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Antioxidant Activity and Phytochemical Screening in *Acacia rigidula* Benth. Leaves

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Abstract

This study aimed to compare the total phenolic content (TPC) and antioxidant activities of *A. rigidula* extracts. It also aimed to identify phenolic acids present in the extracts. The 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and ferric thiocyanate lipid peroxidation antioxidant assays were performed. High performance liquid chromatography was used to identify phenolic acids. There was no solvent effect on TPC nor on scavenging activities, and inhibition of lipid peroxidation ($p > 0.05$) among solvent extracts. On the other hand, 1:1:3 water: acetone: methanol extract (10.22 mg GAE/g sample) had significantly higher reducing potential than 50% ethanol extract (EE) (9.259 mg GAE/g sample) ($p < 0.05$); but EE was not significantly different from 80% methanol extract (9.781 mg GAE/g sample) ($p > 0.05$). Phenolic fraction designated as fraction 4 had the highest antioxidant activity ($p < 0.05$) with 69.49% ABTS scavenging activity and FRAP reducing potential, 22.26 mg of GAE/g sample. DPPH scavenging activities of fractions 4 (55.59%) and 5 (55.64%) were significantly higher than the other fractions ($p < 0.05$). *A. rigidula* extracts contain gallic, caffeic, vanillic, p-coumaric, salicylic acids and vanillin.

Keywords

Acacia, Plant Extract, Antioxidant Activity, Total Phenolic Acid, HPLC, Gallic Acid

1. Introduction

Antioxidants are a family of compounds that protect against oxidation directly or indirectly. Direct antioxidants suppress the formation of reactive oxidative species (ROS) *in vivo* and *in vitro*. Indirect antioxidants, on the other hand, en-

hance the antioxidant response of an organism [1]. Antioxidants slow down or stop the oxidation of a substrate at low doses. They act by a number of chemical mechanisms, such as hydrogen atom transfer (HAT), single electron transfer (SET), and transition metal chelation [2] [3].

ROS are large family of oxidants derived from molecular oxygen [4]; they are oxygen ions (singlet oxygen, superoxide [O_2•−]), or oxygen-containing radicals (hydroxyl, OH•−). High concentrations of ROS alter the structure of proteins, lipids, and nucleic acids [5] [6] which results in adverse and/or detrimental health effects such as early aging, diabetes, cancer, neurodegenerative disease, inflammatory disease, and cardiac disease [6]-[11]. Antioxidants are known to inhibit the enzymes involved in the oxidative stress response, to chelate the trace elements that are involved in free radical formation, and to function as ROS scavengers [12] [13] [14].

There is a continuous search for new sources of biologically active compounds from plants [11] [14] [15]; these include antioxidants. Plants produce secondary metabolites as a defense mechanism against pathogens and other threats [16]. Secondary metabolites are also known to contribute to the antioxidant activity present in a variety and multitude of plant extracts. More specifically, plants containing phenolic compounds are possible sources of natural antioxidants which will stabilize free radicals by hydrogenation or by forming complexes with oxidizing species [17] [18] [19]. Plants in the genus *Acacia* possess several secondary metabolites, that include phenolic acids and flavonoids [20] [21]. Acetone extracts of *A. senegal* and *A. dealbata* were reported to have high concentrations of phenols and flavonoids and exhibited antioxidant activities [22] [23]. *Acacia nilotica* leaf extracts exhibit reducing capabilities (2.57 mg GAE/mg sample ± 0.03) similar to ascorbic acid (2.62 mg GAE/mg sample ± 0.07) at 1000 μg/mL [24]. Various solvent extracts from different plant parts of *A. confusa* have phenolic acid derivatives and flavonoids that also contribute to their antioxidant activities [25].

The genus *Acacia* has an estimated 1200 species distributed all over the world and many pharmacological molecules have been isolated from its diverse species [17] [20]. However, the phytochemicals of only a small percentage of *Acacia* species have been examined so far [21]. The most predominant compounds isolated from the genus *Acacia* are flavonoids, terpenoids and phenolic acids. The most frequently reported flavonoids are catechin and quercetin. Triterpenoids are the reported terpenoids and many gallic acid and coumaric acid derivatives were reported to have been isolated from the genus *Acacia* [20]. Other metabolites known to be present in the *Acacia* species are polysaccharides (gums) and complex phenolic substances (condensed tannins) [26]. *Acacia* leaves have been reported to have antioxidant, antibacterial, antifungal, anticancer, antiparasitic, cytotoxicity, and immunomodulatory properties [17] [20] [27].

The *Acacia* species understudy is *A. rigidula*; also known as *Vachellia rigidula*. It is predominantly found in the southern part of Texas and in the northern states of Mexico [28]. *A. ridigula* is found in the form of a large shrub or small

tree with rigid, thorny branches that display rich, dark-green foliage. It has some degree of drought tolerance and prospers in minimal watering regiments. *A. rigidula* extracts have been extensively used in popular weight-loss supplements [28]. While *A. rigidula's* root and stem extracts have been used as reducing and capping agent to produce silver nanoparticles. These nanoparticles were reported to eradicate pathogenic resistant bacteria *in vivo* [29]. Methanol bark extracts have been reported to have antifungal properties [30]. Cavazos *et al.* (2021) reported the presence of flavonoids and phenolic acids in acetone, methanol, and acetic acid extracts of *A. rigidula* leaves. They also reported *A. rigidula's* potential as a source of antioxidant and antimicrobial compounds [31]. In this regard, additional evidence is needed to confirm the extent of the antioxidant activities of these compounds present in *A. rigidula* leaf extracts. To our knowledge, there are very limited studies on the biological activities of the species understudy.

This present study provides additional evidence needed to confirm the extent of the antioxidant activities of secondary metabolites particularly total phenolic acids (TPAs) present in *A. rigidula* leaves. This present study's main objectives were: 1) To compare the antioxidant activities of the different solvent extracts and the TPA fractions; 2) To identify phenolic acids present in the extract and the TPA fractions. The results of this present study will provide evidence that *A. rigidula* leaves can be a potential and viable source of phenolic compounds with antioxidant activities, and a basis for the potential use of *A. rigidula* leaves for pharmacological purposes.

2. Materials and Methods

2.1. Leaf Collection and Sample Preparation

A total of four trees of similar height (398 cm - 433 cm) and trunk circumference (15.5 cm - 17.2 cm) were selected for leaf collection. These trees are located on the campus of Texas A&M International University in Laredo, Texas, USA and on the following coordinates: 27˚34'25"N 99˚25'56"W, 27˚34'10"N 99˚25'47"W, 27˚34'16"N 99˚25'56"W, 27˚34'25"N 99˚25'55"W. The voucher for this specimen of *A. rigidula* (accession number WSCO 004,010) was deposited in the Mary Carver Hall Herbarium at Weber State University in Ogden, Utah. The specimen's identity was authenticated by Dr. James Cohen, WSU's botanist and herbarium curator.

In preparing the sample, fresh leaves were washed in running tap water and then towel-dried. Leaves with a mass of 150 grams were subjected to oven drying at 80˚C for 120 minutes, while checking on the dried mass weight periodically until there was no longer a change in mass. The dried leaves were then ground into a powder consistency using an electric herb grinder (Cool Knight, M150B) and stored at −20˚C.

