Fractionation using adsorptive macroporous resin HPD-600 enhances antioxidant activity of *Gnetum gnemon* L. seed hard shell extract

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Revised: 23 July 2017 / Accepted: 7 August 2017 / Published online: 4 September 2017
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Abstract In this study, antioxidant activities and identification of the bioactive substances in *Gnetum gnemon* L. (Gg) seed hard shell were evaluated. The seed of *Gnetum gnemon* L., an Indonesian native plant, is commonly consumed as a vegetable or further processed as cracker. Isolated substances from *Gnetum gnemon* seed are mainly stilbenoid derivatives which show potent antioxidant, tyrosinase inhibitor, and antimicrobial activities. Nevertheless, the antioxidant activity of its crude extract is still considered weak. In this study, an effort was made to improve antioxidant potency by fractionation using macroporous adsorptive resin (MAR). This fractionation successfully enhanced antioxidant activity of red Gg seed hard shell extract with efficient adsorption contact time within 30 min. Antioxidant activity of fractions 25–75% v/v ethanol increased three- to sevenfold as compared to crude extract and more importantly resulted in dry product which was easier for further processes. Identification of bioactive compounds in Gg seed hard shell extract with different degrees of ripeness was also performed by HPLC and confirmed the presence of Gnetin C, resveratrol, and other stilbenoid derivatives. These other stilbenoid derivatives could be the main substances contributing in antioxidant action with lower IC₅₀ as compared to both

Gnetin C and resveratrol. In summary, fractionation process using MAR HPD-600 reduced unnecessary sugar molecules from red Gg seed hard shell extract hence resulting to fraction with strong antioxidant activity.

Keywords *Gnetum gnemon* · Seed hard shell · Antioxidant · HPD-600 · Stilbenoid

Introduction

Antioxidant activity is the ability of compound to scavenge free radical or to complex metal ion that driving oxidation process (Tirzitis and Bartosz 2010). Free radicals are unstable substance that can lead to ageing and also serious chronic diseases such as cancer, asthma, atherosclerosis, diabetic, Alzheimer, angiogenesis and hyperuricemia. These chronic diseases are associated with extensive damages as the result of cellular breakage (Lobo et al. 2010). Antioxidant which can be found chemically or naturally, is a stable electron donor compound hence scavenging the free radicals (Fries and Püttmann 2002; Nwokocha and Williams 2011; Saraswaty et al. 2015). Thus, it is a promising substance to delay or to inhibit the progression of chronic diseases.

Gnetum gnemon L. (Gg) or melinjo is an Indonesian origin plant. This plant belongs to the genus of *Gnetum* (*Gnetaceae* family) (Manner and Elevitch 2006). The plant from the family of *Gnetaceae* has been used as folk medicine since many years. Phytochemical studies of the seed, root, and other different parts of Gg reported the presence of stilbenoid derivatives as the main compound which exhibits a wide range of pharmacological activities (Kato et al. 2009, 2011). Amongst others, a Gg seed is the most edible part and traditionally consumed as crackers or included in preparation of

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sour vegetable soup. Several studies on a Gg seed extract reported that the seed has some interesting activities such as antitumor, antioxidant, anti tyrosinase, anti hyperuricemia, lipase inhibitor, anti-diabetes, and also antimicrobial against pathogenic bacteria (Barua et al. 2015; Konno et al. 2013; Yanagihara et al. 2012; Wazir et al. 2011). Despite a screening antioxidant activity from different parts of Gg seed shows that the seed hard shell is a most promising material for antioxidant, its activity is considered weak. It might due to lack in extraction process. (Wazir et al. 2011) Hence, a better strategy in extraction and fractionation processes to enhance its antioxidant activity must be explored, particularly to remove other unimportant substances. It is well known that Gg seed highly contains carbohydrate or sugar in the part of endosperm. Previous study reported that melinjo seed extract contained 79.8 g carbohydrates/100 g melinjo seed (Tatefuji et al. 2014). There is no report describing whether carbohydrate/sugar also presence in the seed hard shell. Commonly, the carbohydrate/sugar creates many problems during stages in the purification process. Removing sugar from seed hard shell extract is suggested as a good approach to enhance the yield of target compounds. For that reason, we are interested in applying MAR HPD-600 for fractionation of crude red Gg seed hard shell extract.

