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Preparation and characterization of nanopowder of *Acalypha hispida* Leaves Extract Using Planetary Ball Milling

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ABSTRACT. *Acalypha hispida* Burm.f. is commonly used as an ornamental plant known for pharmacological effects. The nanoscale extract increases bioavailability and bioactivity. This research aimed to produce and characterize nanopowder extract of *A. hispida* leaves. Powdered leaves were macerated in 96% ethanol, then was evaporated in the spry dryer. Nanopowder extract was produced using planetary ball milling (PBM) at 5000 rpm in different milling times, namely 5 minutes (nanopowder A), 10 minutes (nanopowder B), and 40 minutes (nanopowder C). The nanopowder extracts were evaluated using a particle size analyzer, scanning electron microscope (SEM), and high-performance liquid chromatography. The average particle size of *A. hispida* crude extract was 1271 nm, and nanopowder A, B, and C respectively were 837.1 nm, 803.8, and 512.2 nm. The polydispersity index of *A. hispida* crude extract, nanopowder A, B, and C were 0.754, 0.696, 0.717, dan 0.612. The milling process for 40 minutes reduced the content of 5% gallic acid and 10.3% catechin. The SEM image of nanopowder C was smaller than crude extract. The best average particle size of nanopowder C (512.2 nm) and polydispersity index (0.612) were produced using PBM for 40 minutes at 5000 rpm.

Keywords: *Acalypha hispida*; Nanopowder extract; Planetary Ball Milling; Particle Size Analyzer; Scanning Electron Microscope.

INTRODUCTION

Acalypha hispida Burm.f. is commonly used to decorative plants and taxonomically belongs to the family Euphorbiaceae (Cardiel & Muñoz, 2018). This plant originates from New Guinea, the Malay Archipelago, and other islands East Indies (Bokshi, Sayeed, Ahmed, Karmakar, & Sadhu, 2012). The part of the plants used is leaves and flowers. The bioactivities of *A. hispida* leaves are antimicrobial, antifungal, wound healing, antihyperglycemic, anti-multi drug-resistant food pathogens, antioxidant, and nanosensor (Akinjogunla, Adewumi, & Okon, 2016; Selvakumar, Sithara, Viveka, & Sivashanmugam, 2018; Suminarti & Jualiana, 2020). The *A. hispida* flower is used as cotton dyes because it has a red color (Yasassri, Weerasinghe, & Udayakumara, 2019).

The polyphenol compounds of *A. hispida* leaves are previously well identified by Alfarisi, Sadiah, & Wresdiyati, (2020) and Siraj et al., (2016). The flavonoid contents of *A. hispida* leaves extract are quercetin, catechin, rutin, myricetin, naringenin, and others (Alfarisi et al., 2020; Siraj et al., 2016). *A. hispida* leaves extract contains phenolic acid, such as ellagic acid, gallic acid, syringic acid, rosmarinic acid, sinapic acid, and chicoric acid (Alfarisi et al., 2020; Siraj et al., 2016). Ellagic acid and catechin commonly are used to determine total phenolic in the Folin-Ciocalteu method and total flavonoid (Saeed, Khan, & Shabbir, 2012; Zaiter, Becker, Karam, & Dicko, 2016). Both include a strong natural antioxidant, and their biological activities are antifungal, anti-inflammatory, antivirus, and antibacterial (Choubey, Varughese, Kumar, & Beniwal, 2015; Dos Santos et al., 2020).

