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Evaluation of Polyphenol Composition and Antioxidant Capacity of Leaf and Bark Extracts of *Neolamarckia cadamba*

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Neolamarckia cadamba (Kadamba) is used in ethnomedicine for the treatment of various illnesses. However, there is limited information available regarding its phenolic composition and antioxidant properties. The study assessed the polyphenol profile and phytochemical properties of leaf and bark extracts of *N. cadamba* using attenuated total reflectance-fourier transform infrared spectroscopy (ATR-FTIR) and nuclear magnetic resonance spectroscopy (NMR). The antioxidant properties, including total phenolic content, DPPH free radical scavenging assay, and ferric reducing antioxidant power, were evaluated. Multivariate data analysis (MVDA), via partial least square-discriminant analysis (PLS-DA) and orthogonal partial least square-discriminant analysis (OPLS-DA), was conducted to evaluate the polyphenol profile of the leaf and bark extracts. This study shows that the bark extract has higher TPC value than the leaf extract (58 ± 0.31 mg GAE/g DW and 41±0.36 mg GAE/g DW, respectively). The inhibitory concentration (IC_{50}) values of the *N. cadamba* leaf and bark extracts measured are comparable with the synthetic antioxidants BHT and Trolox. The ferric reducing antioxidant power (FRAP) values for the leaf and bark extracts are in the range of 24 ± 0.936 μM $FeSO_4$/g DW to 72 ± 0.422 μM $FeSO_4$/g DW. These findings suggest that the bark extract has potential in the nutraceutical industry.

Keywords: Medicinal plants; ethnomedicine; Fourier transform infrared; Nuclear magnetic resonance; phytochemicals

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Medicinal plants have boosted the interest of scientists due to their potential as a source of natural biologically active compounds, particularly the antioxidant properties of medicinal plants, which have led to a wide range of applications such as in pharmaceuticals, alternative medicine, and natural therapies [1]. According to Suffredini et al. [2], various studies have shown that many plants are a rich source of antioxidants, such as vitamins A and E and phenolic compounds, including flavonoids, phenolic acids, and tannins.

Neolamarckia cadamba (Roxb.) Bosser (family Rubiaceae), known as 'Kadamba', is a popular Ayurvedic medicinal plant that is native to Australia, China, India, Indonesia, Papua New Guinea, the Philippines, Singapore, Vietnam, as well as Malaysia [3]. Earlier studies have shown that nearly all parts of *N. cadamba* plant have anti-diabetic, antioxidant, antimicrobial, and anti-inflammatory properties [4] and have been used to treat diseases such as hemoptysis, cough, vomiting, ulcers, and debility [5]. Patel et al. [6] discovered that the leaf and bark of *N. cadamba* pose antifungal activities against *Aspergillus fumigatus* and *Candida albicans*.

Antioxidants are required for the protection of living cells from potentially harmful reactive oxygen species (ROS). ROS are constantly produced by metabolic reactions, but they are entrapped by antioxidants. Uncontrolled ROS production can cause dangerous tissue injuries in biological molecules, leading to a variety of diseases [7]. Naturally occurring antioxidants are thus required for the human body to combat oxidative stress caused by disease [8]. Antioxidants are commonly used to protect oxidizable compounds from the harmful effects of oxidation, and additionally being used as dietary supplements to mitigate the negative effects of oxidative stress.

Furthermore, they are used in the food industry to reduce rancidity, protect and stabilize colors and aroma, and extend product shelf life [9], as well as improving food quality [10].

There are numerous natural antioxidant compounds, but polyphenols are without a doubt the most abundant and widespread class in nature [10]. Dzah et al. [11] reported that the polyphenol composition of extracts from plant sources varies from plant to plant, depending on a variety of biotic and abiotic environmental factors. The content of polyphenols and their activity can depend on many factors, including variety and degree of maturity and can be changed as a result of technological processing [12].

Despite the reported benefits of *N. cadamba*, there has been minimal research conducted on the polyphenol composition and antioxidant properties of this plant, particularly from Malaysia as a sampling location. Therefore, it is crucial to investigate the polyphenol composition and antioxidant activities of *N. cadamba* samples in order to improve the exploration of *N. cadamba* as a potential medicinal plant. Plant profiling also plays a significant role in establishing links between phytocompounds and plant biological activities, which can contribute to drug discovery from medicinal plants.

The main objectives of this study are to determine the polyphenol composition of *N. cadamba* leaf and bark extracts using attenuated total reflectance-fourier transform infrared (ATR-FTIR) and nuclear magnetic resonance (NMR) techniques, and to evaluate the antioxidant activity in both leaf and bark extracts through the analysis of total phenolic content, DPPH free radical scavenging assay, and ferric reducing antioxidant power (FRAP).

EXPERIMENTAL

Chemicals and Materials

Analytical grade methanol (CH_3OH), acetone, CH_3OH-d_4 solvent, KH_2PO_4 buffer, TSP, Folin-Ciocalteu phenol reagent, sodium carbonate (Na_2CO_3), DPPH (1,1-diphenyl-2-picrylhydrazyl), sodium carbonate (Na_2CO_3), gallic acid, Trolox (6-Hydroxy-2,5,7,8-tetramethyl chromane-2-carboxylic acid), and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich (Germany). Ultra-pure water was generated using a Milli-Q purification system (Millipore, France). Hydrochloric acid (HCl) 37%, iron(III) chloride hexahydrate ($FeCl_3.6H_2O$), and iron(II) sulphate heptahydrate ($FeSO_4.7H_2O$) were obtained from Merck (Germany).

