

In Vitro Analysis of the Antioxidant Effect of Allspice

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Abstract

Antioxidants are free radical scavengers found in spices which may play a significant role in preventing cell death. Allspice is a dried unripe berry obtained from the *Pimento dioica* plant that may have antioxidant potential. The objective of this study was to determine the total phenolic, flavonoid content and antioxidant capacity of allspice using selected assays such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Potential (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC), Nitric Oxide (NO) and Oxygen Reducing Antioxidant Capacity (ORAC). Total phenolic and flavonoid contents of allspice were determined using both water and methanol extraction. A comparison of antioxidant activity of water and methanol extracts of allspice was conducted using the different assays (DPPH, TEAC, NO, ORAC and FRAP). The total phenolic content (6.9%), NO scavenging (38.8%) and ORAC (35.1%) activity were higher in methanol compared to water extracts of allspice while flavonoids (57%), FRAP (11.2%), and TEAC (1.82%) were higher in water extracts compared to methanol extracts of allspice. The total phenolic and flavonoid content were higher in methanol extracts compared to water extracts of allspice. The IC50 (DPPH), FRAP and TEAC, NO scavenging and ORAC activity were higher in methanol extracts compared to water extracts of allspice. Total flavonoid content, FRAP and TEAC, NO scavenging and ORAC were significantly higher ($p \leq 0.5$) in methanol extracts compared to water extracts of allspice. This shows that allspice has antioxidant potential and that the method of extraction can play a crucial role on the number of phytochemicals extracted from the plant. Utilization of allspice in food products may provide additional functional properties.

Keywords

Allspice Extract (AAE), *Pimenta dioica*, 1,1-Diphenylpicrylhydrazyl (DPPH), Ferric Reducing Antioxidant Potential (FRAP), Phenolics, Flavonoids,

1. Introduction

Allspice is derived from the plant *Pimento dioica*, and is called allspice because of its unique flavor, which is a combination of cinnamon, cloves, ginger, and nutmeg [1]. Allspice is also obtained from another plant closely related to *P. dioica* called *Pimenta racemosa*, mostly found in Central America [2]. Allspice is predominantly found in Jamaica, Mediterranean area and Asia. The allspice grown in Jamaica has the most flavor compared to those grown in other countries, due to high essential oils content. Some of the secondary metabolites include terpenoids, alkaloids, polyphenols, and glycosides [3]. Allspice blends well with ginger, lavender and other spices, making it have different aroma combinations. Allspice has been used for medicinal and non-medicinal purposes [4]. It can also be used for making liqueur, by soaking the berries in rum [5]. Some non-medicinal purposes include uses in the meat industry, as a pesticide, and bakery [6]. Allspice is used to extend the shelf-life of meat and dairy products. In the food industry, it was used synthetically as a preservative in compounds such as butylhydroxyanisole (BHA) and butylated hydroxytoluene (BHT), but these compounds have been shown to induce carcinogenesis [7]. Some medicinal purposes of the extract include analgesic, antibacterial, anesthetic, and anti-neuralgic effects [7]. Allspice can be used in tooth restoration in dentistry [8]. Previous studies have shown the inhibition of prostate and breast cancer development using allspice extracts [9]. Compounds that have been isolated from allspice include epicatechins, anthocyanidins, eugenol and gallic acid have anti-carcinogenic and anti-proliferative effects [10]. Polyphenols are water soluble, highly polar compounds that are secondary metabolites in plants. They are also inhibitors of enzymatic activities in living organisms such as inflammatory enzymes like cyclooxygenase-2 [11]. Eugenol is a phenyl propene predominantly found in clove oil, cinnamon, ginger, nutmeg and has anti-bactericidal, and anti-inflammatory effect [12].

Oxidative stress is the production of free radicals that cannot be neutralized due to insufficient number of antioxidants [13]. Oxidative stress is controlled by a balance between pro-oxidants and antioxidants [14]. Oxidative stress can lead to the development of cardiovascular diseases, cancer and can increase the rate of aging. Free radicals are chemicals or compounds containing an unpaired number of electrons in their outermost shell [15]. Free radicals are produced during aerobic cellular respirations, during which phagocytes phagocytose microorganisms, and during intense physical exercise [16].

