 4 mL of ethyl acetate was added to obtain ethyl acetate extract, then was tested using the Hiai method. Hydrolysed product in ethyl acetate was pipetted and put in a test tube and then added 0.25 mL of vanillin reagent and 2.5 mL of 72% H_2SO_4 (performed in an ice bath). The mixture was heated at a temperature of 60°C for 10 min, then measured at a wavelength of 544 nm. Tests were performed duplicate. It will get the total sapogenin.

***In vitro* of *Eimeria tenella* sporulation assay (Molan *et al*., 2009):** *E. tenella* oocyst was isolated and purified at the Indonesian Research Center for Veterinary Science (IRCVS) Bogor, Indonesia. In vitro of *Eimeria tenella* sporulation assay was conducted following modified method published by Molan *et al*. (2009). This experiment SRP with 75 µm particle size and six different concentrations (100; 50; 10; 1; 0.1; 0.01 mg SRP/mL water) were prepared in duplicates. One hundred oocysts were incubated in Petri Dish with SRP solution at room temperature 25-29°C for 48 h. After incubation, oocysts were poured into a corning tube, then was made to 14 mL with water and centrifuged for 10 min at 2000 rpm. The top of the liquid was aspirated by vacuum pump.

Sediment was stirred after the addition of saturated sugar solution, then centrifuged at 2000 rpm for 10-15 minutes.

Two milliliter of the upper solution was pipetted into another corning tube, then was washed with 12 mL of water and centrifuged for 10 min at 2000 rpm. Supernatant was discarded and the left over approximately 0.5 mL was the oocysts. Oocysts were viewed under Light microscope at 10 x 40 magnification. The number of abnormal (shape and size) oocysts was counted and expressed as the percentage to the normal oocysts (100).

RESULTS

Saponin content of *Sapindus rarak* pericarp powder

Foam height of *S. rarak* pericarp powder: Immediately after being shaken, *S. rarak* pericarp powder in water produced foam, however, only a stable foam height that was measured after 30 min. Table 1 shows that the foam height was higher with smaller particle size but statistically, it was not significantly different among them.

Total *saponins* and *sapogenin*: Type of solvent affected the total saponin content in SRP. Extraction with 70% methanol gave significantly higher total saponin content (35.98%) than that with water (32.53%) (Table 2). The particle size also affected the total saponin content in SRP. Extraction of total saponins from SRP at 75 µm particle size using water or 70% methanol showed the highest level compared to the 300 and 600 µm particle size. But there was no interaction between solvent and particle size on total saponin content.

Sapogenin content extracted with 70% methanol and water was not significantly different (Table 2). Sapogenin content in SRP was significantly higher in the order of 75>300>600 µm particle size.

***In vitro* assay of *E. tenella* sporulation:** Table 3 shows the inhibition percentage of Eimeria oocysts due to the addition of different concentration of *S. rarak* pericarp powder (SRP). The concentration SRP dissolved in water from 100 to 1 mg/mL contained 40.27 to 0.402% of saponin concentration damaged the sporulation of *E. tenella* more than 90%. However, when the concentration was made to 0.01 mg/mL (contained 0.004mg/ml saponin), the activity of SRP toward *E. tenella* sporulation disappeared.

The normal shape of sporulated oocysts (Fig. 1a, yellow arrow) was shown when the sporocyst was not damaged after incubated in the water for 48 h without addition of SRP. The oocysts that incubated in water with SRP showed the irregular or reduced shape and size of sporocysts (Fig. 1b-d) indicating abnormal or damage oocysts. But oocyst's wall of Eimeria (green arrow) was not damaged by the addition of SRP.

Table 1: Foam height of *Sapindus rarak* in different particle size

	Foam height (mm)
Particle size (µm)	After 30'
600	41.03
300	42.33
75	47.67
SEM	3.57
P	0.4293

[a-b]Means sharing similar superscripts within a column do not differ ($p<0.05$)

Table 2: Total saponin and sapogenin contents in *S. rarak* pericarp powder (SRP) with different solvent and particle size

	Total saponin (%)	Sapogenin (%)
Solvent		
Water	32.53[b]	30.36
70% Methanol	35.98[a]	30.55
SEM	0.636	0.216
P	<0.0001	0.5316
Particle size		
600	21.71[c]	22.40[c]
300	37.54[b]	33.46[b]
75	43.52[a]	35.51[a]
SEM	0.779	0.264
P	<0.0001	<0.0001