2.2. Chemicals

Ethanol, methanol, and acetone were used as solvents for phytochemical extrac-

tion. All three solvents were sourced and purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Folin-Cioucalteu (2N) reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium acetate, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), and iron (III) chloride were sourced and purchased from Sigma Aldrich. The phenolic standards gallic acid (B24887), 4-(diphenylhydroxymethyl) benzoic acid (43081-1G), vanillic acid (94770-10G), vanillin (V1104-100G), caffeic acid (C0625-2G), p-coumaric acid (C9008-1G) and salicylic acid (247588-100G) were sourced and purchased from Alfa-Aesar (Ward Hill, Massachusetts) and Sigma-Aldrich.

2.3. Soxhlet Extraction of Secondary Metabolites

Thirty grams of ground leaves were used to fill a 41 mm × 123 mm Whatman cellulose extraction thimble (VWR, 10035-404) and 300 mL of solvent (50% ethanol, water: acetone: methanol (1:1:3), 80% methanol) was added to a round bottom flask. The solvent was heated and allowed to evaporate and reach the Soxhlet condenser. The condenser then released water droplets into the extractor containing the thimble. When the solvent reached the siphon, the solvent and extractant were cycled back into the round bottom flask for additional extraction rounds [32]. To remove excess solvent from the Soxhlet extracts, both rotary evaporation and lyophilization were performed. Rotary evaporation was used to remove the excess alcoholic solvent from the Soxhlet extract. After the alcoholic solvent was removed from the Soxhlet extract, the extract was frozen at −80˚C prior to freeze-drying for 48 - 72 hours. The extract was lyophilized using the Labconco® FreeZone 2.5 Liter Benchtop Freeze Dry System. This method was carried out for each extract.

2.4. Total Phenolic Content (TPC) Determination

TPC of each extract was determined using the Folin Ciocalteu (FC) method described by Bakasso *et al.* (2014) with some modifications. An aliquot of 0.5 mL of the extract (100 µg/mL) was mixed with 2.5 mL of the 2N Folin Ciocalteu reagent. The mixture was allowed to stand at room temperature (RT) for 5 minutes. Then, 2 mL of sodium carbonate (75 g/L) solution was added and incubated in the dark at RT for 2 hours. The absorbance of each sample mixture was measured at a wavelength 760 nm against a blank using a microplate reader (Tecan Infinite M Nano). A calibration curve was plotted using gallic acid in concentrations of 0, 25, 50, 75, 100, 150, and 200 mg/L. The results are expressed as milligrams of Gallic Acid Equivalents (GAE)/gram of extract [33].

2.5. Clean up and Fractionation of Phenolic Compounds

Clean-up and fractionation of phenolic compounds present in the *A. rigidula* water: acetone: methanol (1:1:3 v/v/v) extracts were performed following standard protocols [34] [35] [36] with some modifications. Sephadex LH-20 (Cytiva 51218000-EH) column chromatography was performed using a Biologic LP low

pressure chromatography system (Bio-Rad) with supporting Biofrac fraction collector (Bio-Rad). A Bio-Rad econo-glass column (Bio-Rad 7371532) with dimensions of 1.5 cm × 30 cm was used.

The extract solution (0.5 g of extract dissolved in 2 mL of 50% methanol) was filtered using a 0.45 μm PTFE syringe filter (76479-004) before loading into the column to prevent large particles to clog the column. The Sephadex LH-20 column was conditioned using methanol followed by the methanolic extract solution (0.25 $g{\cdot}mL^{-1}$) and was subjected to methanolic solutions of increasing polarity (50% and 70%) followed by elution with 100% methanol to remove phenolics from the sorbent. Elution was carried out at a flow rate of 1 mL min-1, with 30 mL of 50% methanol, 15 mL of 70% methanol, and 15 mL of 100% methanol.

To confirm the presence of phenolic compounds, the collected cleaned fractions were subjected to the Ferric Chloride test described by Panti *et al.* (2014) with some modifications. Three drops of 10% ferric chloride solution were added to 750 μL of each fraction to determine the presence of phenolic compounds with gallic acid (200 μg/mL) used as a positive control. Positive results were indicated by a black-blue coloring while negative results were indicated by bright yellow coloring [37].

The positive results were then subjected to thin layer chromatography (TLC) using silica gel plates and toluene:acetone:formic-acid (4.5:4.5:1 v/v/v) as a mobile phase to identify similar phenolic compounds in each fraction. Each TLC plate was run allowing the solvent front to reach 8 cm, and the Rf value of each compound was measured. Fractions with compounds of the same Rf values were pooled and further analyzed for antioxidant activity in triplicates.

2.6. Comparison of the Antioxidant Activities of Crude Extracts and Total Phenolic Fractions

The extracts (50% EtOH extract, 80% MetOH extract, 1:1:3 WAM extract, and the total phenolic fractions) were dissolved in 80% EtOH at a concentration of 1 mg/mL and then diluted to prepare the series concentrations for the antioxidant assays [38]. All assays were performed in triplicates for each of four blocks of the experimental design. ABTS scavenging activity (%), DPPH radical scavenging activity (%), Ferric reducing antioxidant power (FRAP) assay (mg GAE/g freeze-dried sample) and inhibition of linoleic acid peroxidation (%) will represent antioxidant activity.

2.6.1. ABTS Radical Scavenging Assay

The 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay was performed following the method of Re *et al.* [38] with modifications. The ABTS radical was generated by the oxidation of ABTS (Roche 10102946001) with potassium persulfate (Sigma-Aldrich 60489-250G-F). The ABTS solution (7 mM) was reacted with potassium persulfate (2.45 mM) solution and it was stored at 4˚C in the dark for 12 - 16 hours to produce a dark co-

lored solution containing the ABTS radical cation. Prior to the assay, the ABTS radical cation was diluted with 50% methanol for an initial absorbance of about 0.70 (±0.02) at 745 nm wavelength. The free radical scavenging activity was evaluated by mixing 3 mL of ABTS working standard with 300 µL of test sample (50, 100, 150, 200, 300 µg/mL) in a 10 mL beaker. The decrease in the absorbance was measured at exactly 1 min after mixing the solution until it reached 6 min. The final absorbance was noted then. The ABTS scavenging activity (%) was calculated based on the following formula [38]:

ABTS scavenging activity (%) = [(absorbance of control − absorbance of sample)/absorbance of control] × 100%

2.6.2. DPPH Radical Scavenging Activity Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay was performed following the method by Zhu [39] with modifications. One mL of DPPH solution (0.1 mM, in methanol) was added to 1 mL of the samples at different concentrations (50 - 300 µg/mL). Then, the reaction mixture was vortexed and incubated in the dark at room temperature for 30 min, and the absorbance was read at 517 nm using a microplate reader (Tecan Infinite M Nano). A solution of 80% ethanol served as the negative control while ascorbic acid (BDH BDH9242-250G) and gallic acid (Alfa Aesar, B24887) served as positive controls (standards). The DPPH scavenging activity (%) was calculated based on the following formula [39]:

DPPH scavenging activity (%) = [(absorbance of control − absorbance of sample)/absorbance of control] × 100%

2.6.3. Ferric Reducing Antioxidant Power (FRAP) Assay

Reducing antioxidant power was calculated using the method by Tomasina and colleagues [40] with modifications. In a 2.7 mL of freshly prepared FRAP reagent (300 mM acetate buffer (pH 3.6), 10 mM TPTZ and 20 mM $FeCl_3$ solution (10:1:1, v: v: v), 0.3 mL of the diluted sample was added. The mixture was incubated for 5 minutes at 37˚C before absorbance was read at 620 nm in a plate reader (Tecan Infinite M Nano). Ethanolic solutions of ascorbic acid and gallic acid served as positive controls and 80% ethanol served as negative control. The results are reported as milligrams of gallic acid equivalents per gram of freeze-dried sample.

