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Antioxidant potential of non-oil seed legumes of Indonesian's ethnobotanical extracts

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Abstract This study investigated the *in vitro* antioxidant properties (DPPH, ABTS, CUPRAC and FRAP), total phenolic content and flavonoid content of extracts from three non-oil seed legumes (*Phaseolus lunatus* red and white, and *Canavalia ensiformis*), local edible seeds from Indonesia, obtained using different solvent system (distilled water, 70% ethanol, and 100% ethanol). The variety of legume was a major source of variation in the phenolic contents, flavonoid content and antioxidant activity. HPLC analysis of the non-oil seed legume extracts identified gallic acid, epicatechin and coumaric acid. Among the varieties of non-oil seed legume extracts, the phenolic content varied from 15.21–38.60 mg gallic acid equivalents/g dry weight and the flavonoid content was 11.73–24.61 mg catechin equivalents/g dry weight. The antioxidant activity of the extracts suppressed the reactive oxygen species (ROS) generation and cellular damage induced by UV-B in HaCaT cells. These results showed that antioxidant activity (1.83–19.42% of inhibition DPPH; 2.99–37.29% of inhibition ABTS; 0.20–2.47 µM CUPRAC value; and 0.96–1.10 µM of FRAP value) of extracts possessed strong radical scavenging activity as well as inhibited ROS generation in a dose-dependent manner without showing any cytotoxicity. Collectively, the data presented that antioxidant of the extracts have potent antioxidant activity and decreasing ROS generation in HaCaT cells. It can be intimately used as alternative criterion for antioxidant and antiradical activities that can be utilized as a functional food and nutraceutical ingredients.

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1. Introduction

Underutilized plants are genetically very diverse groups grown in temperate, subtropical and tropical regions and have been recognized for their human health benefits. Most of the underutilized plants have high content of non-nutritive, nutritive, and bioactive compounds such as flavonoids, phenolics, anthocyanins, phenolic acids, and as well as nutritive compounds such as sugars, essential oils, carotenoids, vitamins,

and minerals (Dogan et al., 2014; Mollica et al., 2017; Tsukamoto et al., 2018; Fazenda et al., 2019; Sadeer et al., 2019).

Non-oil seed legumes are commonly planted (bean) in marginal land with minus of concern, considered to have originated in Southeast Asia, including Indonesia. The young pods are boiled separately as a vegetable, or together with another vegetables as a soup. The dry seeds are cooked together with rice after soaking water. The seeds are utilized as raw materials of tempeh, a traditional Indonesian fermented food. They are ingested by humans throughout the world, including Indonesia, and are an essential commodity in the optimal human diet because their seed structure and composition confer physiological benefits to the total diet. In addition, the consumption of a legume-rich diet has been associated with a decreased prevalence of many chronic diseases (Hayat et al., 2014), including tumor, diabetes, and cancer (Yeap et al., 2014). Some non-oil grain legumes, including lima bean (*Phaseolus lunatus*) and jack bean (*Canavalia ensiformis* [CES]), have been used for thousands of years as a food and therapeutic plant in Indonesia, yet they have not been well-studied and are underutilised.

Besides their biological benefits, such as anti-glycative, anti-angiogenic, and hepatoprotective effects (Sun et al., 2012; Kim O et al., 2013; Kumar and Reddy, 2014), non-oil seed legumes have high nutritional potential. Edible beans contain appreciable amounts of various nutrients, such as protein, carbohydrate, minerals and vitamins (Ekanayake et al., 2000). In recent years, the human health benefits associated with the polyphenols, fibre and various other components in beans have attracted increasing interest (Hayat et al., 2014; Ombra et al., 2016). Natural antioxidants, mainly the polyphenols, are found abundantly in the seed coats than cotyledons and show potent antioxidant and antiradical activities (Diaz-Batalla et al., 2006). Phenolic compounds exert antioxidant action because they participate in redox reactions by donating electrons and hydrogen atoms, and acting as reducing agents, singlet oxygen suppressers and metal chelators (Tsao and Deng, 2004). There are many varieties of non-oil seed legumes, and their chemical composition and nutritive value vary considerably.

The process of extraction is an essential step in analysing the composition of a plant because it allows obtaining a crude extract of the target compounds for further separation and identification. Each plant has unique characteristics, and so the choice of extraction conditions can greatly influence the recovery of the compounds (Chirinos et al., 2007). The traditional solid–liquid extraction with acetone, ethanol, methanol, water, either alone or as mixtures, is commonly utilized for phenolic constituents. The choice of solvent and its concentration will depend on the nature of the target phenolic constituents and the characteristics of the sample (e.g., seeds, leaves, peel). Other factors known to affect the rate of extraction, yield and purity of extracted polyphenolics, include the particle size of the sample, temperature, pH and duration of the extraction (Chew et al., 2011).

Starting from such hypotheses, this research explored the *in vitro* antioxidant properties and polyphenols (including total phenolic content [TPC] and flavonoid contents) of the extracts from three non-oil seed legume (*P. lunatus* red [PLR] and white [PLW], and CES) from Indonesia, obtained using different solvent systems.

2. Experimental

2.1. Materials

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) (ABTS), α,α'-azodiisobutyr amidine dihydrochloride, gallic acid, catechin and Folin–Ciocalteu reagent were procured from Sigma–Aldrich (USA). As the extraction solvents, 70% and 100% ethanol, acetonitrile, formic acid (high-performance liquid chromatography [HPLC] grade), were from Merck (Germany), and distilled water was acquired from a water distillation plant in our laboratory. All other chemicals were of analytical grade. UV–visible spectra were acquired on a Multiskan GO spectrophotometer (Thermo Fisher Scientific, Japan).