The pharmacological effect of the herb is dependent on bioactive compounds and is influenced by physics characteristics (Borhan, Norhidayah, Ahmad, Rusop, & Abdullah, 2013). The administration of herb extract commonly needs high doses to get an effectively pharmacological effect. Therefore, the size reduction of the extract is needed to diminish administrated doses. The fine extract size increases bioavailability and bioactivity, and decreases toxicity (Gunasekaran, Haile, Nigusse, & Dhanaraju, 2014). The method used to reduce extract size is ball milling, a top-down approach. The milling process produces nanoscale material through impact force generated by centrifugal force (Borhan, Ahmad, Rusop, & Abdullah, 2013). The nanoscale materials produced by ball milling are affected by several parameters such as

rotation speed, milling time, and bead quantity. Hence, this research aimed to produce nanopowders of *A. hispida* leaves extract and to characterize using particle size analyzer (PSA), scanning electron microscope (SEM), dan high-performance liquid chromatography (HPLC).

EXPERIMENTAL SECTION

Collection of *A. hispida* Leaves

A. hispida leaves were harvested from the garden of Tropical Biopharmaca Research Center (TropBRC), IPB University. The plant was identified and confirmed by the Indonesian Institute of Science (LIPI). The collection was conducted on July 8, 2020, at 09.00 am (GMT+7) in the dry season. The leaves were dried in the oven for two days and one night at 45 °C. The dried leaves were ground and filtered at a size of 80 mesh.

Extraction of *A. hispida* Leaves

The extraction of *A. hispida* leaves was conducted based on the methods described by Alfarisi et al. (2020). The powdered leaves were macerated in 96% ethanol solution with a ratio of 1:10 (b/v) for 72 hours. Then, the filtrate was processed in a spray dryer to obtain extract powder. The temperature in the spray dryer chamber was set up at 150 °C inlets and 90 °C outlets. The diameter of nozzles was 200 mesh (74 μm).

Extract Characterization

The organoleptic assessment was determined, involve form, smell, color, and taste. Phytochemical compounds (alkaloid, flavonoid, tannin, saponin, steroid, and triterpenoid) were evaluated using Harborne methods (Harborne, 1984). The water and ash contents of leaves powder and extract were measured in duplicates using gravimetric methods (Kartini, Jayani, Octaviyanti, Krisnawan, & Avanti, 2019).

Preparation of Nanopowder Extract

The extract was divided into three groups: (1) nanopowder A extract, (2) nanopowder B extract and (3) nanopowder C extract. Nanopowder A, B, and C extract were milled for 5, 10, and 40 minutes, respectively, at 5000 rpm. The milling process used planetary ball milling (PBM) (FRITSCH, Pulverisette 7, Germany) equipped with two stations. There are 50 beads (stainless steel) with a diameter of 50 mm in every station.

Particle size Analyzer (PSA)

The average size distribution and polydispersity index of nanopowder extract were measured using photon correlation spectroscopy (PCS) (Dynamic Light Scattering, DLS, Zetasizer Nano NS, Malvern, UK) at 25 °C. Nanopowder extract was dissolved in the distilled water, then sonicated for 2 minutes to separate extract aggregation. The refractive index of particles and water was used to measure the average size distribution and polydispersity index of extract (Sadiah, Anwar, Djufri, & Cahyaningsih, 2017).

Scanning Electron Microscope (SEM)

The method of SEM analysis referred to factory procedure. The nanopowder extract was sprinkled as thinly as possible onto the stub specimen's surface coated with carbon tape. Then, it was coated using Sputter Coater Merk Quorum type Q150R ES. This coating used gold material, sputter current 20 (mA), and sputter time 60 (seconds). The coated-nanopowder extract was assembled in the chamber, and the picture was taken using SEM ZEISS EVOIMA10. The instrument was set with the following control: secondary detector electron (SE), the working distance (WD) 7.00 mm, and electron-high tens (EHT) 16.00 kV. The topographical extract was captured in 1000x magnification.