2.6.4. Ferric Thiocyanate Lipid Peroxidation Assay

Percent inhibition of peroxidation in linoleic acid was determined following the ferric thiocyanate method by Sultana and colleagues [41]. In a solution mixture of linoleic acid (0.13 mL), 99.8% ethanol (10 mL) and 0.2 M sodium phosphate pH 7 buffer (10 mL), 5 mg of the freeze-dried sample were added. The mixture was diluted with distilled water to reach a final volume of 25 mL. The solution was then incubated at 40˚C for 15 days. After the incubation period, 0.2 mL of the sample solution was sequentially added with 10 mL of 75% ethanol, 0.2 mL

of 30% ammonium thiocyanate, and 0.2 mL 20 mM of ferrous chloride (Alfa Aesar A16327) in 3.5% HCl. Positive controls were prepared as described above but using ascorbic acid or gallic acid in place of freeze-dried sample. On the other hand, the negative control was prepared as described above but without the freeze-dried sample. After 3 min of stirring, the absorbance of the samples was read thrice using a microplate reader (Tecan Infinite M Nano) at a 500 nm wavelength. Percent inhibition of lipid (linoleic acid) peroxidation was calculated using the following equation [41]:

Inhibition of lipid peroxidation (%) = 100 − [(absorbance of sample)/(absorbance of control)] × 100%

2.7. High Performance Liquid Chromatography (HPLC) Analysis

HPLC was performed using a Shimadzu Prominence-i LC-2030C 3D to identify the phenolic acids present in the three different solvent extracts and their isolates. Following the method reported by Abdelkhalek [42], the samples were separated using an elution gradient with: 1) HPLC grade water 0.1% trifluoroacetic acid (v/v), and 2) HPLC grade methanol. The injection volume was 20 µL. Variable wavelength detector (VWD) was set at 254 nm wavelength to identify the phenolic compounds present in the *A. rigidula* extract. To identify the phenolic acids present in the sample extracts, phenolic acid standards namely gallic acid, 4-(diphenylhydroxymethyl) benzoic acid (43081-1G, Millipore Sigma), vanillic acid (94770-10G, Millipore Sigma), vanillin (V1104-100G, Millipore Sigma), caffeic acid (C0625-2G, Millipore Sigma), p-coumaric acid (C9008-1G, Millipore Sigma) and salicylic acid (247588-100G, Millipore Sigma) were used. The analysis was performed in triplicates for all the *A. rigidula* extracts and the fractions.

2.8. Statistical Analyses

In this present study, the overall experimental design was in the form of a randomized complete blocks design (RCBD) where all treatments were randomly allocated into each of the blocks. The RCBD for TPC and for TFC had two blocks and four treatments. For scavenging activity analysis (SA), the RCBD had four blocks and 11 treatments with an analysis of variance (or anova) and post-hoc test carried for each assay. For percent lipid peroxidation inhibition, the RCBD had three blocks and 24 treatments (each treatment represents an extract and concentration combination). All post-hoc tests were in the form of a Bonferroni test. All test of hypothesis associated with each anova and the post-hoc tests were carried out using a 5% type-I error rate (or $\alpha = 0.05$). SPSS 28 and SAS 9.4 were the statistical software used to perform all statistical analyses.

3. Results and Discussion

3.1. The Optimum Solvent for Extraction of Total Phenolic Acid Content

Previous studies have shown that solvents affect the extraction of plant sec-

ondary metabolites [43] [44] [45]. This study aimed to identify the optimum solvent for extracting total phenolic acid content (TPC) from *A. rigidula* leaves. Table 1 shows mean TPC for the extracts produced from 50% ethanol (EE), 1:1:3 water:acetone:methanol (WAME) and 80% methanol (ME). An anova on TPC revealed no significant differences among the solvent extracts ($p > 0.05$) (Anova table provided as supplementary material).

In comparing this study's results to those of previous studies, some of the most common solvents used for secondary metabolite extraction are: ethanol, methanol, ethyl acetate, acetone, and hexane [21] [46]. Currently, there is no standard method for the extraction of total phenolic acids from plant samples [47]. The solvent used is dependent on the desired chemical composition of the extract; indeed, there is large variability in the chemical composition of phenolic compounds in plants, ranging from simple phenolics (e.g., phenolic acids, flavonoids, and anthocyanins) to complex polymers (e.g., tannins) [48]. The solvent ethanol has been reported to be a good choice for polyphenolic extraction and it has the advantage of being safe for humans [49] [50]. In a study on leaves from *Acacia nilotica* (L.), ethanolic extracts were obtained by Soxhlet extraction with a reported TPC of 536.02 mg of GAE/g of extract [51]. Methanol is also one of the most commonly used solvents for the extraction of plant secondary metabolites, especially smaller molecules such as simple polyphenols [48].

The results of this study showed that there were no significant differences ($p > 0.05$) in TPC among the different solvent (ethanol, water: acetone: methanol, methanol) extracts. In a study comparing TPC of *Acacia senegal* leaves, there were also no observed significant difference between the TPC of extracts prepared by using acetone and methanol as solvents. The TPC from the acetone and the methanol extracts were 0.779 ± 0.313 and 0.842 ± 0.413 mg tannic acid equivalent/mL, respectively [22]. On the other hand, water:acetone:methanol solvent mixture was reported as the most effective solvent at extracting polyphenols like p-aminobenzoic acid, caffeic acid, rutin, and others [52]. In this study—although not statistically different, the WAME solvent mixture was the most effective solvent tested for the extraction of TPC (288.24 mg of GAE/g of extract), when compared to EE (248.37 mg of GAE/g of extract) and ME (279.21 mg of GAE/g of extract). Based on these results, using ethanol as the extraction solvent for future research would be recommended, as it is the most cost effective and a relatively safer solvent compared to acetone and methanol [49] [50].

Table 1. Comparison of the total phenolic content of *A. rigidula* solvent extracts.

Extract	mg of GAE/mL of sample	mg of GAE/g of sample	Sig.
EE	2.484	248.37	ns
WAME	2.882	288.24	ns
ME	2.792	279.21	ns

Values are expressed as the means of four blocks; ns denotes not significant ($p > 0.05$).