Phytochemicals such as ericifolin is extracted from the aromatic berries of *pimenta dioica*, has been shown to prevent prostate and breast cancer [17]. In Caribbean, there is a long history of using allspice berries for folk healing. Jamaicans drink hot tea with allspice for colds, menstrual cramps and dyspepsia

[18]. Costa Ricans are known to use allspice to treat dyspepsia and diabetes and Guatemalans apply crushed Allspice berries to bruises, sore joints and for muscle ache [18]. A rich assortment of secondary metabolites can be obtained from the plant, which can be used for preserving the nutrients and protecting from bacterial and fungal infestation [19]. Some of these secondary metabolites include terpenoids, alkaloids, polyphenols, and glycosides [20]. Compounds isolated from allspice like eugenol and gallic acid have selective anti-proliferative and anti-tumor properties on human cancer cells and animal models [22] [23]. Eugenol could potentially contribute to the anti-inflammatory function associated with allspice in traditional medicine [21]. It is the anti-tumor and anti-inflammatory characteristics of allspice extract (AAE) that has led to the hypothesis of this study.

This study was conducted to determine the antioxidant potential of allspice by the total phenolic and flavonoid content, and conducting different assays such as DPPH (1,1-diphenylpicrylhydrazil, TEAC (Trolox Equivalent Antioxidant Capacity) and FRAP (Ferric Reducing Antioxidant Power), Nitric Oxide (NO) and Oxygen reducing Antioxidant Capacity (ORAC).

2. Materials and Methods

Sample: The allspice sample was obtained in powdered form (Monterey Bay Spice Company). Plant extracts was prepared using a standard protocol described by Chung *et al.* [21] with modifications. Allspice (10 g) was added to 250 ml boiling water and allowed to boil for 2 hours, at a temperature of 100°C and stirred at a speed of 380 rpm. The solution was cooled for 10 minutes and filtered with a whatman filter paper. The residue was used to repeat the extraction process twice. Filtered solutions were pooled and centrifuged at 4000 rpm, for 10 min at 4°C. Supernatent was collected and evaporated to dryness in Rotavapor. The dry sample was reconstituted with water to 10 ml. Extractions were carried out in triplicates.

Methanol Extraction

Extracts were prepared using a standard method described by Przygodzka *et al.* [22]. Methanol (250 ml of 80% methanol) was added to 10 g of the allspice. The mixture was allowed to stir for 2 hours at room temperature. Filtration was carried out using a whatman filter paper and the residue was collected to repeat the extraction process. The filtered solution was then centrifuged and evaporated to dryness using a rotary evaporator, and reconstituted to the desired volume (10 ml) using methanol. Extractions were carried out in triplicates.

3. Antioxidant Assays

3.1. Total Phenolic Content (TPC)

Total phenolic content for allspice extracts was determined according to Ainsworth and Gillespie [23] with some modifications, using the gallic acid as stan-

dard. In a 96-well microplate 12.5 µL of sample (3.75 - 500 mg/mL) and standard was added to wells, after which 50 µL of double distilled (ddH₂O) was added followed by 12.5 µL of Folin-Ciocalteu reagent (FC reagent). After 5 minutes, 125 µL of 7% Na₂CO₃ solution was added and incubated at room temperature for 90 mins. The absorbance was read at 750 nm using a microplate reader (Synergy HT Bioteck Instruments Inc, Winooski, Vermont).

3.2. Total Flavonoid Content TFC

Determination of total flavonoids content in allspice according to Quettier *et al.* [24] with some modifications, using an Aluminum Chloride Colorimetric Assay. In a 96 well microplate, 25 µL of sample extract (3.75 - 500 mg/mL) and standard was added, followed by 125 µL of water and 7.5 µL of 5% NaNO₂ solution and, incubated for 5 min. Next, 15 µL of 10% Aluminium chloride (AlCl₃) was added to the wells and incubated at room temperature for 5 mins. Then 50 µL of 1 M sodium hydroxide was added followed immediately by 27.5 µL of distilled water. The absorbance was read at 510 nm against blank prepared with 125 µL ddH₂O in a microplate reader (Synergy HT Bioteck Instruments Inc, Winooski, Vermont).

