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Free radical scavenging and anti-proliferative activities of avocado (*Persea americana* Mill.) seed extract

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ABSTRACT

Objective: To investigate the chemical components and biological activities of avocado seed extract and fractions in order to determine the nutritional and pharmaceutical values of avocado seed. **Methods:** Various organic solvents were applied for extraction and fractionation of avocado seed. 1,1-diphenyl-2-picryl-hydrazyl, 2,2-azinobis-3-ethyl benzothiazoline-6-sulfonic acid, and DNA oxidation assays were applied for investigation of free radical scavenging activity. Nitric oxide production was measured by Griess reaction assay. Moreover, MTT assay was used to measure cancer cell growth inhibition. **Results:** The result indicated that the avocado seed contains (7.14 ± 0.40) g lipid/100 g, (1.67 ± 0.03) g protein/100 g, (54.0 ± 1.2) g carbohydrate/100 g, and (62.0 ± 2.3) mg gallic acid equivalent/g dried weight extract. Moreover, dichloromethane and ethyl acetate were revealed to be the highest free radical scavenging fractions with IC_{50} values of (48.0 ± 3.4) $\mu\text{g/mL}$ (1,1-diphenyl-2-picryl-hydrazyl assay) and (22.0 ± 1.8) $\mu\text{g/mL}$ (2,2-azinobis-3-ethyl benzothiazoline-6-sulfonic acid assay), respectively. Additionally, the avocado seed extract and fractions were able to protect against H_2O_2 -induced DNA damage at the concentration of 100 $\mu\text{g/mL}$. On the other hand, the ethanol extract was effective in reducing nitric oxide production from lipopolysaccharide-stimulated RAW 264.7 macrophage cells without cytotoxic effect. Notably, the avocado seed significantly inhibited the proliferation of human lung A549 and human gastric BGC823 cancer cells at the concentration of 200 $\mu\text{g/mL}$, especially hexane $(81 \pm 3)\%$ and dichloromethane $(75 \pm 2)\%$ fractions. **Conclusions:** The results of the present study supported the avocado seed as potential by-product source for further development of health beneficial products.

1. Introduction

Food industries are providing a wider range of food products to the consumers. These industries have generated considerable amounts of by-products and wastes, which cause many problems

for environmental pollution[1]. Currently, the utilization of the by-products and discarded materials to develop new value-added products for commercial applications is getting much attention.

Due to high nutritional and bioactive composition, the recovery of bioactive compounds from wastes and by-products is important

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for utilization in development of nutraceutical and pharmaceutical products[2].

Avocado (*Persea americana* Mill.) is a tropical and subtropical fruit with high oil content, native from south central Mexico, but with global consumption[3]. It is not only consumed as a fresh fruit, but also processed for food, cosmetic and pharmaceutical products to increase commercialisation and give a higher added value. Avocado (*Persea americana* Mill.) fruit possesses various beneficial effects due to its nutritional and bioactive compositions[4]. Previously, numerous studies have reported the chemical and bioactivity composition of avocado pulp. Avocado pulp has been determined to possess the anti-cardiovascular, anti-aging[5], anti-cancer[6], antioxidant[7], and anti-inflammatory activities[8]. Besides, 30% of avocado fruits are bio-wastes such as peel and seed that contain potential source of bioactive compounds[9]. Avocado seed has a diverse application in treatment for diarrhea, dysentery, toothache, intestinal parasites, and skin treatment and beautification[10]. Recently, the studies of avocado seed to apply them in functional food or food ingredients have got much attention by many researchers. Following this trend, this study mainly focused on the investigation of avocado seed components and its biological activities including antioxidant, anti-inflammation, and anti-cancer activities.

2. Materials and methods

2.1. Materials

Avocado seeds were collected from Dak Lak province, Vietnam. Solvent was purchased from Xilong (China). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Determination of chemical components

2.2.1. Determination of protein content

The protein content was determined by Bradford method[11]. Five hundred μ L of sample was mixed with 2.5 mL of Bradford reagent for 10 min, and the absorbance of mixture was measured at 595 nm. The protein concentration was identified regarding protein standard curve of bull serum albumin.