3.2. Comparison of Antioxidant Activities of the Different Solvent Extracts

The ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid) assay is based on the reduction of the radical cation (ABTS•+) by the presence of antioxidant compounds [53] [54]. In the ABTS assay, antioxidant compounds served as hydrogen providers or free-radical scavengers [53]. **Figure 1(a)** presents the ABTS scavenging activities (SA; %) of the *A. rigidula* extracts, EE, WAME, and ME at 50, 150, and 250 µg/mL. ABTS SA ranged from 49.85% - 62.53%. Results indicated no significant differences ($p > 0.05$) in scavenging activities (SA) among solvent extracts at all tested concentrations ($p > 0.05$). In addition, there were also no significant differences ($p > 0.05$) in SA among the three solvent extracts and the positive controls (34.20% for ascorbic acid at 100 µg/mL and 65.56% for gallic acid at 100 µg/mL).

Diphenyl-1-picrylhydrazyl assay is another assay that assesses the presence of antioxidant compounds. This assay uses the free radical, diphenyl-1-picrylhydrazyl (DPPH) while the antioxidant served as the hydrogen provider or free-radical scavenger [55]. DPPH scavenging activities (SA; %) in *A. rigidula* extracts are presented in **Figure 1(b)**. The positive controls, ascorbic acid and gallic acid at 100 µg/mL (48.99% and 48.46%, respectively) exhibited the highest activities but were not significantly different ($p > 0.05$) from EE at all levels of concentration (46.33% - 47.85%), WAME, and ME at 150 and 250 µg/mL (46.27% - 47.06%). ME and WAME at 50 µg/mL had the lowest SAs (45.17% - 45.22%) and were significantly lower than the positive controls ($p < 0.05$) **Figure 1(b)**).

In general, it was observed that the scavenging activities (antioxidant activities) of solvent extracts (EE, WAME and ME) were similar to each other. Likewise, the TPC among solvent extracts were not significantly different ($p > 0.05$). It is likely that the TPC of the extracts contribute largely to the observed

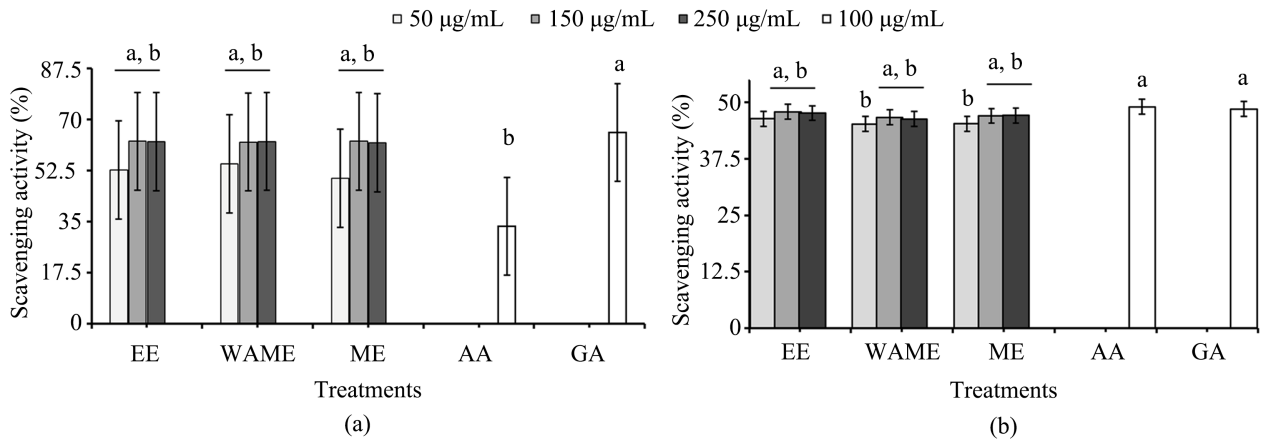
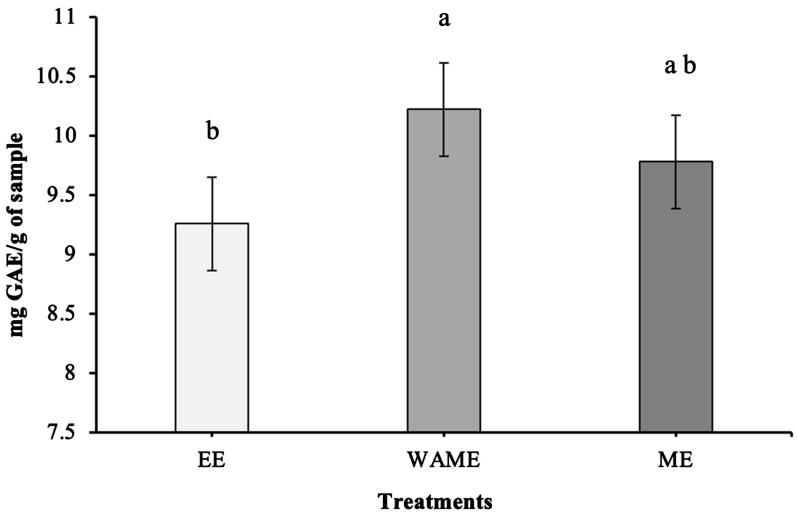


Figure 1. Comparison of the scavenging activities (SA) among *A. rigidula* solvent extracts using (a) ABTS and (b) DPPH assays. Anova performed on SA from the ABTS and the DPPH assays indicated significant differences among the various combinations of treatment and concentration ($p < 0.05$; Tables in SI). A Bonferroni post-hoc test was performed; bars indicate 95% confidence intervals. Means with different letters are significantly different ($p < 0.05$), means with same letters are not significantly different ($p > 0.05$).

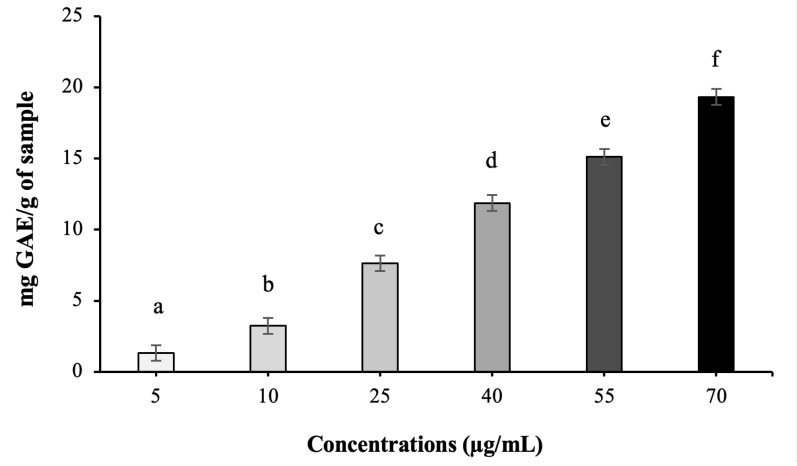
antioxidant activity. The relationship between TPC and antioxidant activities has been reported in multiple works [55] [56] [57]. More specifically, in the work by Bordean *et al.* [58] where they have reported a high correlation ($r = +0.959$, $p = 0.0001$) between TPC and the observed antioxidant activity of different *Artemisia* species. Similar to the results from this study, the antioxidant activities of the different solvent extracts were not significantly different from each other ($p > 0.05$). This is likely due to no significant differences in TPC among solvent extracts ($p > 0.05$).