[a-c]Means sharing similar superscripts within a column do not differ ($p<0.05$)

Table 3: Effect *Sapindus rarak* pericarp powder (SRP) 75 µm particle size on inhibition of *E. tenella* sporulation

SRP conc. (mg/mL water)	Saponin conc. mg/mL	Inhibition (%)	SEM
Control	0.0	0.0	
100	40.27	99.0[a]	0.559
50	20.13	95.5[b]	0.559
10	4.027	94.0[b]	0.790
1	0.402	93.0[b]	0.790
0.1	0.040	50.0[c]	0.559
0.01	0.004	0.0[d]	0.559
P	----------	0.0001	----------

Conc: Concentration

SRP: *Sapindus rarak* pericarp powder at 75 µm particle size

[a-d]Means sharing similar superscript within a column do not differ ($p<0.05$)

DISCUSSION

Saponin is a bioactive compound that capable of producing abundant foam in aqueous solution even at low concentrations because it is able to reduce the surface tension of the solution (Hostettmann and Marston, 1995; Sparg *et al*., 2004; Patrick-Iwuanyanwu and Sodupo, 2007,). Foamability is desired property that needed in some chemical, food, cosmetics, also added to shampoos, liquid detergents, toothpaste and drinks as emulsifiers and foaming agents durable and pharmaceutical processes (Tanaka *et al*., 1996; Karakashev and Manev, 2001; Backleh *et al*., 2004; Khristov *et al*., 2004; Rouimi *et al*., 2005). The ability of a saponin to foam is caused by the combination of the nonpolar sapogenin and the water soluble side chain (Musman, 2010). The highest foam resulting in this study was shown in the particle size of 75 µm but not significantly different with the particle size of 300 and 600 µm. This indicates that the measurement of foam height

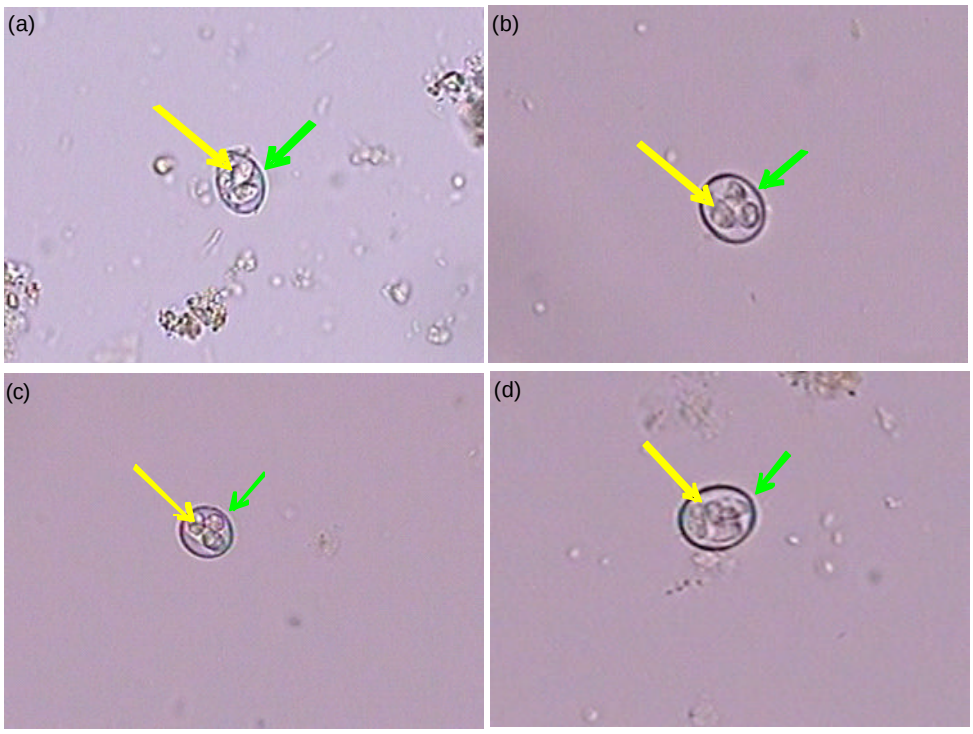

Fig. 1(a-d): Photomicrographs magnification (10x40) of oocysts of *Eimeria tenella*. (a) Normal sporulated oocyst in water, (b-d) different abnormal of sporulated oocysts. Yellow arrow indicates sporocyst. Green arrow indicates oocyst wall.

is not accurate and can only be used for qualitative indication of the presence of saponin compounds in *S. rarak* pericarp. This measurement cannot differentiate the effect of particle size of pericarp *S. rarak* on saponin content.