2.2.2. Determination of lipid content

Lipid content was determined by Soxhlet method[12]. Five gram of avocado seed powder was packed by filter paper and put into Soxhlet distillation system containing ether solvent. It was heated at 40–45 °C for 10–12 h. The lipid content was calculated according to the formula (m_{lipid} is the dried weight of lipid while m_{material} is the dried weight of avocado seed powder):

$$\% \text{ lipid} = m_{\text{lipid}} \times 100/m_{\text{material}}$$

2.2.3. Determination of carbohydrate content

The mixture of 5 g avocado seed powder, 1.5 mL of HNO_3 , and 15 mL of acetic acid was heated for 30 min. Hot water was then added and the mixture was filtered to collect particle. The particle was washed with ethanol and diethyl ether. Finally, the particle was dried at 100 °C until constant weight. The cellulose content was determined by the following formula ($m_{\text{cellulose}}$ is the dried weight of cellulose while m_{material} is the dried weight of avocado seed powder):

$$\% \text{ cellulose} = m_{\text{cellulose}} \times 100/m_{\text{material}}$$

2.2.4. Total phenolic content

The total phenolic content of the extract was determined as described by Le *et al.*[13]. The total phenolic content was calculated from the calibration curve, and the results were expressed as mg of gallic acid equivalent (GAE) per g dry weight extract (DWE).

2.2.5. Determination of ash content

Five gram of avocado seed powder was heated at 600 °C for 6 h (HNO_3 can be added if need). The ash content was calculated by the formula (m_{ash} is the dried weight of ash while m_{material} is the dried weight of avocado seed powder):

$$\% \text{ ash} = m_{\text{ash}} \times 100/m_{\text{material}}$$

2.3. Extraction

The avocado seed powder was soaked into ethanol (1 g/4 mL) for 4 h at 60 °C to achieve ethanol extract (ET). The concentrated ethanol extract was further subjected to partial fractionation with different polarity solvents including hexane, dichloromethane, ethyl acetate, and distilled water. Firstly, ethanol extract was distributed in hexane, filtered and concentrated by removing the solvent under the reduced pressure to obtain hexane fraction (HE). Similarly, the obtained residue was subsequently fractionated with dichloromethane, ethyl acetate, and distilled water to obtain dichloromethane fraction (DM), ethyl acetate fraction (EA), and distilled water fraction (H_2O), respectively.

2.4. Free radical scavenging activity of avocado seed extract and fractions

2.4.1. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assay

The antioxidant activity of extract and fractions was determined by the DPPH assay as described by Vo *et al.*[14]. Vitamin C was used as a reference. The DPPH radical scavenging ability of sample was determined by the following formula:

$$\text{DPPH scavenging effect} = [(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}})/\text{OD}_{\text{control}}] \times 100\%$$

2.4.2. 2,2-Azinobis-3-ethyl benzothiazoline-6-sulfonic acid (ABTS⁺) assay

This assay was performed as described by Vo *et al.*[14]. Vitamin C was used as a reference. The ABTS⁺ radical scavenging ability of

sample was determined by the following formula:

$$\text{ABTS}^+ \text{ scavenging effect} = [(\text{OD}_{\text{control}} - \text{OD}_{\text{sample}})/\text{OD}_{\text{control}}] \times 100\%$$

2.4.3. DNA oxidative assay

DNA oxidation assay was carried out according to Vo *et al.*[15]. Briefly, a mixture of 10 μL of the tested sample at different concentrations (10 and 100 $\mu\text{g}/\text{mL}$) and DNA extract from human liver HepG2 cancer cell line (20 $\mu\text{g}/\text{mL}$) were incubated for 10 min at room temperature followed by the addition of 10 μL of Fenton's reagent (30 mM H_2O_2 , 50 μM ascorbic acid, and 80 μM FeCl_3). The mixture was kept at room temperature for 10 min and the reaction was terminated by adding ethylenediaminetetraacetic acid (10 mM). An aliquot (20 μL) of the reaction mixture was electrophoresed on a 1% agarose gel for 20 min at 100 V. Gels were then stained with 1 mg/mL ethidium bromide and visualised by UV light. Control group was not treated with the the tested sample while blank group was not treated with the tested sample and Fenton's reagent. The relative amounts of DNA were quantified by densitometric scanning (Luminescent image analyzer, Fujifilm Life Science, Tokyo, Japan). Densitometrically calculated expression levels of DNA were given as a percentage compared to blank group.