The similarities in scavenging activities among the solvent extracts and the positive controls ($p > 0.05$) in both the ABTS and DPPH assays provide evidence of the potential of *A. rigidula* extracts as a source of antioxidants. In addition, the observed similarity in the ABTS scavenging activities between the solvent extracts and the gallic acid suggests that phenolic acids are likely contributing to the observed antioxidant activity in *A. rigidula* extracts. Furthermore, the observed scavenging activities of the positive controls suggest that phenolic compounds such as gallic acid, have better scavenging potential against the ABTS radical than non-phenolic compounds like ascorbic acid. Similar to the study on the methanolic extracts of *Vayasthapona rasayana*, ascorbic acid (9% ± 0.5%) had significantly lower ABTS scavenging potential compared to the other positive control, Trolox (95% ± 0.05%) [59]. Another study by Boulebd *et al.* [60] comparing four well-known antioxidants (ascorbic acid, Trolox, BHT, and BHA) reported that ascorbic acid, when subjected to the ABTS assay, required the highest concentration to scavenge 50% of the ABTS radical (23.40 ± 1.06 μM). Other *Acacia* species reported to have scavenging activities against ABTS and/or DPPH radical are *A. nilotica* (L). leaves, *A. caven* flowers and *A. senegal* leaves [23] [24] [50] [61].

The presence of antioxidant activity in *A. rigidula* extracts was also assessed using the ferric reducing antioxidant power (FRAP) assay. In this assay, the antioxidants serve as electron-donors that result to the reduction in ferric iron (Fe^{3+}) in the assay mixture with ferrous ion (Fe^{2+}) [62] [63]. In this study, the FRAP assay results showed a ferric reducing antioxidant power or reducing potential in *A. rigidula* extracts (**Figure 2**). An anova performed on the FRAP results showed no significant differences among the treatment by concentration interactions, but a significant difference among treatments ($p < 0.05$) as well as significant difference among concentrations ($p < 0.001$). Thus, pointing to the main effects of treatments and of concentrations and without any interaction effects (anova table provided as supplementary material). In this regard, the reducing potential of EE, WAME and ME from all concentrations were pooled (**Figure 2(a)**). The highest reducing potential was observed in WAME (10.22 mg GAE/g sample) but was not significantly different from that of ME (9.781 mg GAE/g sample) ($p > 0.05$) (**Figure 2(a)**). On the other hand, the reducing potential of WAME was significantly higher than EE (9.259 mg GAE/g sample) ($p < 0.05$). Statistically significant differences ($p < 0.05$) among all concentrations



(a)



(b)

Figure 2. Comparison of the FRAP scavenging activities (a) among solvent extracts (b) among concentrations. For (a), means were calculated across different levels of concentrations. For (b), means were calculated across treatments. A Bonferroni post-hoc test was performed; bars indicate 95% confidence intervals. Means with different letters are significantly different ($p < 0.05$), means with same letters are not significantly different ($p > 0.05$).

with respect to their reducing ability were observed (**Figure 2(b)**). In comparing the mean reducing potential among six different concentrations, results as expected showed that reducing potential was concentration dependent.

Other studies have also used the FRAP assay to test the reducing power of *Acacia* plant extracts. A study on *Acacia* jacquemontii leaves compared the TPC for methanol and ethanol extracts and found that methanol extract had the highest TPC (271.44 ± 4.41 mg GAE/g of sample) [64]. That study found that the methanol extract of *A. jacquemontii* leaves also produced the highest FRAP reducing power (453.18 ± 5.9 mg TE/g sample) using a 1 mg/mL concentration. In this present study, the concentration of TPC was highest in the WAME sol-

vent extract (288.24 mg GAE/g of sample), as well as the highest reducing power (10.22 mg GAE/g sample).

The ferric thiocyanate lipid peroxidation (LPO) assay measures the linoleic acid peroxidation inhibition. In the assay, the presence of antioxidants will result to an inhibition in the oxidization of ferrous chloride to ferric chloride brought about by the radicals produced from linoleic acid peroxidation. Figure 3 shows the lipid peroxidation inhibition activity of *A. rigidula* extracts, EE, WAME, and ME at 200 µg/mL. LPO inhibition activities were as follows: EE, 70.65% > WAME, 68.99% > ME, 66.64% > ascorbic acid, 62.70% > gallic acid, 52.09%. *A. rigidula* extracts performed similarly as the antioxidant, ascorbic acid ($p > 0.05$) and performed better than the antioxidant, gallic acid ($p < 0.05$) (Figure 3). Once again, these results provide evidence for the antioxidant activity *A. rigidula* extracts.

Compared to this present study, Cavazos *et al.* [31] found lower LPO inhibition activities in *A. rigidula* extracts; *A. rigidula* acetone extract had the higher LPO inhibition activity of 42% while only a 25% was observed for their methanolic *A. rigidula* extract. They also reported the LPO inhibition activity of *A. berlandieri* acetone and methanolic extracts to be at 19% and 13%, respectively. The observed antioxidant activity in their *Acacia* extracts was attributed to secondary metabolites namely flavonoids, terpenes, saponins, and tannins that were qualitatively determined to be present in the *A. rigidula* and *A. berlandieri* extracts. Whereby, solvent extracts (80% ethanol) from *A. nilotica* (L.) leaves, pods, and bark extracts at a concentration of 2000 µg/mL had LPO inhibition activities of 87.39%, 83.72%, and 60.53%, respectively [22]. These extracts were tested at 10× higher concentration than the *A. rigidula* extracts. Based on the results of that study and this present study, *A. rigidula* extracts have higher LPO inhibition activity than *A. nilotica* extracts.

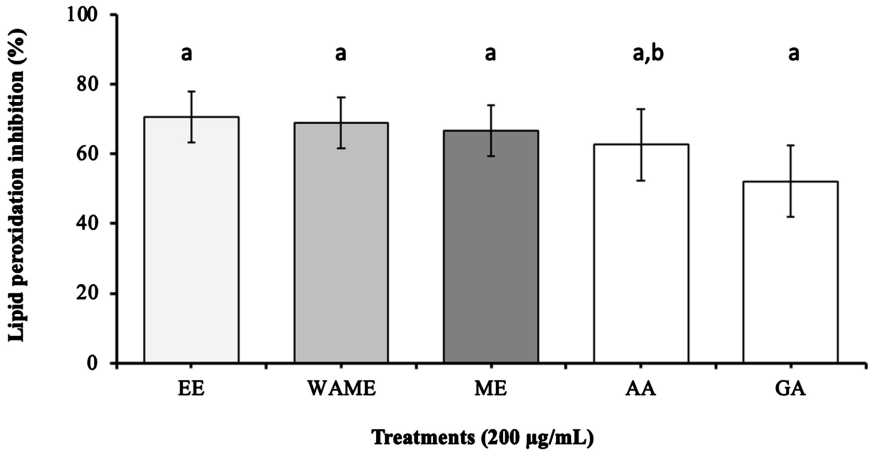


Figure 3. Comparison of the lipid peroxidation inhibition activity of *A. rigidula* solvent extracts. A Bonferroni post-hoc test was performed; bars indicate 95% confidence intervals. Means with different letters are significantly different ($p < 0.05$), means with same letters are not significantly different ($p > 0.05$).