2.2. Sample collection

The non-oil seed legumes (PLR, PLW and CES) (Fig. 1A-C i) were collected from Bondowoso district, East Java, Indonesia. The seeds were collected in polyethylene bags and stored at 4 °C until needed. Seed samples (500 g) were washed, soaked in water for 1 day, peeled, cut, sun-dried, dried in a hot air oven, ground to a fine powder (60-mesh) and stored at 4 °C before extraction. Voucher specimens of the non-oil seed legume samples were deposited in our laboratory (Food Enzyme Biotechnology, Kyungpook National University, Korea) for future reference (2019-Plr, 2019-Plw and 2019-Ce).

2.3. Extraction

The powder of non-oil seeds (30 g) (Fig. 1A-C ii) was extracted three times with i) distilled water, ii) 70% ethanol and iii) 100% ethanol (30 × 300 mL) using an ultrasonic water bath (Powersonic 420, 50/60 Hz) at 40 kHz and 50 °C for 120 min. The supernatants were collected and filtered through filter paper, and the solvent was evaporated using a vacuum rotary evaporator (Eyela N-1000, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The extracts were lyophilised and dissolved in distilled water (Fig. 1A-C iii) to determine their antioxidant activity.

2.4. HPLC analysis

HPLC was conducted to identify the phytochemical characteristics of the non-oil seed legume extracts. The Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) was equipped with an autosampler (SIL-201), an SPD-M20A diode array detector, LC solution 1.22 SPI software and a reverse-phase Phenomenex C18 column (4.6 mm × 250 mm, 5 μm) (Merck, Germany). A stepwise gradient of solvent A (acetonitrile) and solvent B (1% formic acid solution) was utilized with the ration changing each minutes at λ = 280 nm, as described by Brito et al. (2015).

2.5. TPC determination

The crude extracts of PLR, PLW and CES were analysed for TPC by the Folin–Ciocalteu assay as described by Gul et al (2011), using gallic acid as a standard. Specifically, the sample (2 μL) and 100 μL of sodium carbonate (7%) were added to
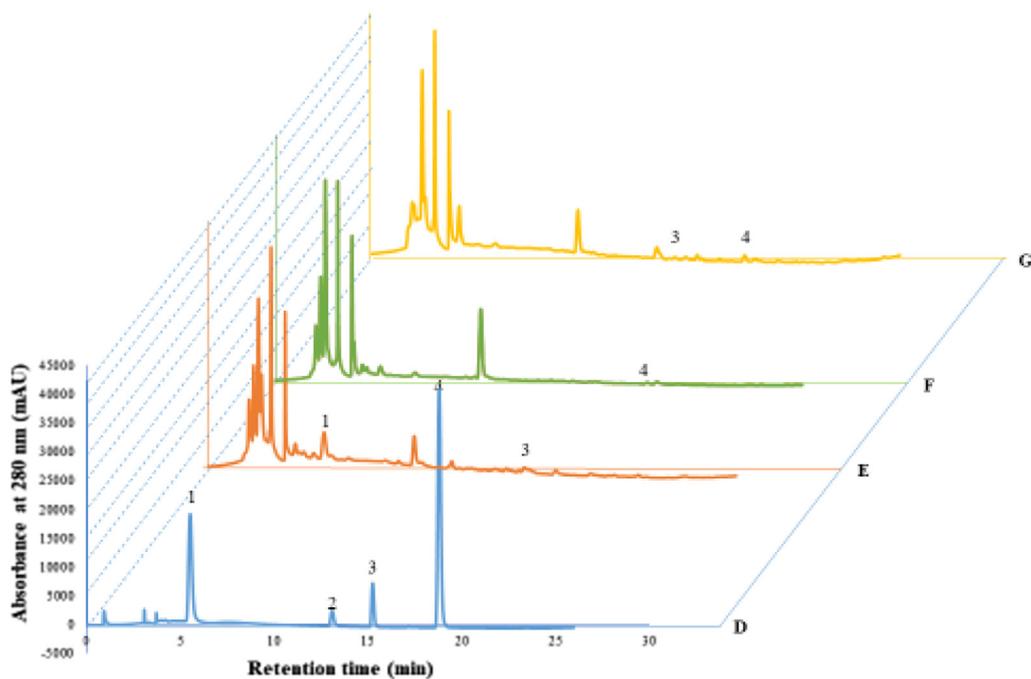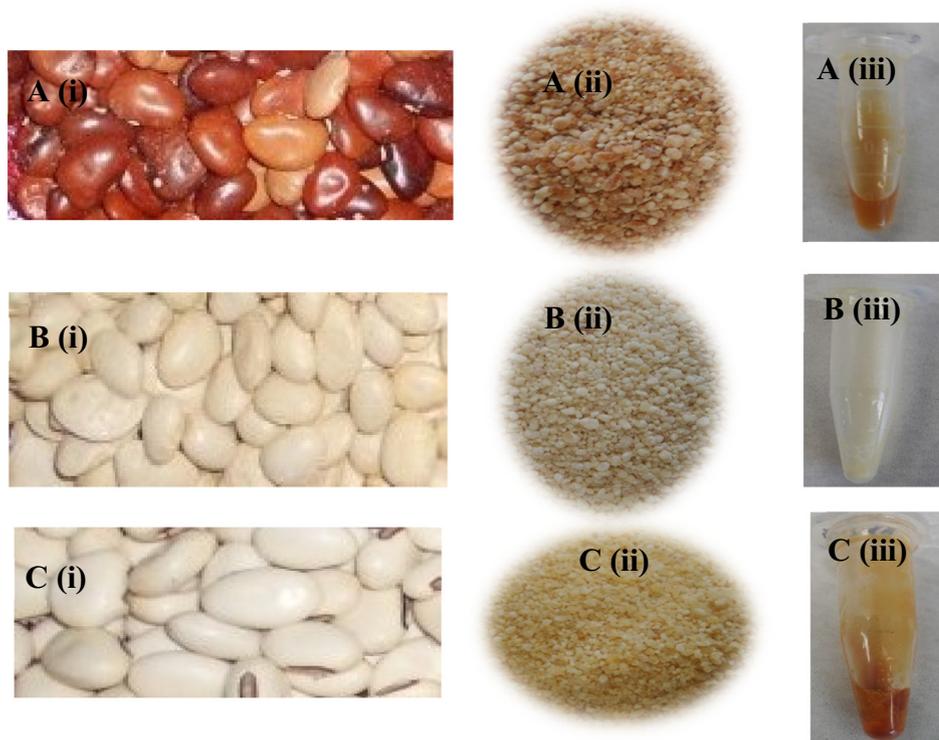
Fig. 1 Photo of non-oil seed legumes varieties, *Phaseolus lunatus* red (PLR) (**A**), *Phaseolus lunatus* white (PLW) (**B**), *Canavalia ensiformis* (CES) (**C**) bean (i), powder (ii), and extract (iii). HPLC (absorbance at 280 nm) – profile of phenolic standards (**D**); *Phaseolus lunatus* red (PLR) (**E**); *Phaseolus lunatus* white (PLW) (**F**); *Canavalia ensiformis* (CES) (**G**). Peaks: gallic acid (1); catechin (2); epicatechin (3); and coumaric acid (4).