3.3. DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) Assay (DPPH)

DPPH, a stable radical was used to measure total antioxidant capability of the extract, using method suggested by Artega *et al.* [25] with some modifications. Briefly, 40 µL of selected sample was combined with 210 µL of 0.1mM DPPH solution. For control, 40 µL of 80% methanol was used instead of the sample. Samples absorbance was read at 0, 30, 60 and 90 minutes at an absorbance of 517 nm using a microplate reader (Synergy HT Bioteck Instruments Inc, Winooski, Vermont). Calculations for DPPH were carried out using the formula:

$$\% \text{DPPH} = (\text{Abs. control} - \text{Abs. sample}/\text{Abs. control}) \times 100$$

3.4. Ferric Reducing Antioxidant Potential (FRAP)

Total FRAP of extracts will be assessed according to the protocol provided by Benziea and Strain [26]. The extract (100 µL) was combined with 3 mL of freshly prepared FRAP reagent [300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ) in 40 mM HCL and 20 mM ferric chloride (FeCl₃·H₂O)]. The mixture was incubated for 10 minutes at a temperature of 37°C. Following incubation, the samples were read at an absorbance of 593 nm in the three replicates. The change in absorbance was compared to the standard ferrous sulfate and expressed as µmol of Fe²⁺/100 grams.

3.5. Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay involves the scavenging of peroxy radicals generated by 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), which prevents the degradation of the fluorescein probe and, consequently, prevent the loss of fluorescence of the probe. ORAC of allspice extract was determined according to

the protocol suggested by Huang *et al.* [27]. 50 µL of ORAC phosphate buffer (PB) (75 mM ORAC-PB) and samples will be added to a 96-well black plate (Fisher Scientific, Pittsburgh, PA., U.S.A.). This was followed by adding 100 µL of fluorescein (20 mM) solution to the mixture. The mixture was incubated (37°C, 10 min) before adding 2,2'-azobis (2-amidinopropane) dihydrochloride (140 mM AAPH). The rate of fluorescence decay (485 nm excitation and 530 nm emissions for 1 min intervals for 40 min) was examined by using a microplate reader (Synergy HT Bioteck Instruments Inc, Winooski, Vermont) and calculations were made using the area under the fluorescent decay curve and a Trolox standard curve. The antioxidant capacities were presented as µmol trolox equivalents (TE)/g extracted samples.

3.6. Trolox Equivalent Antioxidant Capacity Determination

This assay was carried out to determine the inhibition of the absorbance of radical cations of 2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonate) (ABTS) by antioxidants in allspice (metmyoglobin) [28]. ABTS radical cation ($\text{ABTS}^{+}\cdot$) was produced when ABTS solution was added with potassium persulfate (final concentration) and the mixture was allowed to sit in a dark room at ambient temperature for 12 - 16 hours. The $\text{ABTS}^{+}\cdot$ solution was diluted in deionized water or ethanol from which a blank reading was taken. After the addition of the allspice extract solutions to $\text{ABTS}^{+}\cdot$ solution, the absorbance was read every minute over a 6-minute period at 734 nm using a microplate reader.

3.7. Nitric Oxide Radical Scavenging Activity

A colorimetric assay was used to measure nitric oxide which will be read at 546 nm. The results were compared to a standard of diluted ascorbic acid. Initially, 10 mM sodium nitroprusside solution was mixed with diluted samples and standards in a 96 well microplate. The plate was stored for 150 minutes at room temperature. Griess reagent, which contains 1% sulphanilamide, 2% phosphoric acid, and 0.1% naphthyl ethylene diamineichloride, was incorporated into the mixture in the plates and the absorbance read [29].

3.8. Statistical Analysis

All experiments were performed in triplicates and the data are expressed as mean ± standard deviation using SAS system version 9.1. Significance level was determined at $p \leq 0.05$.