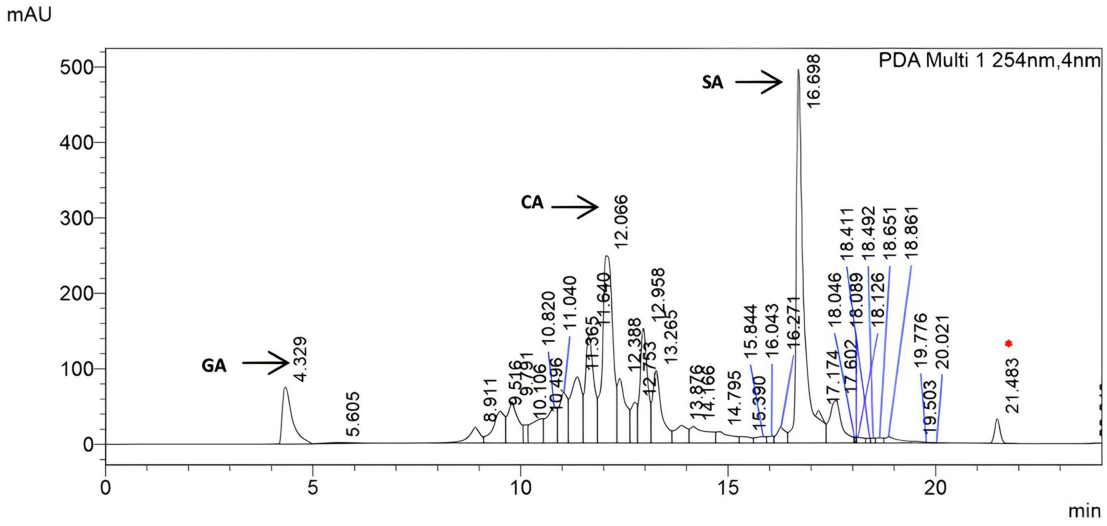
A. rigidula extracts (EE, WAME, and ME) were subjected to two scavenging activity assays (ABTS, DPPH) and two iron reducing assays (FRAP, LPO inhibition) to determine and compare the antioxidant activities of the extracts. The results from all antioxidant assays provide evidence that *A. rigidula* leaf extracts exhibited antioxidant activities comparable to gallic acid and ascorbic acid. In general, there is no difference in antioxidant activities among EE, WAME, and ME extracts as well as in TPC. All extracts contained more than 200 mg GAE/g of sample. Phenolic compounds have been attributed as being powerful antioxidants *in vitro*, greater than well-known antioxidants such as ascorbic acid and vitamin E [13] [65]. It is likely that phenolic compounds contribute greatly to the observed antioxidant activities in *A. rigidula* extracts.

3.3. Isolation and Identification of Phenolic Acids

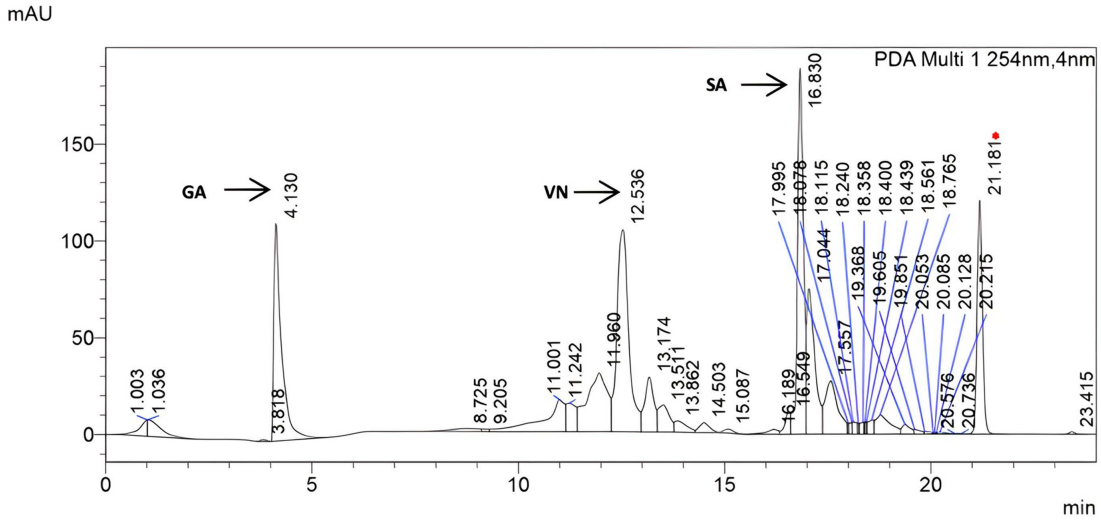
The water: acetone: methanol (1:1:3 v/v/v) (WAME) extract yielded the highest TPC, thus WAME was used as the solvent in the extraction protocol. The isolation of phenolic acids was done using Sephadex LH-20 column chromatography. In total, there were 38 individual fractions collected. These fractions were subjected to the ferric chloride test to detect phenolic compounds. In this test, $FeCl_3$ forms a complex with the hydroxyl group attached to the benzene, creating a dark blue, black or brown coloration. Fractions that were positive in the ferric chloride test were subjected to thin layer chromatography (TLC). Fractions with similar Rf values and banding patterns were pooled together to form the main fractions. This pooling is based on the assumption that fractions with similar Rf values will have the same, or similar, compounds. Five main fractions were obtained and with each of these subjected to High Performance Liquid Chromatography (HPLC) analysis in order to identify the phenolic acids present in the *A. rigidula* extracts.

HPLC is commonly used to determine the phytochemicals present in plant extracts [61] [63] [66] [67]. Antioxidant phenolic compounds: benzoic acid, caffeic acid, p-coumaric acid, gallic acid, salicylic acid, vanillic acid and vanillin [21] [58] [68] frequently reported in plants (*i.e.*, *Acacia*) were used as standards in this present study. **Table 2** shows the retention time and area of each phenolic standard used in this study.

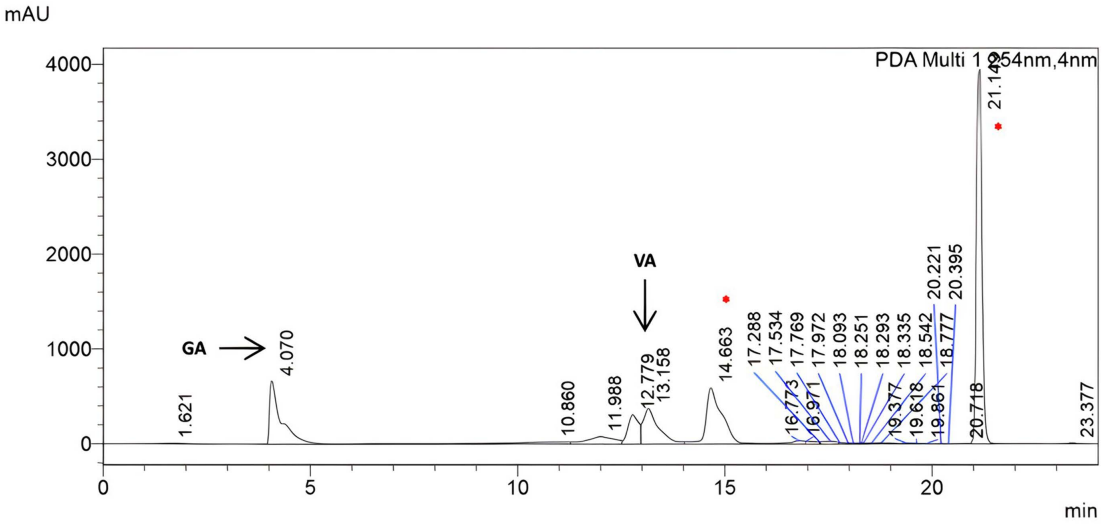
Figure 4 shows the chromatograms for Fractions 1 - 5, with peaks labeled with their phenolic acid identities. Using the retention time obtained from the phenolic standards, gallic acid, caffeic acid, salicylic acid, vanillic acid, vanillin, and p-coumaric acid were identified in these fractions. A study by Mohammed *et al.* [63] also collected five main fractions in investigating the phenolic composition of *Artemisia herba-alba*. That study was able to identify hydroquinone, 4-hydroxy benzoic acid, catechol, quercetin, gallic acid, cinnamic acid, and thymol in its different fractions. Similar to this present study's results, some phenolic compounds were present in multiple fractions, while some compounds only appeared in one fraction. Gallic acid was present in all five fractions in this present study. Salicylic acid was present in Fractions 1 and 2 (**Figure 4(a)** and