2.5. Cell culture

The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 $^\circ\text{C}$ using Dulbecco's modified eagle medium supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES buffer, 100 U/mL of penicillin G, and 100 mg/mL of streptomycin. The cells were maintained *via* two times passage per week, and cells were utilized for experimentation at a density of 70%-80%.

2.6. Nitric oxide (NO) production assay

In order to investigate NO production, the RAW 264.7 macrophage cells were pre-treated with avocado seed extract and fractions (100 $\mu\text{g}/\text{mL}$) before stimulation of lipopolysaccharide (LPS). NO level in the culture supernatant was measured by the Griess reaction as described by Vo *et al.*[16]. The levels of NO production were calculated as a percentage compared to that of control (LPS treatment alone): Release ratio (%) = $(T - B)/(C - B) \times 100$, where B is the group without stimulation as well as sample treatment, C is control group that was stimulated with LPS without presence of the tested sample, and T is the stimulated group with presence of the tested sample.

2.7. Cell viability assay

To exclude the possibility that the inhibition of NO production was due to cytotoxicity effect, MTT assays were performed in RAW

264.7 macrophage cells pre-treated with 100 $\mu\text{g}/\text{mL}$ of avocado extract and fractions for 24 h. The avocado seed extract and fractions were screened for anti-cancer activity *via* investigating the inhibitory effect on the proliferation of human lung A549 cancer cells and human gastric BGC823 cancer cells. The cells were treated with 200 $\mu\text{g}/\text{mL}$ of avocado seed extract and fractions for 24 h and cell viability was then measured by MTT assay. The viability levels of the cells were determined according Vo *et al.*[16] and calculated as a percentage compared to that of blank (no addition of tested sample).

2.8. Statistical analysis

Data were analyzed using the analysis of variance (ANOVA) test of statistical package for the social sciences (SPSS). The statistical differences among groups were assessed by using Duncan's multiple range tests. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Chemical components of avocado seed

In this study, the result of the proximate components of avocado seed was investigated. It showed that the lipid content in the avocado seed was (7.14 ± 0.40) g/100 g, meanwhile the carbohydrate content was (54.0 ± 1.2) g/100 g, and the protein content was (1.67 ± 0.03) g/100 g. Moreover, the ash content of avocado seed was indicated up to (2.60 ± 0.02) g/100 g. Notably, the total phenolic content presented (62.0 ± 2.3) mg GAE/g DWE in the ethanol extract of avocado seed. Accordingly, avocado seed contains substantial amount of nutrients that could warrant its utilization in food.

3.2. Free radical scavenging activity of avocado seed extract and fractions

The antioxidant activity of avocado seed extract and fractions was determined *via* measuring the free radical scavenging ability (Figure 1). Figure 1A showed that DM fraction exhibited the highest DPPH scavenging activity with IC_{50} value of (48.0 ± 3.4) $\mu\text{g}/\text{mL}$, followed by ET extract [$\text{IC}_{50} = (68.0 \pm 4.0)$ $\mu\text{g}/\text{mL}$], EA fraction [$\text{IC}_{50} = (88.0 \pm 2.8)$ $\mu\text{g}/\text{mL}$], HE fraction [$\text{IC}_{50} = (97.0 \pm 5.2)$ $\mu\text{g}/\text{mL}$], and H_2O fraction [$\text{IC}_{50} = (112.0 \pm 4.0)$ $\mu\text{g}/\text{mL}$]. Meanwhile, EA fraction exhibited the highest ABTS⁺ scavenging activity with IC_{50} values of (22.0 ± 1.8) $\mu\text{g}/\text{mL}$, followed by ET extract [$\text{IC}_{50} = (75.0 \pm 5.0)$ $\mu\text{g}/\text{mL}$], HE fraction [$\text{IC}_{50} = (88.0 \pm 4.2)$ $\mu\text{g}/\text{mL}$], H_2O fraction [$\text{IC}_{50} = (151.0 \pm 6.2)$ $\mu\text{g}/\text{mL}$], and DM fraction [$\text{IC}_{50} = (192.0 \pm 3.5)$ $\mu\text{g}/\text{mL}$] (Figure 1B). On the other hand, the free radical scavenging activity of avocado seed extract and fractions was also confirmed *via* investigating the protective effect against H_2O_2 -induced DNA damage. Figure 1C and 1D showed that the significance of the light DNA band was not clear in the control