4. Results

Figure 1 shows the total phenolic and flavonoid content in methanol and water extracts of allspice and that the results were affected by different extraction methods. The results showed that the method of extraction might have played a role in the amount of phytochemical present in the extracts and their antioxidant effect. The phenolic content of methanol extract was 6.9% higher in methanol extracts compared to water extracts, while flavonoid content was 57% in methanol

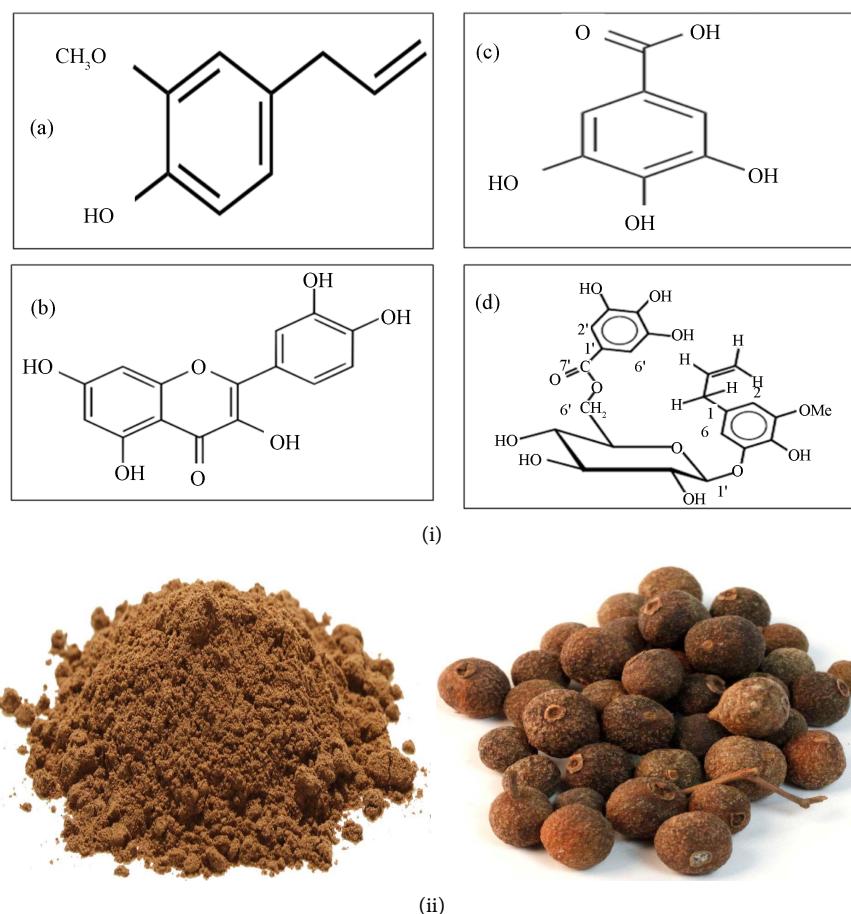


Figure 1. (i) Picture of allspice plant (ii) Different compounds present in *Pimento Dioica* (Allspice). (A) Eugenol: 4-Allyl-2-methoxyphenol; (B) Quercetin: 2-(3', 4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one); (C) Gallic acid: 3,4,5-trihydroxybenzoic acid; (D) Ericifolin: Eugenol 5-O-b-(6'-O-galloylglucopyranoside). (Zhang and Lokeshwar, 2012). Obtained from Riffle and Robert, 1998.

compared to aqueous extracts and the difference might be due to their polarity of methanol compared to water. NO activity and ORAC of allspice were 38.8% and 35.1% higher in methanol extracts compared to water extracts of allspice. TEAC, FRAP and DPPH activity was higher in methanol extracts compared to water extracts of allspice, but the difference was not significant. However, the flavonoid, FRAP, NO and ORAC of methanol extracts was significantly higher in methanol extracts compared to water extract of allspice.

Figure 2 shows the DPPH for methanol and water extracts of allspice. DPPH assay was carried out to determine the ability of the allspice to bind and stabilize the DPPH free radical. The IC^{50} (DPPH assay) for water (and methanol extracts) was 52.5 mg/ml and 48.96 mg/ml.