Macroporous Adsorptive Resins (MAR) is non-ionic resins that is characterized by a large number of permanent pores which are considered to be “spacious” and are accessible to large molecules. The major applications of MAR have expanded from refining sugar to separation and enrichment of pharmacologically active natural products. Separation of pharmacologically active compound using MAR is an advantage due to low cost, less solvent consumption, easy to be regenerated and also green process. (Li and Chase 2010) It also has been reported that yield of some bioactive compounds are successfully enhanced after fractionated using MAR (Fu et al. 2007; Ye et al. 2014).

In present report, we describe the investigation on antioxidant activity from different parts of Gg seed extract and the optimization of fractionation process to enhance bioactivity in detail. In addition, static adsorption kinetic and isotherm for fractionation process of red Gg seed hard shell extract using a polar MAR HPD-600 was also studied. To our knowledge, this is the first study reporting the application of MAR HPD-600 to improve the bioactivity of Gg seed hard shell extract by increasing the yield of target active compound.

Materials and methods

Chemical and reagents

Gnetum gnemon seeds were collected from Imogiri Bantul, Yogyakarta Indonesia, in November to

December 2016. Organic solvents: ethanol from Bratachem, Bandung, Indonesia with technical grade, acetonitrile from Merck (Darmstadt, Germany) with pro analysis grade. 1-diphenyl-2-picryl-hydrazil (DPPH) radicals, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical, Sodium persulphite and Neocuproine were from Sigma Aldrich (St. Louis, MO, USA). Potassium dihydrogen phosphate, Ferric chloride, Trichloroacetic acid, Anthrone, Sulphuric acid and Ammonium acetate are from Merck (Darmstadt, Germany) with pro analysis grade.

Preparation of Gg extract

Gnetum gnemon (Gg) seeds were collected from Imogiri Bantul, Yogyakarta Indonesia, in November to December 2016. The outer skin, hard shell and endosperm were separated manually by hand. Gg seed hard shell powder of 500 g was macerated in 4 L ethanol 50% v/v for 3 × 24 h at ambient temperature. The filtrates were collected and concentrated with evaporator under vacuum by Heidolph rotary vapour (Heidolph Instrument GmbH and Co.KG, Schwabach, Germany). Concentrated extract was used for further fractionation and analysis.

Carbohydrate/sugar assay by anthrone method

The presence of carbohydrate or sugar in Gg seed hard shell extract was determined qualitatively using Anthrone method with small modification. One g of Anthrone was dissolved in 500 mL 72% v/v sulphuric acid. One mL of sample in a test tube was mixed with 5 mL of Anthrone reagent. Subsequently, the mixture was incubated in water bath at 80 °C for 10 min. Positive carbohydrate or sugar was indicated by blue green color development (Morse 1947).

Preparation of adsorbent

Macroporous resins HPD-600 (Cang Zhou Bonchem Co., Ltd., Hebei, China) was used as adsorbent. The MAR was extensively washed with aquadest. Prior to adsorption experiments, MAR HPD-600 was washed subsequently using ethanol then followed with aquadest.

Static adsorption kinetic experiment

Adsorption kinetic curve of total phenolic content (TPC) from red Gg seed hard shell extract on MAR HPD-600 was studied according to Fu et al. (2007 and Wang et al. (2015). Pre-weighed amount of MAR HPD-600 equal to 0.5 g dry MAR-HPD-600 was put in Erlenmeyer flask

with 5 mL of crude Gg seed hard shell extract (30 mg/mL, in aquadest), followed by shaking at 25 °C for 210 min (150 rpm). Total phenolic content (TPC) was then determined spectrophotometrically using HITACHI U-2800 spectrophotometer (HITACHI High-Technologies, Tokyo, Japan) at certain time intervals (30, 60, 90, 120, 150, 180, and 210 min). TPC was determined from linear regression of Gallic Acid as reference standard (Gallic Acid Equivalent/GAE). Initial concentration of TPC (GAE) in crude red Gg seed hard shell extract was also determined. Adsorption capacity (Q_e) was then calculated with Eq. 1:

$$Q_e = \frac{(C_o - C_e) \cdot V}{m} \quad (1)$$

Q_e is Adsorption capacity (mg/g dry resin), C_o is initial Total Phenolic Content (Gallic acid equivalent /GAE) (mg/mL), C_e is total phenolic content after adsorption at equilibrium (mg/mL), V is working volume (mL), and m is weight of dry MAR HPD-600 (g).