group exposed by H₂O₂ alone. However, DM and EA fractions were observed to be effective in protection against H₂O₂-induced DNA damage at the concentration of 100 µg/mL. Meanwhile, ET extract as well as HE and H₂O fractions possessed the moderate protective effect at the same concentration.

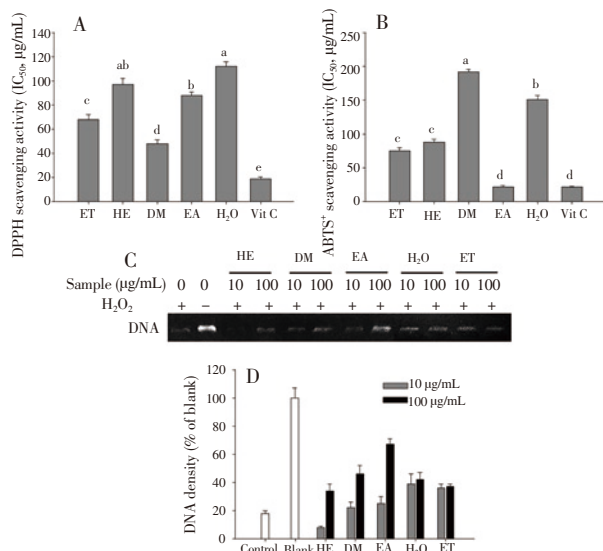


Figure 1. Free radical scavenging activities of avocado seed extract and fractions.

The DPPH (A) and ABTS⁺ (B) scavenging activity of avocado seed extract and fractions. IC₅₀ is the half maximal inhibitory concentration of extract or fractions on DPPH or ABTS⁺ radicals. (C) Protective effect of avocado seed extract and fractions against H₂O₂-induced DNA damage. (D) Densitometrically calculated levels of DNA which were given as a percentage compared to the blank group. Ethanol extract (ET), hexane (HE), dichloromethane (DM), ethyl acetate (EA), and H₂O fractions. Different letters a-e indicated significant difference among groups ($P < 0.05$) by Duncan's multiple-range test.

3.3. Inhibitory activity of avocado seed extract and fractions on NO production

As shown in Figure 2, NO production levels were observed to decrease as exposed to the extract and fractions. It indicated that ET extract was the strongest inhibitor of NO production, followed by EA, DM, HE, and H₂O. Moreover, Figure 3 showed that ET extract and H₂O fraction have slight cytotoxicity effect, while HE, DM, and EA fractions possess the moderate cytotoxicity effect on RAW 264.7 macrophage cells. According to the result, the inhibitory effects of avocado seed extract and fractions were not due to cytotoxicity on RAW 264.7 macrophage cells.

3.4. Growth inhibition effect of avocado seed extract and fractions on cancer cell proliferation

The result showed that avocado seed extract and fractions had significant inhibition on both human lung A549 and human gastric BGC823 cancer cells at the concentration of 200 µg/mL (Figure

4). The highest inhibition on human lung A549 cancer cells was observed by HE fraction, followed by ET, DM, EA, and H₂O. Meanwhile, DM exhibited the strongest inhibition on human gastric BGC823 cancer cells, followed by EA, ET, HE and H₂O. The inhibition of avocado seed on human lung A549 cancer cells was slightly higher than that of human gastric BGC823 cancer cells.