The FRAP assay is based on the ability of the allspice extract to reduce colorless Fe^{3+} to blue colored Fe^{2+} (**Figure 3**). Methanol extract (4.5 ± 0.03 mmol Fe/g) showed similar result to water extract (5.07 ± 0.1 mmol Fe/g) of allspice.

Figure 4 shows the TEAC assay. This assay was conducted to determine the ABTS radical scavenging ability of allspice extract. The result showed that water

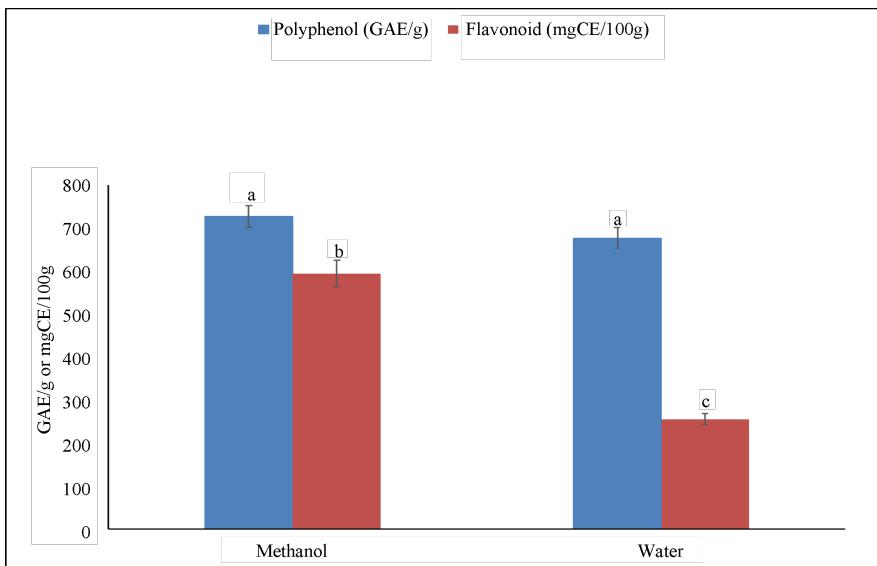


Figure 2. Total Phenolic and Flavonoid Content of Allspice. (GAE-Gallic Acid Equivalent, CE-Catechin Equivalent). Values are expressed as means \pm SEM. ^{abc}Means with different superscripts are significantly ($p \leq 0.05$) different using Tukey's studentized range test.

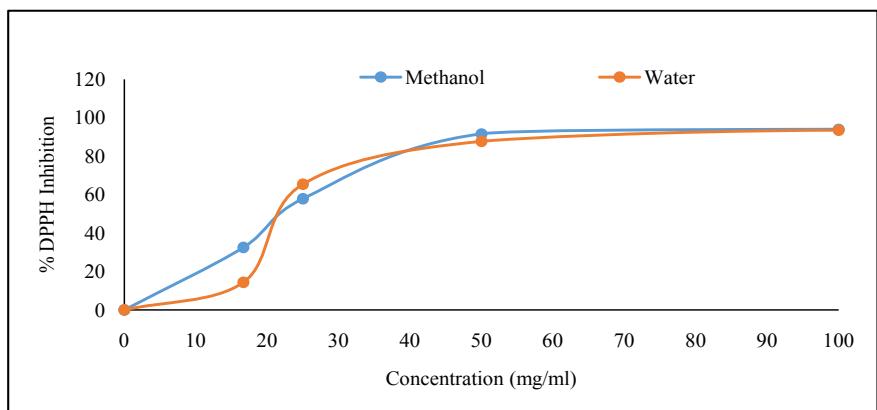


Figure 3. Ferric Reducing Antioxidant Power (FRAP) of Allspice. Values are expressed as means \pm SEM. ^{abc}Means with different superscripts are significantly ($p \leq 0.05$) different using Tukey's studentized range test.