% Adsorption ratio was calculated using Eq. 2:

$$\% \text{Adsorption ratio} = \frac{(C_o - C_e)}{C_o} \times 100\% \quad (2)$$

C_o is initial TPC (GAE), C_e is TPC (GAE) after adsorption at equilibrium.

Static adsorption isotherm experiment

Adsorption equilibrium data were obtained by introducing pre-weighed amount of MAR-HPD-600 equal to (0.5 g dry MAR HPD-600) into four different volume solutions of crude red Gg seed hard shell extract ranging from 2.5, 5, 7.5 and 10 mL at concentration of 30 mg/mL and shaking at 150 rpm at ambient temperature. The equilibrium concentration of total phenolic content in adsorption solution was determined spectrophotometrically using Hitachi U2800 spectrophotometer. Freundlich and Langmuir equations were used to describe adsorption behavior of MAR-HPD-600 (Fu et al. 2007; Wang et al. 2015).

Freundlich equation is shown by Eq. 3:

$$q_e = K_F \cdot C_e^{1/n} \quad (3)$$

Meanwhile for Langmuir equation is described as Eq. 4

$$\frac{C_e}{q_e} = \frac{1}{Q_o \cdot b} + \frac{C_e}{Q_o} \quad (4)$$

where q_e is Adsorption capacity (mg/g dry resin), K_F is Freundlich constant, $1/n$ is adsorption intensity, C_o and C_e are initial and equilibrium TPC (GAE) (mg/mL), Q_o is maximum adsorption capacity (mg/g dry resin), and b is Langmuir constant.

Scale up fractionation using MAR HPD-600

MAR HPD-600 was activated in ethanol 95% then washed with aquadest. Water soluble concentrate extract was packed into MAR HPD-600 column and pre-eluted with aquadest for sugar refining. Further, the column was eluted with various ratios of ethanol/water: 25, 50, 75 or 95% v/v. The filtrates for each fraction were collected and dried with evaporator rotary under vacuum.

Antioxidant activity determination

1-diphenyl-2-picril-hydrazil (DPPH) assay

DPPH assay was performed according to Saraswaty et al. (2015) and Herald et al. (2012). A sample with 50 µL of volume at various concentrations was mixed with 200 µL of DPPH free radicals (5 mg in 100 mL methanol). The mixture was incubated at room temperature for 30 min. The absorbance was then measured at λ 517 nm. Percentage of inhibition was calculated using Eq. 5:

$$\% \text{Inhibition} = \frac{[C - S]}{C} \times 100\% \quad (5)$$

C is Absorbance of control (DPPH), S is Absorbance of sample. IC_{50} was determined based on linear regression of the concentration versus % inhibition curve. Ascorbic acid and α -tocopherol were used as standards. Antioxidant Activity Index (AAI) was calculated using Eq. 6:

$$AAI = \frac{[DPPH]}{IC_{50}} \quad (6)$$

$[DPPH]$ is concentration of DPPH (ppm) and IC_{50} is Inhibition concentration 50% (ppm) (Scherer and Godoy 2009).

2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay

ABTS assay was performed based on the protocol described by Büyüktuncel et al. (2014) and Thaipong et al. (2006). A sample of 20 µL at various concentrations was mixed with 180 µL of ABTS radical (Absorbance at λ 734 nm of 0.7 ± 0.02). The mixture was incubated at room temperature for 7 min. The absorbance was then measured at λ 734 nm. Percentage of inhibition was calculated using Eq. 5. Ascorbic acid and α -tocopherol were applied as standards. IC_{50} was determined based on linear regression of the concentration versus % inhibition curve.