Type of solvent is one of the important things that were chosen to optimize the extraction of many plant bioactives. The solvent commonly used in the bioactives extraction from plants were water, methanol, ethanol, acetone or their mixtures (Lafka *et al*., 2007). The extraction yield and saponin content highly depended on solvent polarity. The result of this experiment showed that 70% methanol gave higher saponin content suggesting that *S. rarak* pericarp has bioactive saponins that have the same polarity with 70% methanol. Similar to this experiment, the highest amount of total saponins was obtained when the *Codonopsis lanceolata* roots were extracted by 70% ethanol (Zhao *et al*., 2012). Beside extracted saponin, the other scientist used 70% methanol to extract bioactive compounds (total phenol and total flavonoid) from papaya and gave higher level than 100% methanol and water (Addai *et al*., 2013). For the safety of the animal or human, it is better to use water than 70% methanol for SRP application. However, if the purpose is to obtain the saponin fraction of SRP, it is better to use 70% methanol than water.

Grinding plant material (dry or wet) is the basic principle to increase the surface area for extraction thereby increasing the rate of extraction (Tiwari *et al*., 2011). The smaller the size of raw material the easier to extract the bioactive compounds and the higher the yield of extract could be obtained. This experiment was in agreement with Tiwari *et al*. (2011) as SRP at smaller particle size (75 μm) contained higher saponin and sapogenin levels than SRP at bigger particle size (300 or 600 μm). Zubairi *et al*. (2014) also reported that smaller particle size (0.5-2 mm) of *Derris elliptica* produced higher yield of rotenone than that of bigger particle size (2-5 mm) (Zubairi *et al*., 2014). Similar results were reported by Zhang *et al*. (2009) in *Zingiber officinale* root and Hu *et al*. (2006) in *Astragalus membranaceus* root.

S. rarak pericarp powder with particle size 75 μm at a concentration of 1 mg SRP/mL of water containing 0.402 mg saponin /ml was still able to damage the sporocyst more than 90% but not the wall of *E. tenella* oocysts under laboratory conditions. The oocysts have remarkably strong wall and extremely robust that able to survive in the environment for prolonged period of time. Unsporulated or sporulated oocyst wall of *E. tenella* consisted of 19% carbohydrate, 14% lipid and 67% peptide (Stotish *et al*., 1978), while Mai *et al*. (2009) reported that oocyst wall of *E. tenella* consisted mainly protein (90.4-98.3%) with low carbohydrate content

(0.6-2.0%) and lipid content (1.4-7.6%). As other research showed that saponin extract of *S. rarak* pericarp has the ability to reduce protozoa population in the rumen (Wina *et al*., 2005), this experiment showed that saponin in SRP has also the ability to damage oocyst of *E. tenella* (shown in Fig. 1). The mechanism of saponin to destroy oocyst might be different from the mechanism of saponin destroying protozoa in the rumen. Saponin directly bound with the cholesterol of the protozoa membrane, hence, lysis the protozoa membrane in the rumen (Wina *et al*., 2005). Saponin of SRP which easily dissolved in water perhaps enter the oocyst through micropyle cap that present in the polar end of oocyst or through the gap between cap and oocyst wall. Wiedmer *et al*. (2011) showed the presence of micropyle and gap of Eimeria oocyst using SEM. After the saponin entered the inside of oocyst, it might damage the sporozoite directly since in Fig. 1, it was shown that the sporocyst had irregular (abnormal) shape or reduced shape. The experiment to study the exact mechanism of saponin destroying the oocyst is warranted.

Conclusion: In conclusion, total saponin from *Sapindus rarak* pericarp powder at 75 µm has saponin content higher than 300 and 600 µm particle size. Seventy percent methanol extracted higher total saponin than water. *Sapindus rarak* pericarp powder at 75 µm particle size with concentration of 1 mg/mL contained 0.402 mg/mL saponin damaged the number of oocysts more than 90%. *Sapindus rarak* pericarp has the ability to prevent the development of *Eimeria tenella*. Further *in vivo* evaluation using *S. rarak* pericarp powder as feed additive to prevent coccidiosis is suggested.

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