High Performance Liquid Chromatography (HPLC)

The analysis of HPLC chromatographic was performed using Dionex UltiMate 3000 (Thermo Scientific, USA) equipped with quaternary rapid separation pump (LPG-3400RS) and rapid separation diode array detector (DAD-3000RS). The chromatographic separation was accomplished at HITACHI LaChrom C18 (4.6 x 250 mm; 5μm), and its temperature was constantly controlled at 40 °C. The separation was carried out using a gradient elution program (0 min 0.05%A, 15 min 0.05%A/10%B; 15 min, 0.05%A/60%B; 25.1-40 min, 0.05%A/10%B, where A is H_3PO_4 in H_2O, B is acetonitrile-methanol (3:2). The injection volume was 10 μl, and the flow rate was 1.0 mL/min. The detection was made at a wavelength of 280 nm. The solution of test extract was prepared in ethanol with a crude extract concentration of 20 mg/ml and a nanopowder C extract of 5 mg/ml. The standard solution was prepared in methanol to give a gallic acid concentration of 50 μg/ml and a catechin concentration of 100 μg/mL. The chromatographic analysis was done using Chromeleon (c) Dionex Version 7.2.4.8179.

RESULTS AND DISCUSSION

Characterization of *A. hispida* Leaves Crude Extract

Powdered leaves and crude extract of *A. hispida* were shown in **Figure 1**. Powdered leaves showed bright green color, and the crude extract showed strong green. The water and ash content of powdered leaves were 5.27% and 11.63%, respectively (**Table 1**). The characteristic of *A. hispida* leaves extract previously was reported. The ash content (11.63 %) of powdered *A. hispida* leaves in this research (**Table 1**) was lower compared to that of previous research of *A. hispida* (15% ash content) from Bogor, Indonesia (Bay, Hermanu, & Sinansari, 2020). The extract using a spray dryer (4.5%) in this research to evaporate the solvent showed less yield compared to that of extract using an evaporator (17.43%) (Alfarisi et al., 2020).

A. hispida is not yet listed in the monograph of Indonesian herbal pharmacopoeia 4th edition, so that the standard of ash and water content is not yet available (KEMENKESRI, 2017).

The extract exhibited a content of 7.12% water and 6.99% ash. The yield of the crude extract was 4.5% (**Table 2**). An organoleptic assessment revealed that the extract was in powder form, with a specific smell, strong green color, and bitter taste. According to the phytochemical test, the extract contained flavonoid, saponin, and steroid. However, alkaloid, quinone, and triterpenoid were absent from the crude extract.

The organoleptic assessment and phytochemical test of 96% ethanolic extract of *A. hispida* leaves in this research confirmed the result of previous studies (Alfarisi et al., 2020; Febriyanti, Sanjaya, Supriyatna, Diantini, & Subarnas, 2013). The secondary metabolites which were always consistently identified in ethanol, methanol, and distilled water from Indonesia, Nigeria, and India were flavonoid, saponin, and steroid (Akinjogunla et al., 2016; Alfarisi et al., 2020; Evanjelene & Karthiga, 2018; Febriyanti et al., 2013; Manikanta, Varma, Krishna, & Kumar, 2014). Several publications reported the presence of alkaloid and quinone in extract's methanol and distilled water of *A. hispida* leaves. (Akinjogunla et al., 2016; Evanjelene & Karthiga, 2018).

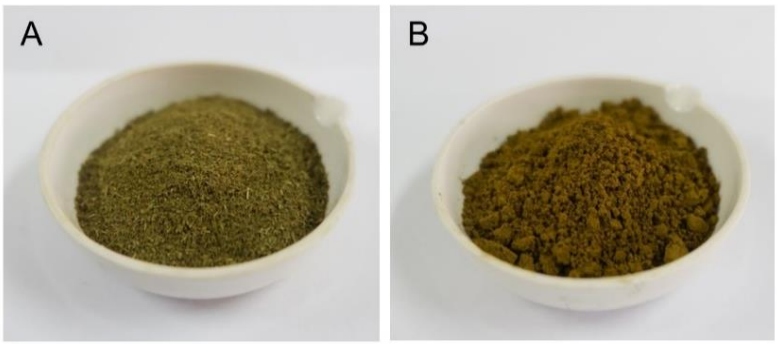

Figure 1. Powder (**A**) and extract (**B**) of *Acalypha hispida* leaves

Table 1. The water and ash content of powdered and crude extract of *A. hispida* leaves.