Cupric reducing activity capacity (CUPRAC)

CUPRAC assay was performed based on the protocol described by Badarinath et al. (2010) A sample of 250 µL

was mixed with 500 μL of 10 mM Cooper chloride, 500 μL of 7.5 mM neocuproine and 500 μL of 1 M ammonium acetate buffer pH 7. The mixture was incubated for 30 min at room temperature and measured against blank at λ 450 nm. Reducing power activity was then determined based on linear regression of α -tocopherol and ascorbic acid as positive reference standards.

Ferric reducing activity power (FRAP) assay

FRAP assay was performed according to Nishaa et al. (2012). A sample of 100 μL at concentration of 0.1 mg/mL was mixed with 1250 μL phosphate buffer (pH 6.6, 20 mM) and 1250 μL Potassium ferricyanide (1% w/v in aquadest). The mixture was incubated at temperature of 50 °C for 30 min. Further, the mixture was added with 1250 μL Trichloro acetic acid (10% w/v in aquadest) and 250 μL FeCl_3 (0.1% w/v in aquadest). The reaction was allowed for 10 min. Subsequently, the absorbance was read at λ 700 nm using Hitachi U2800 Spectrophotometer. Reducing power activity was then determined based on linear regression of α -tocopherol as a positive reference standard.

Total phenolic content determination

Determination of total phenolic content was conducted using Folin Ciocalteu method (Saraswaty et al. 2015; Javanmardi et al. 2003). A volume of 300 μL of aliquot was put in test tube. Then 1200 μL of Folin Ciocalteu (10% v/v) and 1500 μL of Na_2CO_3 7.5% (w/v) were added sequentially. The test solutions were incubated (50 °C, 15 min) and then vigorously shaken. Absorbance at λ 760 nm was recorded with Hitachi U2800 spectrophotometer. A standard calibration plot was generated using known concentration of gallic acid. The concentration of total phenolic content in the test samples were calculated from the calibration plot and expressed as Gallic Acid Equivalent (GAE) per g of sample (concentrated extract).

High performance liquid chromatography (HPLC) analysis

HPLC analysis was performed to identify the bioactive compound in the extract, using Waters 1525 and 2487 instrument (Waters, Milford Massachusetts, USA). The separations were performed on a C-18 column (250 mm $\times$ 4.6 mm, 5 μm , Phenomenex: Torrance, USA). The mobile phase was acetonitrile:water (50:50, v/v), with consistent flow rate at 0.8 mL/min. The injection sample volume for each run was 50 μL . Detection of the main suggested active compound Gnetin C and resveratrol was carried out at maximum absorption wavelength of 280 nm.

Statistical analysis

Samples were analyzed in triplicates. All data were presented as average $\pm$ Standard deviation (SD). Microsoft Excell 2013 was applied for one-way analysis of variant (ANOVA) in order to assess the significant difference ($p < 0.05$).

Results and discussion

Antioxidant activity of different part of Gg seed extract and solvent for extraction

Gg seeds consist of outer skin, hard shell and endosperm (Fig. 1a–c). Gg seeds are also available in different color based on degree of ripeness (Fig. 1d–f). Previous studies done by Barua et al. (2015) and Wazir et al. (2011) explored the antioxidant activity of different parts of Gg extracts but not the Gg seed hard shell. They detected weak activity of these part extracts to inhibit free radical reaction by DPPH assay ($< 50\%$ inhibition using 300 ppm of each part extract). Therefore, we focused to explore the Gg seed only and the screening of antioxidant activity from different parts of Gg seed extract was shown (Table 1). It revealed that red/old seed hard shell exhibited the highest antioxidant activity for scavenging DPPH free radical cation with IC_{50} of 162.12 ± 1.46 ppm (significant difference $p < 0.05$) with AAI index of 0.30. This potency was higher as compared to previous reports. We therefore suggest that Gg seed hard shell is a potential source for antioxidant.

In crackers home industry of Gg, outer skin and endosperm of Gg seeds are used for production of edible crackers, the seed hard shell is unused and left as a waste. The successful outcome we reported here encourages further investigation as this part will gain big interest, not only in term of health but more importantly to safe environment from bio-waste.