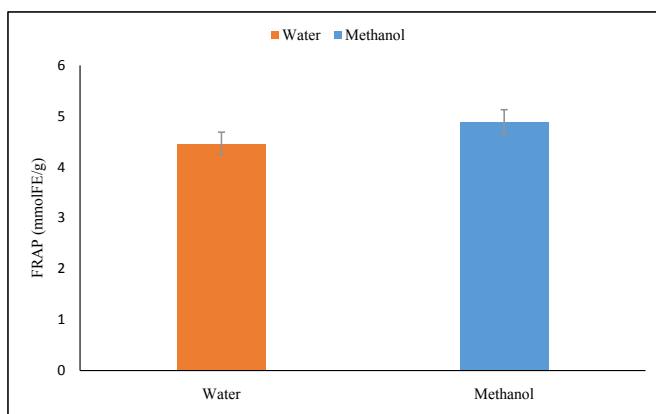


Figure 4. Percentage DPPH Inhibition of Water and Methanol Extracts of Allspice.

extract (362.4 ± 7.2 mmolTE/g) of allspice was higher than methanol extract (355.8 ± 9.7 mmolTE/100 g), however, this difference was not significant.

Figure 5 shows the result of NO free radical scavenging ability of allspice extract. The result shows that methanol extract (326.2 ± 54.4 $\mu\text{g/ml}$) has a significantly higher activity compared to water extract (199.7 ± 50.3 $\mu\text{g/ml}$) of allspice.

Figure 6 shows the ORAC activity of allspice. In this assay the ability of allspice to inhibit the degradation of fluorescein, by inactivating peroxy radicals generated by AAPH. Methanol extract (44.1 ± 5.8 $\mu\text{molTE}/100$ g) of allspice showed a significantly higher ORAC activity compared to water extract (28.6 ± 7.5 $\mu\text{molTE}/100$ g) of allspice.

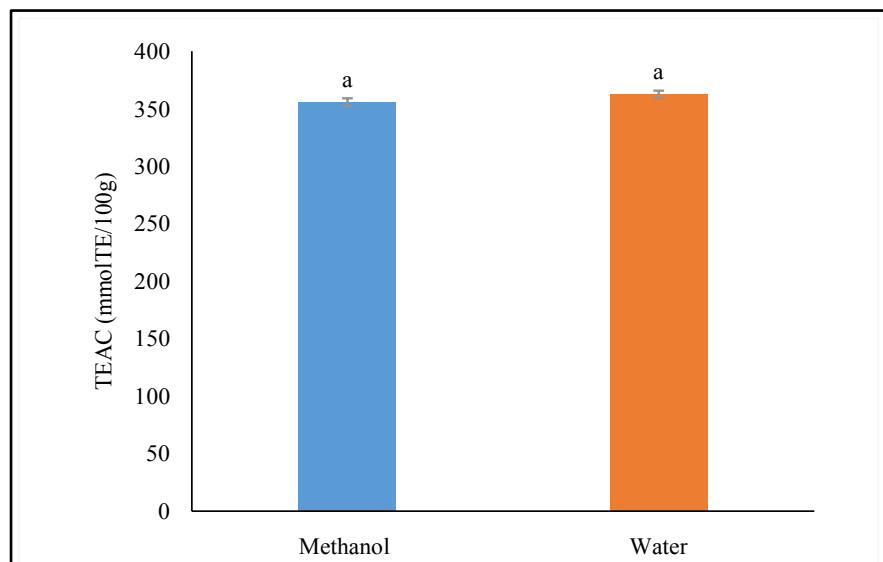


Figure 5. TEAC (Trolox Equivalent Antioxidant Capacity) of Allspice. Values are expressed as means \pm SEM. ^aMeans with different superscripts are significantly ($p \leq 0.05$) different using Tukey's studentized range test.