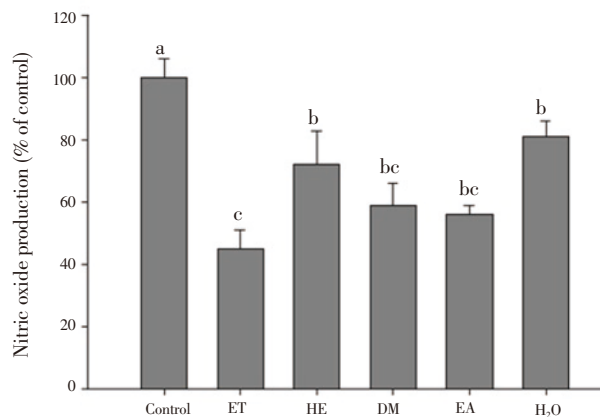


Figure 2. The inhibitory effect of avocado seed extract and fractions on NO production from LPS-stimulated RAW 264.7 macrophage cells.

NO level was measured using the Griess reaction, and the results were expressed as percentage of control. Ethanol extract (ET), hexane (HE), dichloromethane (DM), ethyl acetate (EA), and H₂O fraction. Different letters a-c indicated significant difference among groups ($P < 0.05$) by Duncan's multiple-range test.

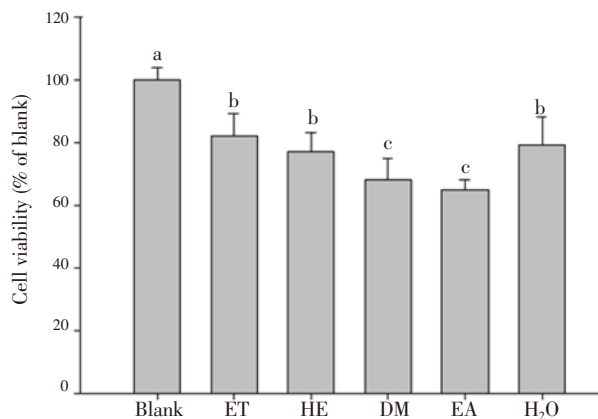


Figure 3. The effect of avocado seed extract and fractions on RAW 264.7 macrophage cell viability.

Cell viability was assessed by MTT method, and the results were expressed as percentage of surviving cells over blank cells. Ethanol extract (ET), hexane (HE), dichloromethane (DM), ethyl acetate (EA), and H₂O fraction. Different letters a-c indicated significant difference among groups ($P < 0.05$) by Duncan's multiple-range test.

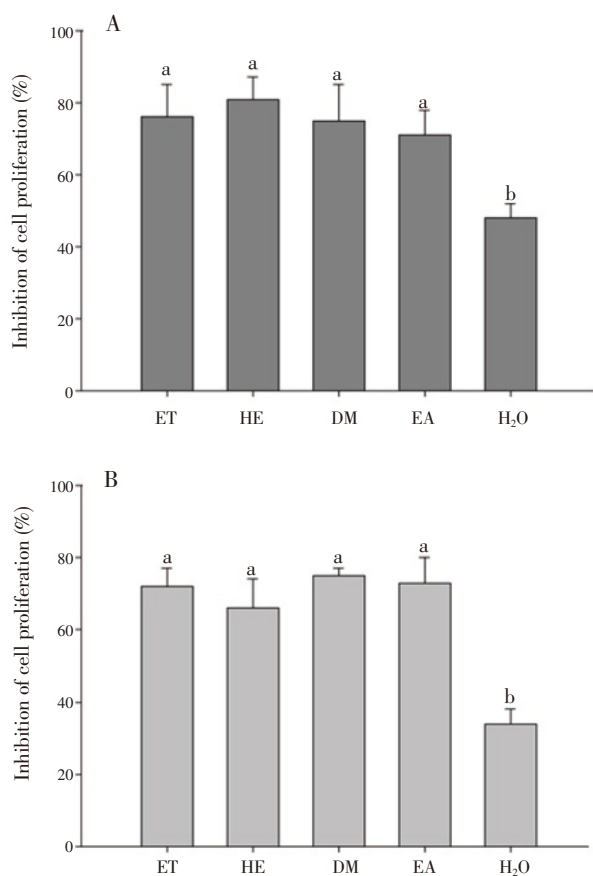


Figure 4. The inhibitory effects of avocado seed extract and fractions on proliferation of human lung A549 cancer cells (A) and human gastric BGC823 cancer cells (B).