mAU
PDA Multi 1 254nm,4nm
GA
CA
SA
4.329
12.066
16.698
min

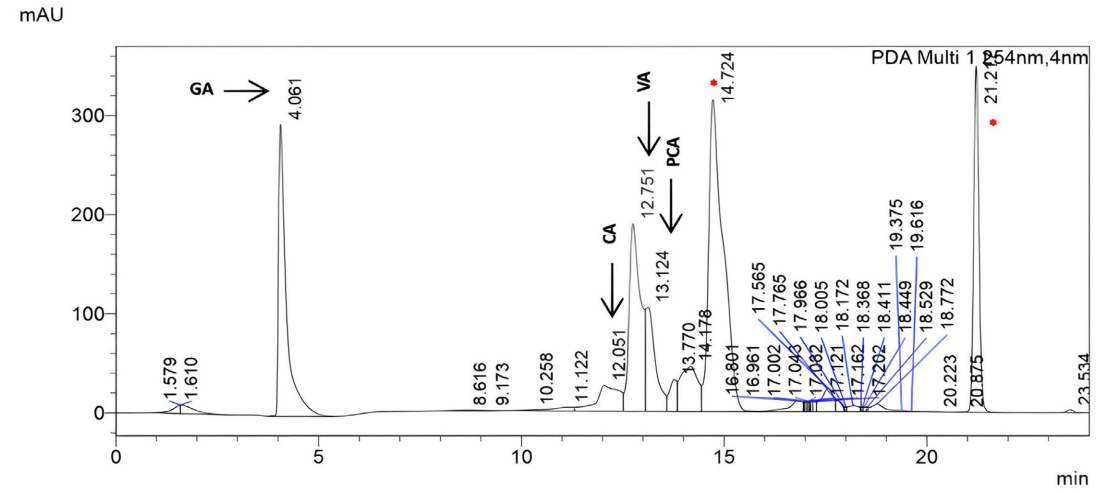
(a)


mAU
PDA Multi 1 254nm,4nm
GA
VN
SA
4.130
12.536
16.830
21.181
min

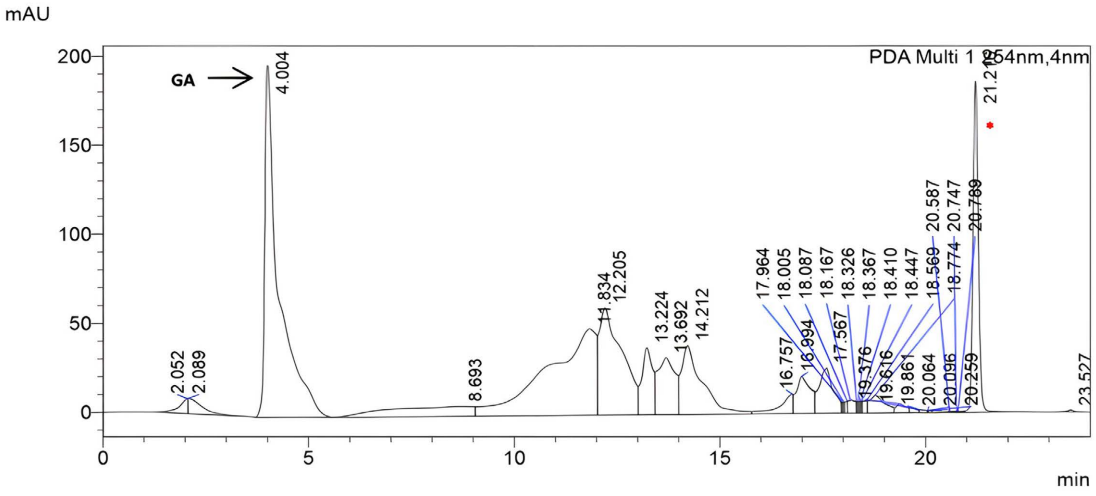
(b)


mAU
PDA Multi 1 254nm,4nm
GA
VA
4.070
14.663
21.142
min

(c)



(d)



(e)

Figure 4. HPLC chromatogram of Fractions 1 - 5. (a) Fraction 1; (b) Fraction 2; (c) fraction 3; (d) Fraction 4; (e) Fraction 5. GA = gallic acid, CA = caffeic acid, SA = salicylic acid, VN = vanillin, VA = vanillic acid, PCA = p-coumaric acid. Red asterisk denotes a peak with unidentifiable compound.

Table 2. Standards of phenolic compounds and their retention times.

Standards	Retention Time (min)	Concentration (ppm)	Area
4-(diphenylhydroxymethyl) benzoic acid	18.02	1000	55,292,075
Caffeic acid	12.08	1000	68,926,704
p-coumaric acid	13.08	1000	69,474,605
Gallic acid	4.35	1000	121,965,654
Salicylic acid	16.50	1000	2,128,743
Vanillic acid	12.83	1000	75,722,335
Vanillin	12.62	1000	63,944,221

All phenolic standards were 1000 ppm. All fractions and standards were ran using a linear gradient of methanol (0% - 50% for 10 min, 50% - 70% for 10 min, and 100% for 5 min) for the elution and identification of the chemical components.

Figure 4(b)). Vanillin was present in Fraction 2 only, and vanillic acid in Fraction 4 only. Caffeic acid was observed in both Fractions 1 and 4. The p-coumaric acid was only visible in Fraction 4, which was the fraction with the most identifiable compounds present (Figure 4(d)). Additionally, there were two peaks observed in all fractions, but were most intense in Fractions 3, 4, and 5, with retention times averaging 14.69 and 21.24 min for these two peaks. These peaks did not fall within the range of any of the phenolic standards and hence remained unidentified.