10 µL of Folin–Ciocalteau regent (10% v/v). The absorbance was monitored at 595 nm after 10 min of reaction at room temperature. Result were expressed as gallic acid equivalent (GAE) per gram of extract.

2.6. Determination of total flavonoids

Total flavonoid content of PLR, PLW and CES were determined by aluminium chloride colourimetric method, as

described by Zengin et al. (2015), 2 µL of sample and 5% sodium nitrite (5 µL), 10% aluminium chloride (10 µL), 1 M sodium hydroxide (40 µL) and distilled water (43 µL) were mixed. The absorbance was measured at 405 nm after 10 min at room temperature. Catechin was used to construct the calibration curve. Flavonoid content was expressed as milligrams of catechin equivalents per gram of extract (mg CE/g).

2.7. DPPH• free radical-scavenging activity

To evaluate the free radical scavenging activity of extracts we used DPPH method as described by Alam et al (2017). The non-oil seed legume of extracts of different concentrations (3, 10, 30 and 100 µg/mL, respectively) and ascorbic acid (as the standard) were dispensed into a 96-well plate. The volume of each well was adjusted to 200 µL by adding 4% methanolic solution of DPPH. The plate was left at 37 °C for 10 min in the dark before the absorbance was measured at 520 nm. An appropriate reagent blank was run simultaneously in each assay.

2.8. ABTS•+ free radical-scavenging activity

The evaluation of ABTS•+ scavenging by the extracts was assayed, as described previously (Mocan et al, 2016) with modification. ABTS•+ chromogenic radical reagent solution was prepared by reacting aqueous ABTS (7 mM) with $K_2S_2O_8$ to produce a final concentration of 2.5 mM persulphate, then kept for 12–24 h in the dark at room temperature before use. Ethanol (50% v/v) was added to the blue–green solution at a 1:10 ratio, and the absorbance was 1.28 ± 0.04 at 595 nm. Various concentrations of the sample extract (2 µL) were added to 198 µL of final ABTS•+ solution, and the change in absorbance at 595 nm was recorded for 20 min.

2.9. Cupric ion-reducing antioxidant capacity (CUPRAC)

CUPRAC was evaluated by following the method of Uysal et al. (2017) with some modification, utilising copper (II)– neocuproine reagent as the chromogenic oxidising agent, and ascorbic acid as the standard. In this assay, 2 µL of extract was carefully mixed with 198 µL of working reagent of neocuproine (75 mM) and $CuCl_2$ (10 mM), and the absorbance read at 450 nm after 20 min.

2.10. Ferric-reducing antioxidant power (FRAP)

The FRAP assay of Dezsi et al (2015) with modification was carried out on the sample extracts diluted appropriately with distilled water to provide an absorbance in the linear range. The FRAP reagent was freshly-prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ (10:1:1 v/v/v). The sample extracts (2 µL) at various concentrations were added to 198 µL of working FRAP reagent and incubated at 37 °C for 10 min. The absorbance was read at 520 nm, and the FRAP value was expressed as micromoles of Fe^{2+} equivalents per gram of extract or gram of seeds.