Parameter	Content	
	Powdered (%)	Extract (%)
Water	5.27	7.12
Ash	11.63	6.99

Data was duplicate

Table 2. Characteristics of *A. hispida* leaves extract

Parameter			Result
Yield (%)			4.5
Organoleptic:			
	Form		Powder
	Smell		Specific
	Color		Dark green
	Taste		Bitter
Phytochemicals			
	Alkaloids:	Wagner	-
		Mayer	-
		Dragendorff	-
	Flavonoids		+
	Tannins		+
	Saponins		+
	Quinones		-
	Steroids		+
	Triterpenoids		-

Plus (+) means contain
Minus (-) means not contain

Preparation and Evaluation of Nanopowder Extract

Crude extract that had been milled for 5, 10, and 40 minutes at 5000 rpm, as shown in **Figure 2**. The results of crude and nanopowder extract analyzed using a particle size analyzer were displayed in **Figure 3**. The average particle size of crude and nanopowder extracts and its polydispersity index (PDI) were exhibited in **Table 3**.

The average size of crude extract was 1271 nm. The smallest average size of diameter was shown by nanopowder extract C (512 nm), then was followed by nanopowder B (803.8 nm) and A (837.1 nm), respectively. This result was proven by SEM images of crude and nanopowder extract C (**Figure 4**). The SEM image revealed the topographical nature of crude extract from spray dryer and nanopowder extract C. **Figure 4** displayed that the average particle size of crude extract was reduced after 40 minutes of milling time.

The nanopowder extract was produced using top-down methods. The top-down approach is principally the breakdown of bulk material to get the nano-sized material desired. (Bayda, Adeel, Tuccinardi, Cordani, & Rizzolio, 2020; Iqbal, Preece, & Mendes, 2012). Ball milling is a top-down approach commonly used to produce nanomaterial, nanocomposite, nanocarbon, and nano-quasicrystalline material (Yadav, Yadav, & Singh, 2012). There are different definitions of a nanoparticle. National Nanotechnology Initiative (NNI) defines that a nanoparticle in nanoscience and nanotechnology studies is material at the nanoscale (1-100 nm) (Bayda et al., 2020; Iqbal et al., 2012). According to The European Commission on the definition of nanomaterials, there is no scientific data to validate the appropriateness of this value (1-100 nm by general consensus) (EU, 2011).

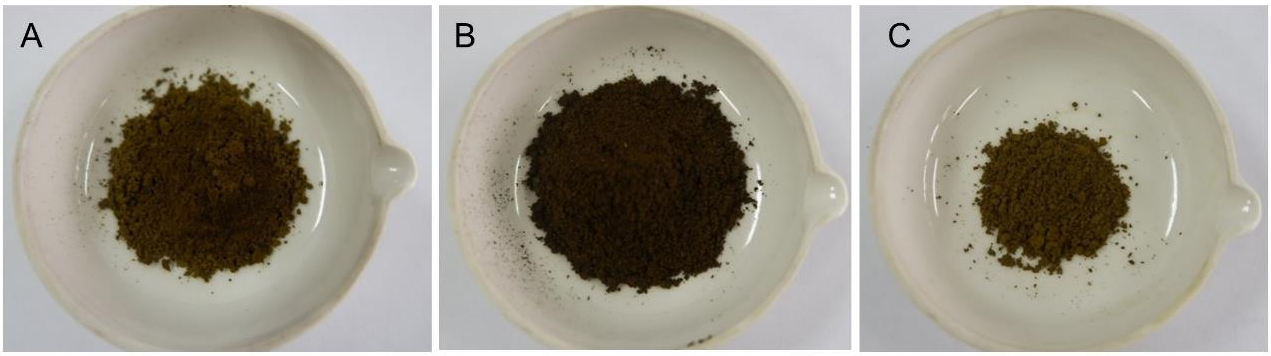

Figure 2. Nanopowder extract of *A. hispida* leaves at 5000 rpm for 5 minutes (**A**), 10 minutes (**B**), and 40 minutes (**C**).