We report the use of mild process on extraction by using ethanol, the most common organic solvent for pharmaceutical products. The antioxidant activity of Gg seed hard shell ethanol 50% extract was greater than ethanol 95% extract. It is well known that ethanol is flammable and potentially explosive solvent. Yet, ethanol is used on a large scale for natural product extraction, because it is easily available, has a low price, completely biodegradable, and regulatory comply. In green extraction concept, diluted ethanol is more environmentally friendly (Valavanidis and Vlachogianni 2012). According to safety consideration for human being and environmental issue as well as based on antioxidant activity screening, we selected mixture of ethanol-water (50% v/v) as a solvent for Gg seed hard shell

Fig. 1 Different parts and colour of Gg seed (outer skin peel (a), hard shell (b), endosperm (c) young/green seed (d), medium/orange seed (e), old/red seed (f)) (color figure online)

Table 1 Screening of antioxidant activity based on DPPH assay for different parts of Gg seed

Parts of seed	Solvent for extraction	IC ₅₀ (ppm)	AAI	Concentration (µg/mg extract)	
				Resveratrol	Gnetin C
Outer skin	95% ethanol	338.26 ± 7.69 ^A	0.15	n.d	n.d
Hard shell	95% ethanol	327.69 ± 6.78 ^B	0.15	n.d	n.d
Endosperm	95% ethanol	1549.90 ± 70.29 ^C	0.03	n.d	n.d
Hard shell	50% ethanol	200.44 ± 2.98 ^D	0.25	n.d	n.d
Red/old hard shell	50% ethanol	162.12 ± 1.46 ^E	0.30	0.02 ± 0.06	4.44 ± 0.32
Orange/medium hard shell	50% ethanol	200.93 ± 8.93 ^F	0.25	0	11.48 ± 0.39
Green/young hard shell	50% ethanol	374.39 ± 18.61 ^G	0.13	0.62 ± 0.04	21.54 ± 1.82

Data presented as average (n = 3) ± standard deviation (SD)

AAI antioxidant activity index, n.d not determined

Different subscript letter (A–G) showed significant difference (*p* values < 0.05)

extraction. However, the lack of using ethanol:water in this extraction process is co-presence of sugar (Chavan and Amarowicz 2013), as also positively detected in this report by using Anthrone assay. The high content of sugar in some cases disturbing further steps when the end product is required in dry state. This matter leads to solid paste form after dried Gg seed hard shell extract. This further on will reduce the bioactivity such as antioxidant.

Adsorption kinetic of TPC from Gg seed hard shell extract on MAR HPD-600

Adsorption ratio and capacity of TPC from Gg seed hard shell at a various contact times is listed in Table 2. As presented, adsorption capacity of phenolic compound from red Gg seed hard shell extract on MAR HPD-600 increased

rapidly at first 5 min and reached equilibrium in 30 min (Fig. 2a) with adsorption ratio of 95.29%. Adsorption capacity of phenolic compound from red Gg seed hard shell extract at minute 30 was 13.36 mg/g dry resin. The static adsorption kinetic of crude red Gg seed hard shell extract on MAR HPD-600 showed fast adsorption characteristic.

Adsorption isotherm of TPC from Gg seed hard shell extract on MAR HPD-600

Adsorption isotherms curves of TPC from Gg seed hard shell extract on MAR HPD-600 at 25 °C is depicted in Fig. 2b. TPC of red Gg hard shell crude extract increased along with extract weight and adsorption capacity, yet the adsorption ratio was decreased.