2.11. Cell culture and 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay

The human keratinocyte cell line (HaCaT) was prepared at 37 °C in an incubator with 5% CO_2 humidified atmosphere. Cells were cultured in high-glucose-containing Dulbecco's modified Eagle's medium supplemented with penicillin–streptomycin mixture (100 U/mL) and 10% foetal bovine serum. HaCaT cells were seeded in 96-well plates (1×10^5 cell/mL), incubated for 24 h, and treated with extracts of the non-oil seed legumes at various concentrations, and gallic acid as a standard, respectively. MTT solution was added to each well after 1 h incubation, followed by dimethyl sulphoxide, and the absorbance read at 595 nm. The MTT colourimetric assay is described elsewhere (de Oliveira et al., 2014).

2.12. Detection of intracellular reactive oxygen species (ROS)

The fluorogenic substrate 2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) was used to detect intracellular ROS generated by UV-B (Bender et al., 2014) with modification. HaCaT cells were seeded in a 96-well plate at a density of 1×10^5 cell/mL and treated for 24 h with gallic acid and the extracts of non-oil seed legumes, respectively. Cells were exposed to UV-B (60 mJ/cm^2) after incubation for 1 h at 37 °C in a 5% CO_2 incubator. Afterwards, the cells were washed with PBS, then H_2DCFDA solution was added. 2′7′-Dichlorofluorescein fluorescence was detected 10 min later, using a spectrofluorometer (PerkinElmer, Waltham, USA) at 485 nm excitation and 535 nm emission.

2.13. Statistical analysis

All data are represented as mean and standard deviation of three parallel measurements and were analysed by SPSS 10.07 (SPSS, Chicago, IL, USA). Differences between means were calculated by one-way analysis of variance and considered significant if $p < 0.05$. Antioxidant activity assays and HPLC separations were conducted at least in three replications. Pearson's correlation coefficient (r) was determined to establish the relationship between the phenolic and flavonoids contents and the antioxidant activity.

3. Results

3.1. HPLC analysis of 70% ethanolic extracts

In this study, the HPLC phenolic profiles of the non-oil seed legume extracts (PLR, PLW and CES) were analysed by comparison of the retention times with those of known standard antioxidants. A representative chromatogram of these samples and standards is shown in Fig. 1D. Notably, the retention times of 5.536, 13.109, 15.276 and 18.822 min corresponded to the presence of gallic acid, catechin, epicatechin and coumaric acid, respectively (Fig. 1E-G). By employing the peak areas of notable concentrations of standards, the amounts of these polyphenols in each of the non-oil seed legume extracts were determined. As shown in Fig. 1E-G, peaks for gallic acid and epicatechin were identified in PLR extract, and coumaric

acid in PLW extract, while CES extract contained epicatechin and coumaric acid.

3.2. Measurements of TPC and total flavonoid content

The TPC of the extracts (Fig. 2A) was based on the Folin–Ciocalteu method, using gallic acid as the standard. Among the aqueous and ethanolic (70% and 100%) extracts, the 70% ethanolic extract of CES at 100 μg/mL presented the highest TPC (38.60 mg GAE/g dry weight), and the lowest TPC was represented by the 70% ethanolic extract of PLW (15.21 mg GAE/g dry weight). The quantitative differences in TPC for CES (16.27–38.60 mg GAE/g dry weight), PLW (15.21–31.68 mg GAE/g dry weight) and PLR extract (15.33–23.16 mg GAE/g dry weight) were both dose- and solvent-dependent. The TPC values differed significantly according to the solvent and variety of non-oil seed legume ($p < 0.05$).

The flavonoid contents of the extracts were determined by the aluminium chloride colourimetric method and are described in Fig. 2B. The total flavonoid contents in the extracts of the non-oil seed legumes, PLR, PLW and CES, varied in the ranges 12.64–19.63, 11.73–23.39 and 12.72–24.61 mg CE/g dry weight, respectively. The lowest (11.73 mg CE/g dry weight) and highest flavonoid contents (24.61 mg CE/g dry weight) were detected in the extracts of PLW and CES, respectively. Similarly to the TPC, the flavonoid contents were significantly influenced by the solvent and variety of non-oil seed legume ($p < 0.05$). The TPC and flavonoid contents data promoted the analysis of the free radical-scavenging and reducing properties of the extracts.
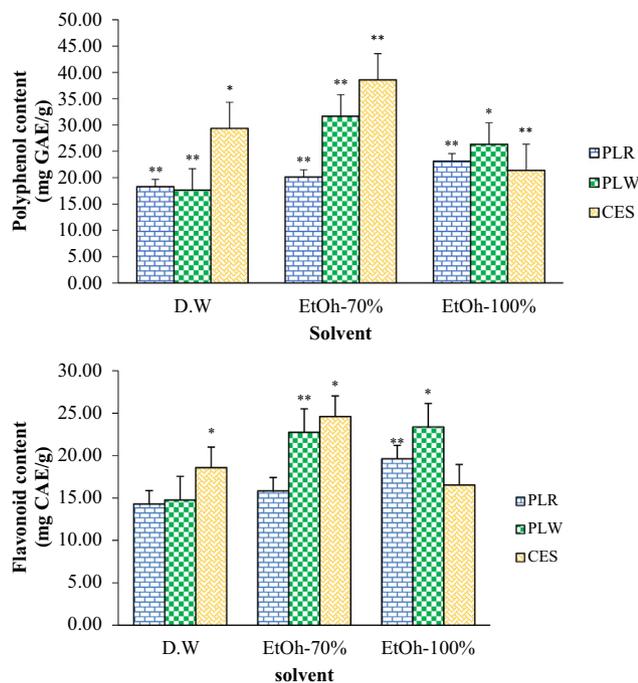

Fig. 2 Total phenolic content (**A**) and flavonoids content (**B**) in extracts of *Phaseolus lunatus* red (PLR), *Phaseolus lunatus* white (PLW) and *Canavalia ensiformis* (CES) in different solvents (distilled water, D.W; ethanol 70%, EtOH-70%; ethanol 100%, EtOH-100%).