Cell viability was assessed by MTT method, and the results were expressed as percentage of surviving cells over blank cells. Ethanol extract (ET), hexane (HE), dichloromethane (DM), ethyl acetate (EA), and H₂O fractions. Different letters a-b indicated significant difference among groups ($P < 0.05$) by Duncan’s multiple-range test.

4. Discussion

Avocado fruit processing leads to the release of high amount of by-products such as seed and peel that are normally discarded. These by-products cause environmental problems and economic losses due to waste disposal[17]. Therefore, it is important to utilize these by-products as sources of food supplements or medicinal products. It showed that the lipid content was (7.14 ± 0.40) g/100 g that was higher than that $[(1.87 \pm 0.31)$ g/100 g] reported by Bora *et al.*[18]. However, it was slightly lower than lipid contents reported by Oluwole *et al.*[19] $[(12.24 \pm 0.07)$ g/100 g] and Arukwe *et al.*[20] $[(16.54 \pm 2.10)$ g/100 g]. Furthermore, the carbohydrate content was determined to be (54.0 ± 1.2) g/100 g that was slightly higher than carbohydrate reported by Arukwe *et al.*[20] $[(48.11 \pm 4.13)$ g/100 g]. In addition, the avocado seed presented a protein content of (1.67 ± 0.03) g/100 g that was similar with the protein amount reported by Bora *et al.*[18] $[(1.95 \pm 0.16)$ g/100 g].

Besides, the ash content, which is considered as minerals in a plant,

was also investigated. The ash content of avocado seed in the present study was slightly higher than that reported by Arukwe *et al.*[20] $[(2.40 \pm 0.19)$ g/100 g] and significantly higher than that recorded by Oluwole *et al.*[19] $[(1.15 \pm 0.03)$ g/100 g] and Bora *et al.*[18] $[(1.87 \pm 0.24)$ g/100 g].

Especially, the total phenolic content of ethanol extract of avocado seed was found to be higher than that reported by Tremocoldi *et al.*[21] $[(57.0 \pm 2.3)$ mg GAE/g DWE], Rodríguez-Carpena *et al.*[22] $[(35.0 \pm 9.8)$ mg GAE/g DWE], and Githinji *et al.*[23] $[(18.0 \pm 2.8)$ mg GAE/g DWE]. Phenolic compounds are known to act as antioxidants due to donating hydrogen or electrons[24]. With the total phenolic content value of $[(62.0 \pm 2.3)$ mg GAE/g DWE], avocado seed was suggested to be a rich source of phenolic compounds, thus bringing about its high antioxidant activity.

A free radical is considered as a molecule that contains one or more unpaired electrons in its outermost atomic or molecular orbital. It is generated from endogenous sources such as intracellular auto-oxidation and inactivation of small molecules or from exogenous sources such as tobacco smoke, certain pollutants, organic solvents, and pesticides[25]. The overproduction of free radicals can result in oxidative stress, causing the destructive process due to harming certain cell structures such as cell membranes, DNA, and proteins[26]. It leads to human aging and various diseases such as atherosclerosis, inflammation, and certain cancers[25]. Thus, the high antioxidant agents from natural products would contribute an important role in prevention and treatment of free radical-caused diseases[27]. In this study, DM and EA were found to be effective in scavenging DPPH and ABTS⁺ radicals. Moreover, these fractions exhibited the highest protective effect against H₂O₂-induced DNA damage. So far, various solvents with different polarities were applied for isolation of different polarity antioxidants. In this study, the solvents with moderate polarity such as dichloromethane and ethyl acetate were suitable for extracting potential DPPH and ABTS⁺ radical scavenging components. On the other hand, Antasionasti and colleagues have reported that the fractionation of methanolic extract of avocado peel was able to scavenge DPPH and ABTS⁺ radicals with IC₅₀ values of $(4\ 221 \pm 137)$ µg/mL and (855 ± 13) µg/mL, respectively[28]. Moreover, Melgar and colleagues have shown that avocado peel and seed scavenged 50% DPPH radical at the concentration of 149 and 220 µg/mL[29]. As indicated in the result, the scavenging activity of avocado seed in the present study was significantly stronger than that of avocado peel and seed in the previous studies. It indicates that avocado seed may contain various active components, especially polyphenols with moderate polarity and high antioxidant activity.