Table 2 Total phenolic content (GAE), adsorption capacity and adsorption ratios of phenolic content in red Gg hard shell extract on MAR HPD-600 at various contact time

Time (min)	TPC (GAE) (mg/mL)	Adsorption capacity (Qe) (mg/g dry resin)	Adsorption ratio (%)
0	1.40	0.00	0.00
5	0.10	12.98	92.58
10	0.10	12.99	92.67
15	0.10	13.06	93.17
20	0.09	13.10	93.63
30	0.07	13.36	95.29
60	0.07	13.35	95.27
90	0.08	13.25	94.70
120	0.08	13.20	94.19
150	0.07	13.28	94.79
180	0.08	13.22	94.27
210	0.07	13.31	94.92

Fig. 2 Adsorption kinetics curves (a) and adsorption isotherms curves (b) for TPC (GAE) on MAR HPD-600 at 25 °C

Based on Fig. 2b, it can be determined the linear equation of Freundlich and Langmuir models are $y = x + 0.0301$ ($R^2 = 1$) and $y = 0.00007x + 0.5068$ ($R^2 = 0.7663$) respectively. The theoretical maximum adsorption capacities Q_0 of phenolic compound from red Gg hard shell extract on MAR HPD-600 was determined from the Langmuir equation accounted as 14.285 mg TPC (GAE)/g dry resin MAR HPD-600. Based on the correlation coefficient value (R^2), adsorption of phenolic compound from red Gg hard shell extract onto MAR HPD-600 followed the Freundlich model. In regard to the adsorption isotherms curves (Fig. 2b), it is suggested that MAR HPD-600 formed multilayer adsorption of TPC.

Effect of fractionation process of red Gg seed hard shell extract onto MAR HPD-600

MAR has demonstrated a highly potent and effective process to produce numerous bioactive fractions (Fu et al. 2007; Ye et al. 2014). MAR HPD-600 has polystyrene functional group with surface area of 550–660 m²/g. The small average pore diameter of 8 nm and particle diameter in the range of 0.3–1.2 mm lead to high adsorption

capacity for polar compound (Yue et al. 2016; Du et al. 2012). Based on this characteristic, we applied MAR HPD-600 for fractionation process of red Gg seed hard shell extract. As confirmed by our assay using Anthrone method, Gg seed hard shell extract was proven containing carbohydrate/sugar. The water fraction that gave positive results on carbohydrate/sugar was obviously inactive. Ethanolic fractionation process of red Gg seed hard shell extract using MAR HPD-600 significantly reduced these unnecessary sugar molecules hence enabling to produce the dry powder which made easier for further processes. Fractionation process using MAR HPD-600 eluted with 25–75% v/v ethanol produced dry powder with yield of fractionations are 4, 8 and 1.3% w/w, respectively as compared to crude extract. Among these ethanol fractions, fraction of 50% v/v ethanol produced the highest yield of dry powder.

The antioxidant activity of both red Gg seed hard shell extract and its fractions is presented in Table 3. As clearly shown, fractionation process of red Gg seed hard shell extract using MAR HPD-600 successfully enhanced the antioxidant activity. As also seen, using different antioxidant assays, the results were different (significant

difference p value 0.05). Fraction 75% v/v ethanol exhibited the greatest antioxidant activity for scavenging DPPH cation and ABTS anion, with IC_{50} of 37.15 ± 1.08 and 5.59 ± 0.50 ppm respectively. While, the strongest reducing power was shown by fraction with 50% ethanol.

Antioxidant activity index (AAI) can be classified into poor (AAI < 0.5), moderate (AAI between 0.5 and 1.0), strong (AAI between 1.0 and 2.0), and very strong (AAI > 2.0) (Scherer and Godoy 2009; Faustino et al. 2010). AAI of red Gg seed hard shell ethanol fractions is in the range of 0.7–1.7, indicating all ethanol/water fractions exhibited moderate to strong antioxidant activity. By referring to DPPH and ABTS assay, the highest antioxidant activity increased 5- and 3.8-fold respectively (Fractions of 75% v/v). Meanwhile, the strongest reducing power activity increased seven and three-fold respectively (50% v/v fraction) by CUPRAC and FRAP assays. As fraction with 75 and 50% v/v ethanol showed different activity, it is suggested that both fractions contained different bioactive compounds. The compounds showing reducing power activity has more polar characteristic than that act as free radical scavenger.