3.3. Antioxidant assays

In the present study, the DPPH, ABTS, CUPRAC and FRAP antioxidant abilities of the non-oil seed extracts were explored. All four assays were simple, reproducible and inexpensive, accordingly employed together to estimate the antioxidant capacity *in vitro*.

The DPPH and ABTS antioxidant capacities of the extracts were fundamentally based on their ability to quench free radicals by donating a hydrogen atom (DPPH assay) or an electron (ABTS assay) (Re et al., 1999). For these assays, the antioxidant capacity is related to the degree of the decolourisation of the free radical (Prior et al., 2005), while the CUPRAC method is based on the principle of utilizing copper (II)–neocuproine reagent as the chromogenic oxidising agent (Apak et al., 2008). The non-oil seed extracts (PLR, PLW and CES) displayed electron-donating potential and reducing power capacity, and these effects were concentration- and solvent-dependent (Fig. 3A–D). Statistical assessments highlighted significant differences in the DPPH• and ABTS•+ antiradical activities among the non-oil seed varieties and solvent systems ($p < 0.05$). The 70% ethanolic extract of CES exhibited both the highest DPPH and ABTS antioxidant capacity, respectively (19.42% DPPH inhibition and 37.28% ABTS inhibition; Fig. 3A, B). Another mode of action of an antioxidant is its capability to engage in redox reactions. Based on the CUPRAC and FRAP value, PLR, PLW and CES all possessed a notable ability to act as reducing agents (Fig. 3C, D). The FRAP assay defines an antioxidant as any substance in the reaction medium that has reducing power (Benzie and Strain, 1996). Considering this procedure, the 70% ethanolic extracts of CES (100 μg/mL) had the highest reducing power, and the aqueous extract of PLW had the lowest FRAP (Fig. 3D).

3.4. Pearson's correlation analysis

Pearson's correlation analysis was performed to assess the relationship between the antioxidant activities of the extracts and their TPC and flavonoid content (Lesaffre et al., 2009). Pearson's correlation coefficients between these variables are provided in Table 1. Significant positive correlations were observed between the TPC and antioxidant activity of the aqueous, 70% ethanolic and 100% ethanolic extracts measured by the ABTS ($r = 0.9013, 0.9327$ and 0.9182), CUPRAC ($r = 0.9424, 0.9485$, and 0.8369) and FRAP assay ($r = 0.7861, 0.8531$ and 0.8767), respectively. Moderate correlations were established between the DPPH antioxidant activity of the 70% ethanolic ($r = 0.6608$) and 100% ethanolic extracts ($r = 0.6834$) and their TPC when compared with the aqueous extract ($r = 0.8740$). In addition, inferior correlations were noticed between the DPPH• scavenging activity and the flavonoid contents of the 70% and 100% ethanolic extracts ($r = 0.5110$ and 0.4766, respectively). On the contrary, however, there were strong positive correlation between the flavonoid content of the aqueous, 70% ethanolic and 100% ethanolic extracts and the ABTS ($r = 0.8554, 0.9413$ and 0.9424), CUPRAC ($r = 0.9731, 0.9144$ and 0.9448), FRAP activities ($r = 0.7568, 0.8685$ and 0.8834), respectively.
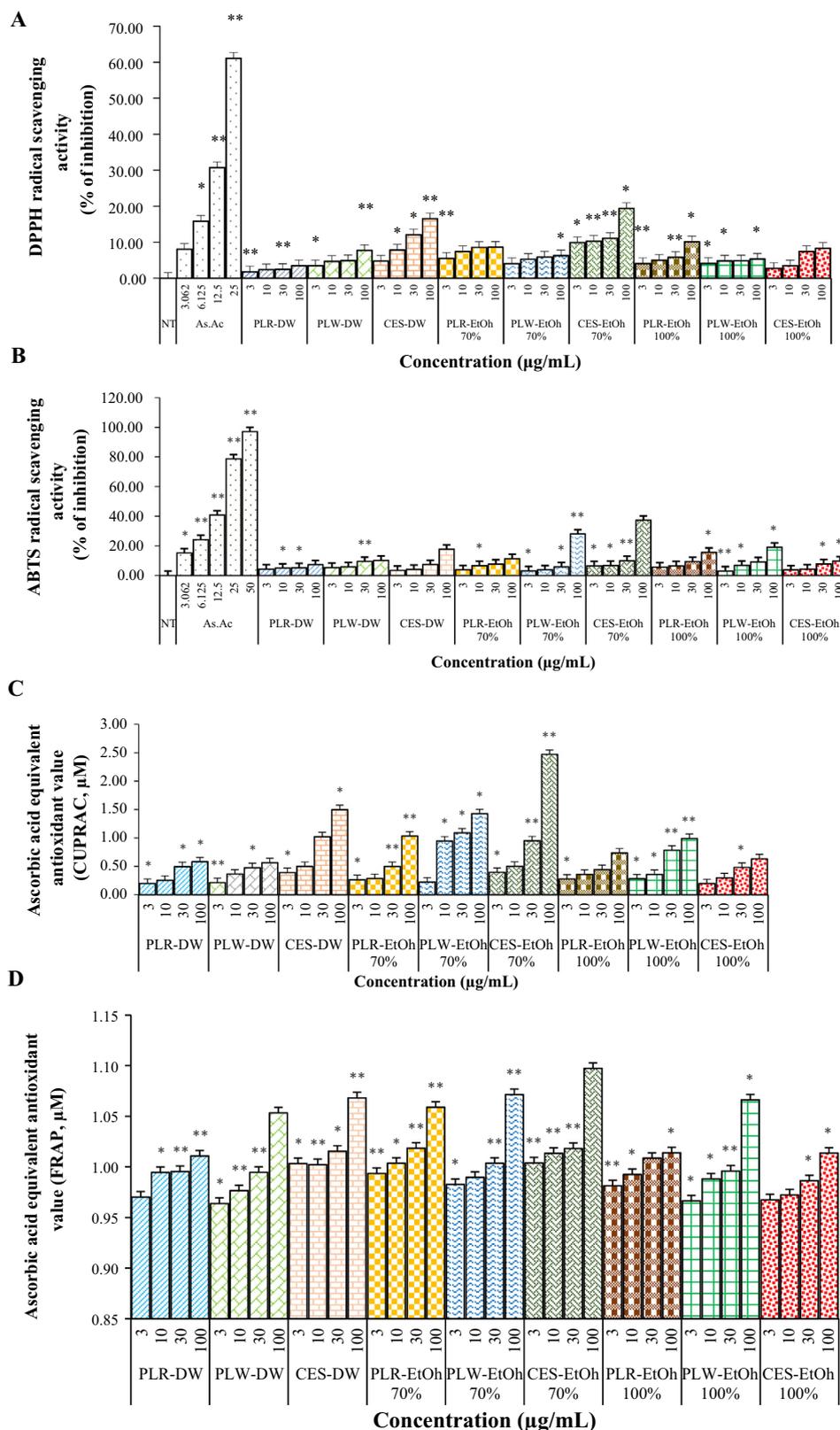
Fig. 3 DPPH (A), ABTS (B), CUPRAC (C) and FRAP (D) antioxidant activity of *Phaseolus lunatus* red (PLR), *Phaseolus lunatus* white (PLW) and *Canavalia ensiformis* (CES) extracts in different solvents (distilled water, D.W; ethanol 70%, EtOH-70%; and ethanol 100%, EtOH-100%).