NO is known as an important mediator in inflammatory response[30]. The activation of macrophages in inflammatory reaction leads to the release of a variety of effector molecules, including NO[31]. The treatment with NOS inhibitors reduces the degree of inflammation in rats with acute inflammation[32]. Therefore, NO is indicated to play a part in the acute and chronic inflammation. Herein, ET extract was showed to be the strongest inhibitor of NO production without significant cytotoxicity on RAW 264.7 macrophage cells. According Kim and colleagues, Persenone A and

B from avocado fruit were determined to be novel inhibitors of NO production from RAW 264.7 macrophage cells[33]. Their inhibitory activities on NO production ($IC_{50} = 1.2$ and $3.5 \mu\text{M}$, respectively) were comparable to or higher than that of a natural NO production inhibitor, docosahexaenoic acid ($IC_{50} = 4.3 \mu\text{M}$). Accordingly, ET extract was considered to contain the potential components such as polyphenols that was able to down-regulate the inflammatory response *via* decreasing the pro-inflammatory mediator production.

Cancer is one of the major reasons for death due to its rapid pace of development and the lack of efficient treatments. The breast, lung, gastric, colorectal, and esophageal cancers are the most common types of cancer nowadays[34]. Notably, proliferation plays an important role in cancer development and progression[35]. Thus, the important parameter for screening anti-cancer agents is suppressing cancer cell proliferation. In this study, the avocado seed extract and fractions have significant inhibition on both human lung A549 and human gastric BGC823 cancer cell proliferation, except H_2O fraction. Abubakar and colleagues have determined that triterpenoid compound from ethanol extract inhibited cell proliferation of human breast cancer cell line (MCF-7) and human hepatocellular carcinoma cell line (HepG2) with the IC_{50} values of $62 \mu\text{g/mL}$ and $12 \mu\text{g/mL}$, respectively, and was safe to normal cells[36]. Moreover, Alkhalif and colleagues also reported the inhibition of avocado fruit and seed on the cell proliferation of HepG2 and colon cancer cell line (HCT116)[37]. In addition, avocado fruit was showed to inhibit the cell proliferation of HepG2 and HCT116 with IC_{50} values of $58 \mu\text{g/mL}$ and $14 \mu\text{g/mL}$, respectively. Meanwhile, avocado seed significantly suppressed the cell proliferation of HepG2 and HCT116 with IC_{50} values of $12 \mu\text{g/mL}$ and $3 \mu\text{g/mL}$, respectively. According to the results of the present study, avocado seed may be considered as potential materials for isolation of the effective anti-cancer compounds that can be applied as natural chemotherapy of various cancer types.

Natural polyphenols are widely distributed in the plants, foods, and beverages that have been shown to possess numerous biological activities and health benefits[38]. Moreover, it was evidenced that polyphenols have strong antioxidant capacities due to scavenging of free radicals, inhibiting lipid oxidation, and reducing hydroperoxide formation[39]. Especially, polyphenols exhibited potential immunomodulatory and anti-inflammatory effects[40]. Additionally, the anti-cancer activity of polyphenols was also proved at mouth, stomach, duodenum, colon, liver, lung, mammary gland or skin[41]. Notably, avocado seed was revealed to be potential source of phenolic compounds. Therefore, the biological activities of avocado seed extract in the present study may be related to phenolic compounds.

In conclusion, this study has indicated the chemical components and the potential health beneficial effects of avocado seed such as antioxidant, anti-inflammatory, and anti-cancer activities. The chemical components of avocado seed have been determined to be high in carbohydrates and total phenolic contents. Notably, the avocado seed effectively scavenged various free radicals and significantly inhibited cancer cell proliferation *in vitro*. These promising pharmaceutical properties indicate the values of the

waste parts of the avocado fruits. However, the further studies on identifying the active compounds and determining the spectrum of biological activities of avocado seed are necessary. Moreover, the additional researches will reveal whether these promising results are able to translate into clinically or nutritionally useful agents.

Conflict of interest statement

There are no conflicts to declare.

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