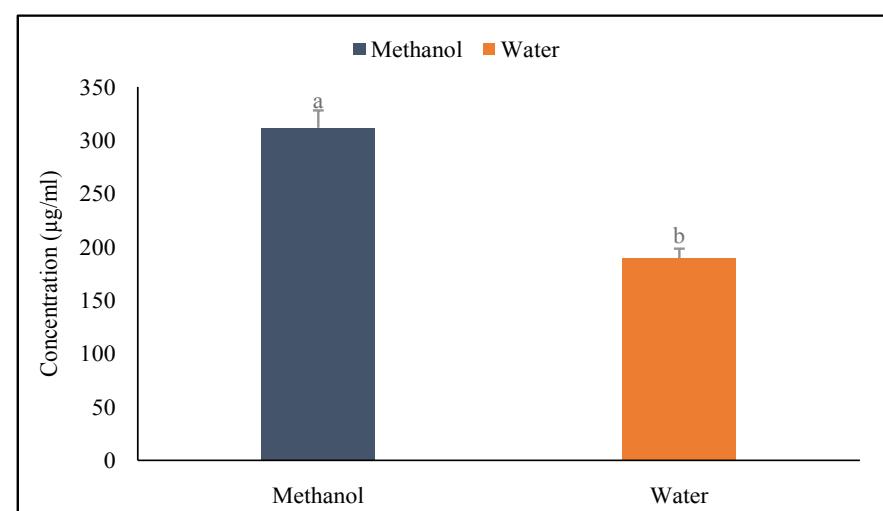


Figure 6. NO (Nitric Oxide) Scavenging Activity of Allspice. Values are expressed as means \pm SEM. ^{ab}Means with different superscripts are significantly ($p \leq 0.05$) different using Tukey's studentized range test.

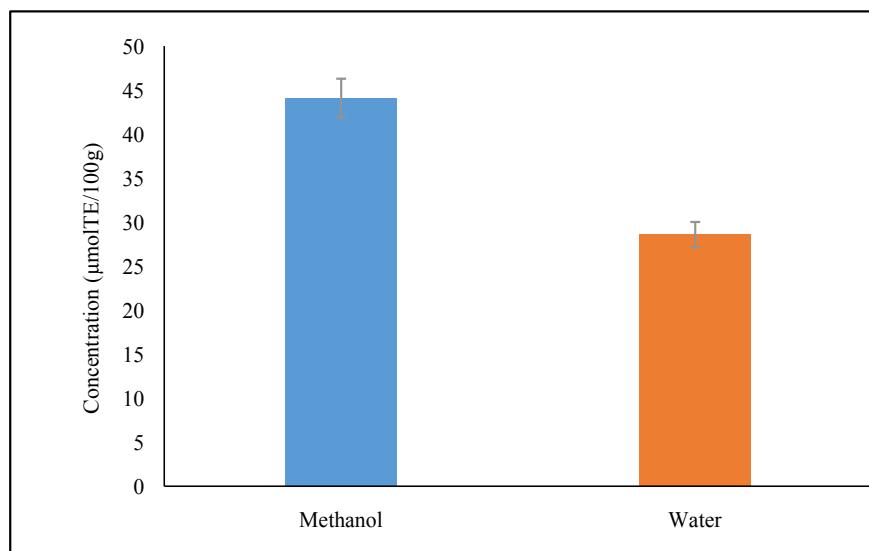


Figure 7. ORAC (Oxygen Reducing Antioxidant Capacity) of Allspice. Values are expressed as means \pm SEM. ^{abc}Means with different superscripts are significantly ($p \leq 0.05$) different using Tukey's studentized range test.

5. Discussion

Plants produce secondary metabolites which have both pharmacological and biological functions such as antioxidative, anticarcinogenic, and antimicrobial effects [30]. The 3 major chemicals extracted from plants include alkaloids, terpenoids and phenolic compounds [31]. Extensive studies have been carried out on phenolic compounds compared to alkaloids and terpenoids due to their potential benefits [32]. These phenolic compounds include polyphenols, flavonoids and phenolic acids [33]. Some of the methods utilized for extraction of these compounds include, water extraction, ultrasound based extraction and maceration [34]. Polar solvents frequently used for extracting polyphenols include ethanol, ethyl acetate, methanol and acetone [35]. Acetone is better suited for extracting flavonoids, while methanol is better suited for extracting polyphenols [36]. These solvents can also be combined to provide better extraction of phenolic compounds [37]. Example the greatest antioxidant activity was observed when using 50% acetone and 50% ethanol to extract polyphenols from black tea [38]. Phenolic compounds are composed of aromatic rings with hydroxyl groups that are able to neutralize free radicals by forming a stable phenoxy radical and the higher the hydroxyl groups, the stronger the antioxidant effect [39]. Antioxidants derived from plants have more beneficial effects compared to the synthetic ones, because they do not produce side effects such as genotoxicity [40]. The determination of the antioxidant potential of a plant extract does not only depend on its composition, but also on the method of extraction [41]. Methanol has the ability to extract more polyphenol from the sample compared to water extraction method [41]. Methanol has a low polarity, which makes it easier to extract the most polyphenols from plants compared to other polar compounds [42]. The determination of antioxidant potential can be divided into two classes;