Table 1 Pearson's correlation coefficient between the variables.

In vitro assay	Flavonoid (R value)			Polyphenol (R value)		
	D.W	EtOH-70%	EtOH-100%	D.W	EtOH-70%	EtOH-100%
DPPH	0.9465*	0.5110	0.4766	0.8740*	0.6608*	0.6834*
ABTS	0.8554*	0.9413*	0.9424*	0.9013*	0.9327*	0.9182*
CUPRAC	0.9731*	0.9144*	0.9448*	0.9424*	0.9485*	0.8369*
FRAP	0.7568*	0.8685*	0.8834*	0.7861*	0.8531*	0.8767*

* Correlation is significant at the 0.05 level.

3.5. Cytotoxicity and decrease of ROS by the extracts

The MTT assay was carried out to explore the cytotoxic effects of extracts on UV-B-irradiated. Exposure to UV-B caused cell damage. HaCaT cells are well-established cell line used to explore the protective effect of extract compounds against oxidative stress-induced conditions. Here, the viability of HaCaT cells exposed to UV-B following pre-treatment with PLR, PLW and CES extracts, respectively, were analysed using the MTT assay. Pre-treatment with PLR, PLW and CES significantly protected HaCaT cells against cell death from oxidative stress induced by UV-B (60 mJ/cm^2), in a dose-dependent manner (Fig. 4A). Antioxidants may inhibit cellular damage induced by oxidative stress immediately or progressively. As described in Fig. 4B, UV-B irradiation significantly enhanced ROS generation compared non-irradiated cells. Pre-treatment of PLR, PLW and CES extracts significantly lessened ROS generation compared with the UV-irradiated control.