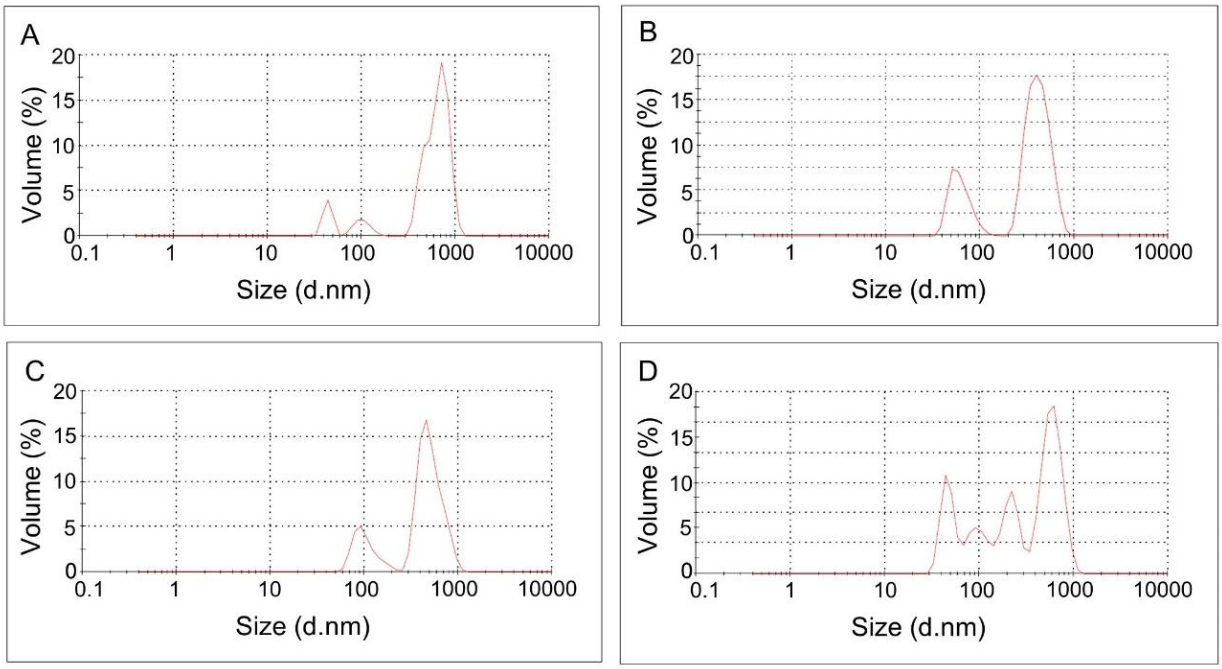

Figure 3. Size distribution by volume of nanopowder extract of *A. hispida*. **A**: crude extract, **B**: nanopowder A, **C**: nanopowder B, **D**: nanopowder C

In nanomedicine, nanoparticles generally are accepted in the range size of a few nanometers to <1000 nm (Garnett & Kallinteri, 2006; Kettler, Veltman, van de Meent, van Wezel, & Hendrik, 2014). In addition, researchers also defined a nanoparticle as a solid or dispersal particle with a scale of 10-1000 nm (Mohanraj & Chen, 2007; Rizvi & Saleh, 2018). The particle size of 100-1000 nm includes nanoscale and not microscale because microparticle is a term to describe particles in diameter of 1-1000 µm (Stack et al., 2018). Many researchers used the term "nanoparticle" to their materials in size >100 to 1000 nm, such as 150-250 nm (Wang et al., 2017), 234.7 nm-892.6 nm (Imansari, Sahlan, & Arbianti, 2017), 333 nm (El-Far et al., 2017), and 680 and 974 nm (Venkatachalam, Thiyagarajan, & Sahi, 2015).