procedures based on transfer of hydrogen atoms (HAT), and procedures based on electron transfer (ET) [42]. HAT assays include ORAC (Oxygen Reducing Antioxidant Capacity), where the sample extract and substrate compete for peroxyl radical generated thermally [43]. ET assays deal with colorimetric change due to the reducing power of the plant extract [43]. There is a positive correlation between the antioxidant power of the extract and the intensity of the color change in all assays carried out [44]. Examples of ET based assays include total phenolic content assay, DPPH, FRAP etc. [45]. There is no single assay specific enough to determine the antioxidant potential of a sample; therefore, multiple assays need to be performed [46]. This experiment focused on determining the total antioxidant potential of allspice extract using DPPH, FRAP, TEAC, NO and ORAC assay. ORAC assay is time consuming and difficult to implement correctly, however it is the only method that is able to completely inhibit all antioxidants present in a sample [47]. DPPH and FRAP assays are easy to implement and yield the fastest results that are easy to reproduce [48]. In the DPPH assay, the higher the concentration of the allspice extract, the faster the rate at which DPPH is inactivated. There was a faster rise in DPPH inhibition in water compared to methanol, however both levelled off at similar level at 91% for methanol, and 87% for aqueous extracts. A study was conducted by Nayak *et al.* [49] to determine the effect of leaf extracts of allspice on DPPH. Different concentrations of allspice were added to DPPH solution and the result showed there was increased scavenging of DPPH radicals with increasing concentration of allspice extracts [50]. The results show that *Pimento dioica* leaves contain less polyphenols compared to berries of allspice since 80 µg/ml was required to reach IC₅₀, while in this study, only 21.5 µg/ml was required to reach IC₅₀ [51]. The concentration required for 50% inhibition of allspice in both extraction methods was similar. FRAP and TEAC assays showed a slightly higher antioxidant activity in water extracts of allspice compared to methanol extracts [52]. Another study was conducted by Ilhami *et al.* [53] to investigate the reducing effect of clove oil on the ferric ion (Fe³⁺) to convert it to ferrous ion (Fe²⁺). Allspice also contains clove, and had a similar effect by reducing Fe³⁺ to Fe²⁺.

Other factors that might have also influenced the different results include sample size, storage length and presence of substances that can interfere with the degree of extraction of phytochemicals [54]. Some phenolic compounds can exist in complex forms with carbohydrates and proteins, which can make them insoluble and decrease amount that can be extracted [55]. Phenolic extracts contain a mixture of wanted and unwanted phenolic compounds and there is no method to phenolic compounds with 100% purity from plant materials [56]. Extraction time can also affect the phenolic content of a sample, because the longer the extraction time, the higher the rate of oxidation of the phenolic compounds in the sample [57]. This can be prevented by adding a reducing agent to the extraction mixture [58]. A study conducted by Naczk and Shahidi [59] showed that increasing the liquid content of methanol or water can lead to increase in total polyphenols extracted from the sample mixture.

6. Conclusion

In this study, we were able to determine the phenolic and flavonoid content of allspice using both methanol and water extraction methods. The antioxidant potential of allspice was determined using the two extraction methods. Phenolic and flavonoid content were higher in methanol extracts of allspice compared to water extracts which might be attributed to the lower polarity of methanol, sample size, amount of extraction solvent utilized and extraction time. All assays carried out were able to show reducing and free radical scavenging ability which supports the view of allspice as a good source of antioxidants. Additional assays need to be carried out to further show the specific compounds present and their activity in order to be incorporated into different foods formulations, as well as in vivo and animal studies to see if there is any anticarcinogenic effect of the extract.

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