4. Discussion

There are many varieties of non-oil seed legumes in Indonesia that can grow in high productivity even in a marginal area.
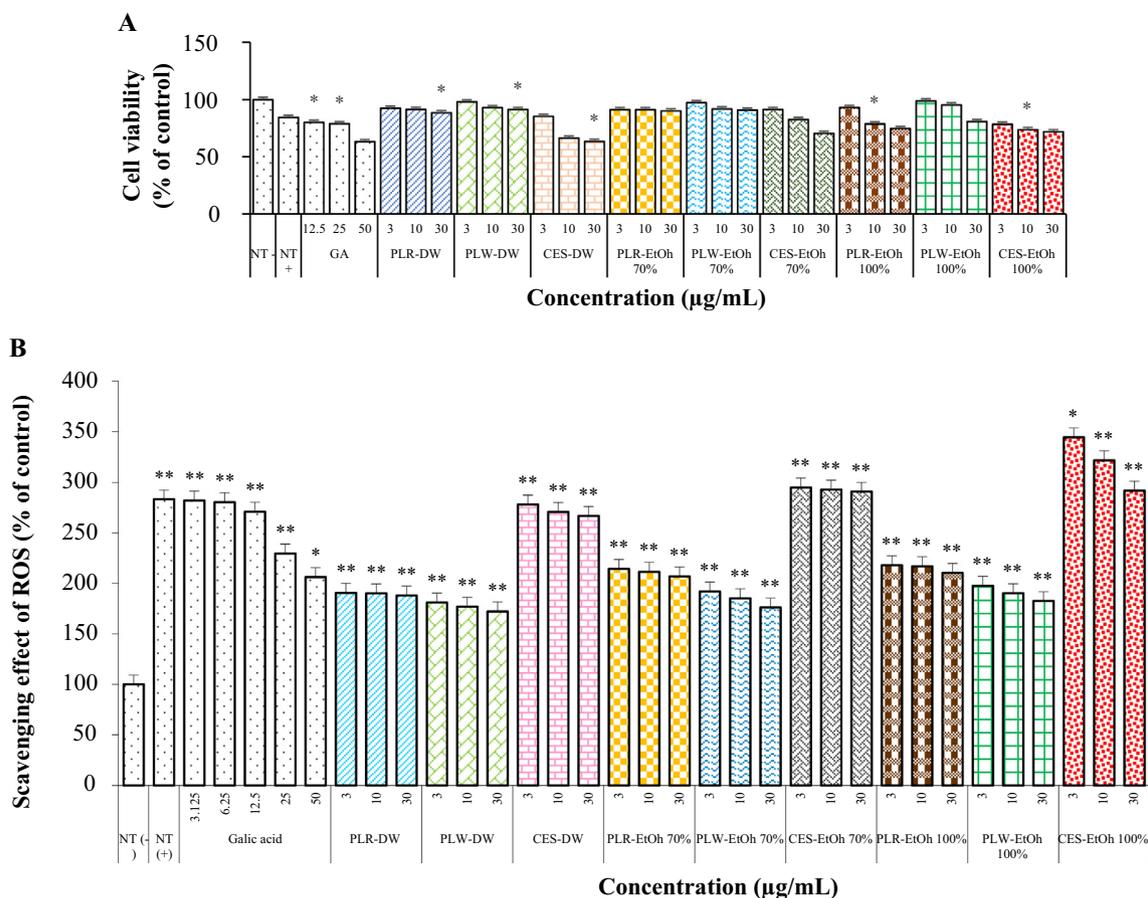

Fig. 4 HaCat cell viability. HaCaT cells were seeded at a density of 1×10^5 cell/mL, and the MTT assay was performed (**A**). *Phaseolus lunatus* red (PLR), *Phaseolus lunatus* white (PLW) and *Canavalia ensiformis* (CES) extracts in different solvents (distilled water, D.W; ethanol 70%, EtOH-70%; 100% ethanol, EtOH-100%) were evaluated for their ability to scavenge intracellular ROS generated by UV-B 60 mJ/cm^2, by the H$_2$DCF-DA assay (**B**).

Nonetheless, the non-oil seed legumes are currently underutilised. Here, three varieties of non-oil seed legumes, namely PLR, PLW and CES, were collected from a farm in Indonesia and studied for their polyphenolic contents and antioxidant properties. All three varieties of non-oil seed legumes are commonly consumed as food sources in the local region. The profile of the bean (i), powder (ii) and extracts (iii) of these varieties were shown in Fig. 1A–C.

The HPLC phenolic profiles of the 70% ethanolic extract varieties (Fig. 1E-G) were notable for the polyphenolics, gallic acid and epicatechin (PLR), coumaric acid (PLW) and epicatechin and coumaric acid (CES). Correspondingly, Luthria and Pastor-Corrales (2006) identified p-coumaric acid, ferulic acid and sinapic acid in the extracts of 15 varieties of *P. vulgaris*, mainly from the base-hydrolysed fraction, and the TPC was 19.1–48.3 mg/100 g of bean. These same three polyphenolics were also established in seven different *P. vulgaris* cultivars after alkaline hydrolysis of the extracts (Chen et al., 2015). Several researcher isolated and identified gallic acid as a major compound in the legume. Gallic acid and its derivatives have potent antioxidant capacity and are therefore thought to be largely responsible for the high antioxidant capacity of the red and black sword bean coats (Badhani et al., 2015; Gan et al., 2017).

Phenolic compounds are known as free radical scavengers and account for the majority of the antioxidant activity of a plant. The antioxidant mechanism of polyphenolic compounds is mostly derived from their metal ion-chelating and hydrogen-donating abilities (Jacobo-Velazquez and Cisneros-Zevallos, 2009). The TPC and flavonoid contents differed significantly ($p < 0.05$) among the extracts of the non-oil seed legume varieties (Fig. 2A, B). In the present study, the 70% ethanolic PLR, PLW and CES extracts presented the highest amount of phenolic compounds. According to the results, 70% ethanol was the most powerful solvent for phenolic extraction, and distilled water was the least potent. In a previous study, Abdille et al., (2005) reported the antioxidant activity of *Dillenia indica* fruit extracts decreased in the order of methanol extract > ethyl acetate extract > water extract, as analysed through various *in vitro* assays. Additionally, Wijekoon et al., (2011) notified that for phenolic extraction from *Etlingera elatior* Jack inflorescence, water was the least powerful solvent in comparison to methanol and acetone (50%, 90% and 100% v/v). Although there is no common appropriate solvent, 70% ethanol is usually preferred for extracting phenolics from plants (Prior et al., 2005).

In general, some preliminary comparative research of numerous legumes and bean varieties showed that the TPC of part bean have different value (Gan et al., 2017). It was further suggested that the solvent polarity and bean variety affected the extractability of polyphenols and flavonoids (Agostini-Costa et al., 2015; Orak et al., 2016). Some studies demonstrated that the bean cultivar was the main source of variance in the yield of tannins, and TPC and *in vitro* antioxidant activity (Gan et al., 2017).