3.4. Comparison of Antioxidant Activities of Phenolic Fractions against the Crude Extract

Comparing the antioxidant activities of the different fractions, it can be observed that Fractions 4 and 5 showed the highest antioxidant activities in all antioxidant assays performed (Table 3). More specifically, Fraction 4 had significantly higher ABTS scavenging activity as well as ferric reducing power (FRAP) than Fraction 5 ($p < 0.05$). In terms of DPPH scavenging activity, Fractions 4 and 5 had no significant difference ($p > 0.05$). In comparison with the crude extract, both Fractions 4 and 5 has significantly higher ($p < 0.05$) ABTS scavenging activity than the crude extract but no significant difference ($p > 0.05$) in DPPH scavenging activity among Fraction 4, Fraction 5 and the crude extract (Table 3). On the other hand, a statistically higher ($p < 0.05$) reducing power was observed in the crude extract (23.61%) compared to Fraction 4 (22.26%) and Fraction 5 (16.54%). Fractions 1, 2 and 3 showed lower antioxidant activities (with Fraction 1 being the lowest) in all assays performed compared to Fractions 4, 5 and the crude extract.

Table 3. Results of ABTS, DPPH, and FRAP assays for fractions and crude extracts.

Sample	ABTS	DPPH	FRAP
(100 μg/mL)	% Inhibition	% Inhibition	mg GAE/g of sample
Fraction 1	25.27 ± 3.20^{i}	51.66 ± 2.76^{b}	6.61 ± 0.19^{h}
Fraction 2	46.16 ± 3.20^{e}	53.98 ± 2.76^{b}	10.60 ± 0.34^{f}
Fraction 3	59.24 ± 3.20^{c}	53.36 ± 2.76^{b}	10.91 ± 0.05^{f}
Fraction 4	69.49 ± 3.20^{a}	55.12 ± 2.76^{a}	22.26 ± 0.67^{b}
Fraction 5	63.61 ± 3.20^{b}	55.59 ± 2.76^{a}	16.52 ± 0.25^{c}
Crude Extract	60.19 ± 3.20^{c}	55.64 ± 2.76^{a}	23.61 ± 0.37^{a}
Gallic Acid	62.82 ± 3.20^{b}	54.43 ± 2.76^{a}	-
Ascorbic Acid	63.44 ± 3.20^{b}	56.45 ± 2.76^{a}	-

Values are expressed as mean ± 95% CI of three trials. *A. bonferroni* post hoc test was performed. Means in the same column with different letters are significantly different ($p < 0.05$), means with same letters are not significantly different ($p > 0.05$).

Fraction 4 is the most interesting fraction given that among all fractions, Fraction 4 had the significantly highest ($p < 0.05$) ABTS scavenging activity (69.49%) and an activity higher than the positive controls: gallic acid (62.82%) and ascorbic acid (63.44%) as well as with the crude extract (60.19%). In addition, in terms of the DPPH scavenging activity, Fraction 4 (55.12%) had no significant difference ($p > 0.05$) in scavenging activity when compared to gallic acid (54.43%), ascorbic acid (56.45%) and the crude extract (55.64%). The results from the antioxidant assays imply that there is a difference in the compounds extracted at different times during the column chromatography methanol gradient. The comparison of the antioxidant activities of the collected fractions implies that most of the compounds contributing to the antioxidant activity were isolated in Fraction 4 using a mostly 100% methanol solvent followed by Fraction 5 eluted with a mostly 80% methanol solvent. Based on these results, Fraction 4 had the best antioxidant activities when compared to the rest of the fractions for the ABTS, DPPH, and FRAP assays.

Results from HPLC analysis (Table 4) showed that when compared to Fraction 5, Fraction 4 had detectable presence of gallic acid, caffeic acid, vanillic acid, and coumaric acid while Fraction 5 contained gallic acid only. Gallic acid was also detectable in all fractions but was higher in Fractions 4 and 5. It is most likely that gallic acid contributed majorly to the antioxidant activities observed in all fractions especially to the higher activities observed in Fractions 4 and 5. Vanillic and p-coumaric acids were detected in Fraction 4 only. Thus it is likely that these phenolic acids contribute to the higher antioxidant activity observed in Fraction 4 compared to Fraction 5. Based on the combined results of the HPLC analysis and the antioxidant assays; the phenolic compounds in *A. rigidula* extracts responsible for the antioxidant activities are: gallic acid > coumaric acid > vanillic acid > vanillin > salicylic acid > caffeic acid.

Phenolic compounds have been reported to have high antioxidant activities [69] [70] [71]. High correlations between total phenolic content (TPC) values and antioxidant activities were reported in several studies *i.e.*, Artesemia species, Western Australian honeys, *A. chasmamtum*, kiwi and cucumber, *C. didymus* [2] [18] [58] [70] [71]. Reports of the antioxidant activity in *Acacia* extracts have

Table 4. Phenolic compounds identified in fractions 1 - 5.

Fraction	GA	CA	VA	VN	4-BA	PCA	SA
1	+	+					+
2	+			+			+
3	+						
4	+	+	+			+	
5	+						

GA—gallic acid; CA—caffeic acid; VA—vanillic acid; VN—vanillin; 4-BA-4 (diphenylhydroxymethyl) benzoic acid; PCA—*p*-coumaric acid; SA—salicylic acid.

been reported by various studies which have been consolidated in a review by Batiha [21]. The investigation of an extract of *A. hydaspica* produced from ethyl acetate showed high concentrations of polyphenolic compounds exhibited great antioxidant activity *in vivo* [17]. Further studies on the antioxidant activity of 5 different *Acacia* species (*A. mangium*, *A. auriculiformis*, *A. crassicarpa*, *A. leucophloea*, and *A. deccurens*) showed that *A. crassicarpa* had the highest antioxidant activity along with a high content of phenolic compounds present in the methanolic extract [72]. A correlation between TPC and antioxidant activity is further supported by the results of Prayogo *et al.* [72]. They observed lower antioxidant activities in extracts of *A. mangium* and *A. decurrrens* with lower phenolic acid content.

Phenolic compounds possess excellent antioxidant potential due to their high redox reactivity to reduce free radicals and prevent destructive cascade reactions [73] [74]. The antioxidant activity of the phenolic compounds is attributed to its reducing hydrogen and singlet oxygen quenching properties [74]. Furthermore, phenolic compounds have been identified to be excellent antioxidant compounds due to their chemical structure. Phenolic compounds are composed of a 6-membered phenyl ring where one or more double bonds could be present. Due to the large delocalization of electrons of the compound, phenolic acid and other phenolic compounds are able to donate electrons to free radicals [47] [74]. Furthermore, the resonance stabilized structures terminate the propagation of additional radical formation [73] [74]. Phenolic acids and other phenolic compounds can also act as antioxidant compounds due to their ability to form chelation complexes with metals. Transitional metals can remove or donate electrons resulting in the formation of free radicals. Phenolic acids with two or more hydroxyl groups can conjugate with metals thus limiting the formation harmful free radicals [73] [74].

4. Conclusion

This study demonstrated that *Acacia rigidula* leaves possess antioxidant activities. There were no significant differences in antioxidant activities among solvent extracts. It was observed that type of extraction solvent does not significantly impact TPC extraction. Based on the combined results of the HPLC analysis and the antioxidant assays; the phenolic compounds in *A. rigidula* extracts responsible for the antioxidant activities are: gallic acid > coumaric acid > vanillic acid > vanillin > salicylic acid > caffeic acid. The results of this present study provide evidence that *A. rigidula* leaves can be a potential and viable source of phenolic compounds with antioxidant activities, and a basis for the potential use of *A. rigidula* leaves for pharmacological purposes.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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