The application of nanotechnology has rapidly grown in various fields of science, such as chemistry, physics, biology, biomedicine, technic, and electronic (Bayda et al., 2020; Contreras, Rodriguez, & Taha-Tijerina, 2017; El-Sayed & Kamel, 2020). In the biomedicine field, nano-sized particles affect stability, drug profile release, biodistribution, mucoadhesive, drug delivery systems, and uptake cellular (internalization) (El-Sayed & Kamel, 2020). Cellular internalization or endocytosis in the biological systems is through phagocytosis or pinocytosis. Phagocytosis is mainly achieved by a material larger than 1 μm, and pinocytosis is achieved by a material smaller than 1 μm (Danaei et al., 2018). Nanopowder C extract in the size of 512.2 nm possible enter the cells by pinocytosis mechanism (**Table 3**). In addition to size particle, physicochemical character influencing endocytosis mechanism is polydispersity index (PDI) (Sahay, Alakhova, & Kabanov, 2010).

The distribution of size population (polydispersity index, PDI) in the crude and nanopowder extract was shown in **Table 3**. The PDI of nanopowder A, B, and C extract, respectively, was 0.696, 0.717, and 0.612. The time milling for 40 minutes indicated the best PDI compared with 5 and 10 minutes. These results revealed that the duration of time milling at the same speed rotation reduced size particle and PDI value.

PDI is used to describe the grade of non-uniformity of the size distribution of particles (Bera, 2015). The technique to maintain the size and size distribution of particles is extrusion, sonication, homogenization, and freeze-thawing (Bahari & Hamishehkar, 2016; Danaei et al., 2018). The high monodisperse (homogenous) size distribution is shown by PDI's value smaller than 0.7; however, the heterogeneity size distribution is determined by PDI's value larger than 0.7 (Danaei et al., 2018). As shown in that criteria, nanopowder C extract by PDI 0.612 is in the range of monodisperse size distribution (0.005-0.7) (**Table 3**).

Table 3. The average size distribution and polydispersity index of *A. hispida* leaves extract

Nanopowder extract	Milling time (Minutes)	Average size distribution (d.nm)	Polydispersity index (PDI)
Crude	-	1271.0	0.754
A	5	837.1	0.696
B	10	803.8	0.717
C	40	512.2	0.612