The determination of free radical-scavenging activity of natural antioxidants by DPPH and ABTS analyses provides simplicity and high sensitivity. In this analysis, the DPPH radicals receive a hydrogen atom or an electron from an antioxidant donor (Moon and Shibamoto, 2009). The colour changes from purple to yellow upon the reduction of the DPPH$^{\bullet}$ to the stable diamagnetic molecule. Our results showed that the PLR,

PLW and CES extracts exhibited a dose-dependent scavenging activity (Fig. 3A). It means that the antioxidants of non-oil seed legumes extracts were competent to decolourise DPPH$^{\bullet}$ and had free radical-scavenging potential. The phenolic compounds are indicated as the antioxidants of non-oil seed legumes with scavenging potential, due to their hydrogen-donating abilities. The free radical-scavenging potential was further verified by the ABTS method (Fig. 3B). In this present study, the CES 70% ethanolic extract had the highest antioxidant activity. Regarding the hydroalcoholic extracts, the most effective scavenger against ABTS$^{\bullet+}$ was obtained when 70% ethanol was the solvent. Prior et al. (2005) suggested that the sample's antioxidant capacity correlated with the degree of cation radical decolourisation. Based on of these results, it should be noted that hydrophilic compounds are effective free radical scavengers. Moreover, like the literature studies discussed above, the antioxidant properties depended on the bean variety, the solvent and the concentration of the extract.

In the present study, it has been suggested that the type of antioxidant compound, antioxidant activity and capacity being dissolved in the solvent also varies with the change of solvent polarity, different concentration of solvent and solvent system. A solvent with low viscosity, low density and high diffusivity can easily diffuse into the pores of the plant materials to extract the plant components (Alothman et al., 2009).

The reducing ability of antioxidants is evaluated in the FRAP assay. In the presence of an antioxidant, the yellow Fe^{3+}–TPTZ complex is reduced to the intensely blue Fe^{2+}–TPTZ complex, in an acidic medium (Moon and Shibamoto, 2009). In this study, all non-oil seed legumes extract presented Fe^{3+}-reducing power and Fe^{2+}-chelating activity. These properties were higher for the 70% ethanolic than aqueous extracts (Fig. 3D). Although the FRAP antioxidant method is well-established, the CUPRAC method is a relatively new method expanded by Apak et al. (2008). This assay is based on the cupric ion-reducing capability in the presence of copper (II)–neocuproine. From the CUPRAC antioxidant activity results of the evaluated non-oil seed legume extracts, the cupric ion-reducing capability increased in a concentration-dependent manner and was in the order of 70% ethanol > distilled water > 100% ethanol (Fig. 3C), consistent with the trend in the free radical-scavenging activity (DPPH$^{\bullet}$ and ABTS$^{\bullet+}$)

UV-B was used treat HaCaT cells to analyse oxidative damage. Several studies have verified the protective influences of phytochemicals against oxidative stress-induced cytotoxicity. Likewise, in our study, PLR, PLW and CES pre-treatment attenuated UV-B induced cell death and ROS formation in HaCaT cells (Fig. 4A, B). Flavonoids have the ability to inhibit ROS-producing enzymes, as well as the potential to immediately scavenge these ROS through the up-regulation of antioxidant enzymes (Alam et al., 2017). A healthy cell condition was maintained in the control samples, and cell viability decreased after UV-B exposure. The antioxidant pre-treatments significantly reduced cell death.

Pearson's correlation coefficients were evaluated to indicate the extent to which the antioxidant activities displayed by the extracts was particularly induced by the phenolic compounds and associated with the great number of flavonoids present in the extracts. A remarkable correlation between the antioxidant activities and the phenolic compounds and flavonoid content in plants has been confirmed in another research (Chaudhari and Mahajan, 2015; Teixeira et al., 2017). Several

studies have identified a direct and positive correlation between these variables (Marathe et al., 2011; Quiroga et al., 2013). Phenolic compounds other than flavonoids are likely to be the greatest contributors to the antioxidant capacity of the non-oil seed legume extracts studied in this research because the flavonoids content was inferiorly correlated with the antioxidant activity in comparison to the TPC. Generally, the extracts with the highest TPC showed the highest antioxidant activities.

In this study, we investigated the TPC, flavonoid content and antioxidant capacity of the extracts (aqueous, 70% ethanol, 100% ethanol) obtained from three non-oil seed legumes (PLR, PLW and CES). Generally, the ethanolic 70% extracts had the greater level of *in vitro* antioxidant capacity, TPC and flavonoid content. Collectively, the data indicated that the non-oil seed legumes could provide health advantages against oxidative stress-related chronic diseases and could be used as a functional food, nutraceutical ingredient and as cosmetic agents. Many chronic diseases derive from molecular inflammation in the tissues in the body. Identifying natural sources of antioxidants represents an important step in confronting various oxidative stress conditions. Further studies are required for the isolation and identification of the individual phenolic compounds in the studied legumes, and *in vivo* studies are required for supporting their mechanism of action as an antioxidant.

Author contributions

N.D.D. performed the experiments. N.D.D., M.B.A., and S.-H.L. designed the research and analyzed the data. N.D.D. and S.-H.L. wrote the paper. N.D.D., M.B.A. and S.-H.L. revised the paper.

Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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