Gnetin C and resveratrol content in Gg seed hard shell extract

Gg seeds contain high amount of stilbenoid derivatives which major compounds are Gnetin C and resveratrol (Kato et al. 2009; Yanagihara et al. 2012). However, they did not describe the influence of the ripeness of the seed on the content of these substances. In present study, HPLC method was applied to identify the bioactive substances of Gg seed hard shell extract with different degrees of ripeness (Fig. 3). The presence of resveratrol and Gnetin C were clearly detected at retention time (Rt) of 4.9–5.0 and

6.1–6.3 min, respectively and others not identified yet (Fig. 3). The content of Gnetin C in Gg seed hard shell extract was more dominant than resveratrol and significantly different with reverse correlation to the ripeness. However, presence of high content Gnetin C in the young Gg seed hard shell extract was not confirmed by strong antioxidant activity (Table 1).

The content of resveratrol and Gnetin C were highest in young/green hard shell extract accounted respectively as 0.62 and 21.54 $\mu\text{g}/\text{mg}$ concentrate extract (Table 1). Although this part contained the highest Gnetin C and resveratrol level, its antioxidant activity was the lowest among others. It is clearly depicted on HPLC chromatograms that degree of ripeness of Gg seed contributed to the quantity of Gnetin C, a phenolic compound in the form of stilbenoid dimer (Yanagihara et al. 2012). Yet, content of Gnetin C decreased along with the ripeness. For that reason, we suspect Gnetin C in the Gg seed hard shell is a precursor for biosynthesis of other antioxidant compound detected at Rt of 2.5 min. This unidentified compound was allegedly having better antioxidant activity and need to be explored and elucidated.

Conclusion

Application of MAR HPD-600 in fractionation process as reported in this study successfully enhanced antioxidant activity of Gg seed hard shell extract. MAR HPD-600 provides a good material for fractionation to remove unnecessary sugar molecules from red Gg seed hard shell extract. The fraction contained gnetin C, resveratrol, and other stilbenoid derivatives. However, both gnetin C and resveratrol did not contribute directly to the antioxidant activity. The presence of gnetin C, in particular, could be

Table 3 Antioxidant activity of red Gg hard shell extract and various fractions

Sample	DPPH (IC_{50} , ppm)	AAI	ABTS (IC_{50} , ppm)	FRAP TEC	CUPRAC	
					AAEC	TEC
Crude ethanol 50% extract	162.12 ± 1.46	0.30	18.73 ± 0.35	2.62 ± 0.2	89.75 ± 11.52	217.86 ± 13.73
Water fraction	1453.64 ± 37.40	0.03	n.d	n.d	n.d	n.d
Fraction 25%	70.16 ± 2.63	0.70	11.80 ± 0.68	6.54 ± 0.9	218.68 ± 16.25	371.58 ± 19.38
Fraction 50%	37.15 ± 1.08	1.40	5.59 ± 0.50	19.76 ± 1.5	306.60 ± 1.62	587.26 ± 1.94
Fraction 75%	29.88 ± 1.69	1.70	4.90 ± 0.59	19.14 ± 0.8	297.39 ± 5.60	465.42 ± 6.67
Fraction 95%	42.05 ± 1.09	1.20	n.d	13.97 ± 0.9	276.42 ± 21.90	440.42 ± 26.07
Ascorbic acid	8.41 ± 0.46		3.93 ± 0.03			
α -Tocopherol	60.7 ± 1.74		8.1 ± 0.05			

Data presented as mean ($n = 3$) $\pm$ standard deviation (SD)

TEC tocopherol equivalent capacity ($\mu\text{g}/100 \mu\text{g}$ sample), AAEC ascorbic acid equivalent capacity ($\mu\text{g}/100 \mu\text{g}$ sample), n.d not determined

All data showed significant difference (p values < 0.05)

Fig. 3 HPLC chromatogram of Gg seed hard shell extract from different colour of seed. Red (a), orange (b) and green (c). Resveratrol and Gnetin C are clearly detected at Rt of 4.9–5.0 and 6.1–6.3 min respectively

the precursor for biosynthesis of the other unknown stilbenoid derivatives which play role as antioxidant.

Acknowledgements This project is financially supported by Ministry of Research Technology and Higher Education of Republic of Indonesia, under scheme of Consortium of Incentive of National Innovation System Research, year 2016.

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