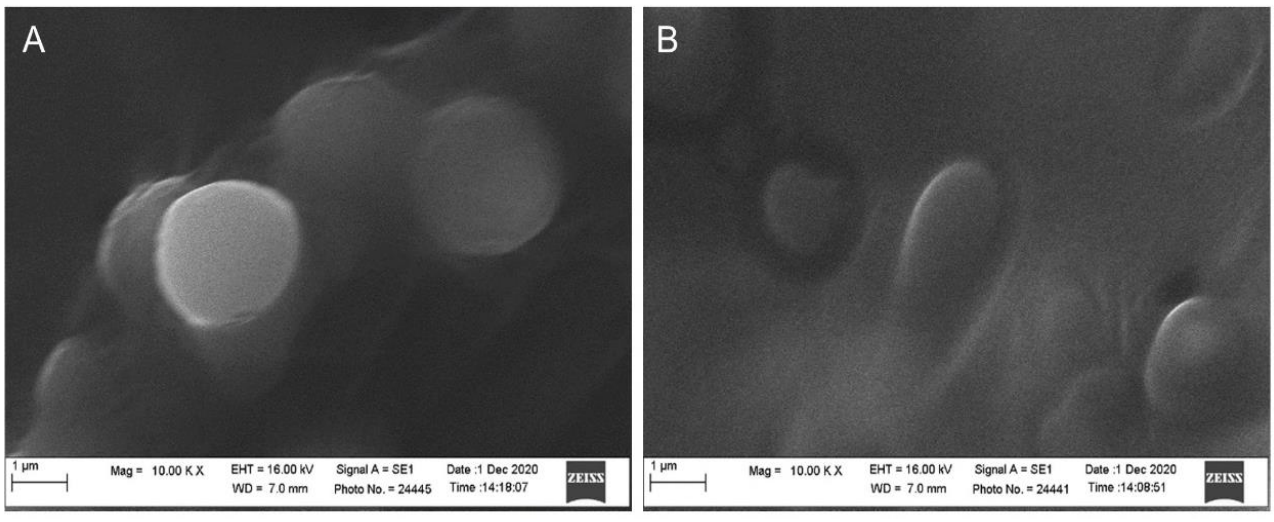

Figure 4. The SEM images of crude extract (**A**) and nanopowder C extract (**B**) of *A. hispida* leaves at magnification 10000x.

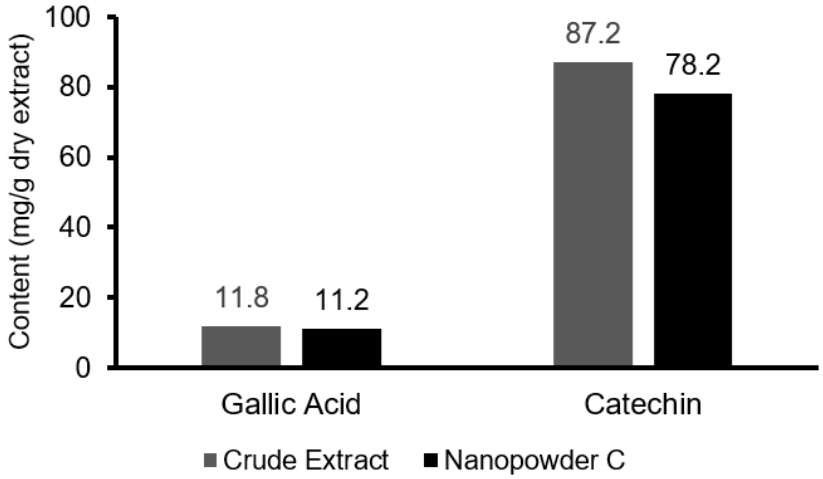

Figure 5. The gallic acid and catechin content of crude and nanopowder C extract of *A. hispida* leaves.

Gallic Acid and Catechin Concentration

The contain of gallic acid and catechin of crude and nanopowder C extract was shown in **Figure 5**. The crude extract of *A. hispida* contained 11.8 mg/g dry extract of gallic acid and 87.2 mg/g dry extract of catechin. The gallic acid and catechin content of nanopowder C extract were 11.2 mg/g dry extract and 78.2 mg/g dry extract, respectively. Milling time of extract for 40 minutes at 5000 rpm reduced 5% of gallic acid and 10.3% catechin.

As shown in **Figure 4**, the gallic acid and catechin compound of crude extract of *A. hispida* leaves in the present study confirmed previous research (Alfarisi et al., 2020). The reduced level of gallic acid and catechin was maybe caused by the heat and pressure effect in the station during the milling process. The previous research showed that the preparation of herbal nanoparticles sometimes increased or decreased bioactive compounds (Borhan et al., 2013). However, the reduced compounds of *A. hispida* leaves extract were not necessarily lowered efficacy because nano-sized particles increased biodistribution and uptake cellular as described before. So that, further research *in vivo* was needed to study its biological effect.

CONCLUSIONS

In conclusion, nanopowder C extract of *A. hispida* leaves, shown by the average distribution size of 512.2 nm and polydispersity index of 0.612, was successfully produced using planetary ball milling for 40 minutes at 5000 rpm. The increasing milling duration decreases the average size distribution and polydispersity index of particles. The milling process for 40 minutes reduced 5% gallic acid and 10.3% catechin in the crude extract.

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