Sample Collection and Preparation

Leaves and bark of *Neolamarckia cadamba* were collected from mature plants in Mentakab, Pahang, Malaysia. The plant samples were identified and voucher specimen has been deposited in an herbarium (Voucher No. UniSZA/A/000000004). The leaf and bark samples were freeze-dried and pulverized into fine powder.

Ultrasound-assisted Extraction

Powdered samples of bark and leaves of *N. cadamba* were extracted using ultrasound assisted extraction method with 80% methanol (v/v) at a ratio of 1:10 (w/v). A sample mixed with the solvent was sonicated for 30 min (37 kHz, 26ºC) using a sonicator (Branson 5510, USA). The extract was then filtered and the residue was re-extracted twice. Filtrates were combined and evaporated to dryness using a rotary evaporator (Rotavapor® R-300, Buchi, Switzerland) to obtain the crude extracts. Crude extracts were stored in -20ºC until further use.

Polyphenol Analysis by ATR-FTIR

The *Neolamarckia cadamba* leaf and bark extracts (0.4 mg/mL in acetone) were analyzed on a Thermo Scientific FT-IR Spectrometer (Thermo Fisher, USA) using a diamond single reflection attenuated total reflectance (ATR) accessory equipped with a zinc selenide crystal. Spectral acquisition was acquired by using Lab Solution software (Thermo Fisher, USA). Infrared spectra were obtained in the range of 4000-600 cm^{-1} with the resolution of 4 cm^{-1} and 64 scans.

Polyphenol Analysis by Nuclear Magnetic Resonance (NMR)

Sample preparation and Nuclear Magnetic Resonance (NMR) measurement were performed based on the protocols described by Kim et al. [13]. Each crude extract was mixed with CH_3OH-d_4 solvent and KH_2PO_4 buffer in D_2O (pH 6.0) containing 0.1% TSP. The solutions were centrifuged to separate the supernatant from the residue. The supernatant was transferred to an NMR tube and subjected to ^{1}H-NMR analysis (400 MHz Bruker NMR machine). All the NMR spectra's baseline and phase were corrected manually by removing noise and improving background in order to optimize the spectra for the analysis. 2D-J resolved and heteronuclear multiple bond correlation (HMBC) were used as additional support for the identification of the compounds. For each sample, a NOESY Presat 1D NMR experiment with 256 scans of 64 K data points was acquired by using a spectral width of 20.5536 ppm, an acquisition time of 34 minutes and 47 seconds, and a relaxation delay of 2.00 s. All ^{1}H-NMR spectra were pre-processed and binned to ASCII files using NMRProcFlow open-source software (https://nmrprocflow.org/).

Multivariate Data Analysis

Metabolite identification of the *N. cadamba* leaf and bark extracts was accomplished by comparing

chemical shifts in the 1H-NMR spectra to references in the Chenomx compound library, Human Metabolome Databases (HMDB), and published literature data. The chemical shifts were also double-checked and compared to published results. SIMCA-P software (version 12.0.1.0, Umetrics AB, Umea, Sweden) was used to examine the standardized bucketed data with the Principal Component Analysis (PCA) and partial least-squares analysis discriminant analysis (PLS-DA). The relative quantification of the metabolites of interest was carried out using Graph Pad Prism (Version 9, CA, USA) and the ^{1}H-NMR signals.

Determination of Total Phenolic Content

Total phenolic content of the bark and leaf extracts was determined spectrophotometrically according to the Follin-Ciocalteu's method based on Indiarto et al. [14], with slight modifications. The assay involves the Folin-Ciocalteu reagent as the oxidizing agent and gallic acid as the standard, and the result is expressed as milligram of gallic acid equivalent (GAE) per gram dry extract. Briefly, 200 µL of a sample solution that has been diluted with 80% methanol at 4 mg/mL was mixed with 1.5 mL of 7.5% (w/v) of Na_2CO_3 and 1.0 mL of 0.10M Folin-Ciocalteu reagent in a test tube. The mixture was vortexed and allowed to stand at room temperature for 30 minutes in dark environment. The absorbance was taken at 765 nm using a UV-VIS spectrophotometer (UV-1800, Shimadzu, Japan). The calibration curve obtained (y= 7.729x + 0.0737; R^2= 0.9938) was prepared using gallic acid at the concentration in the range of 0 - 0.2 mg/mL. The total phenolic content of the extracts was calculated using Equation 1 below:

$$C = cV/m \qquad \text{Equation 1}$$

Where,

C = total content of phenolic compound in mg/g in gallic acid equivalent (GAE),

c = concentration of gallic acid established from calibration curve in mg/mL,

V = volume of extract in mL,

m = weight of extract in g.

Antioxidant Activity

The *Neolamarckia cadamba* leaf and bark extracts were assessed for antioxidant activity using DPPH radical scavenging activity and ferric reducing anti-oxidant power.

DPPH Free Radical Scavenging Activity

The radical scavenging activity of the bark and leaf extracts of *N. cadamba* was estimated according to Mubarak et al. [15], with slight modifications. Briefly, 1.5 mL of sample solution at 500 ppm was added into a test tube before the addition of 1.5 mL of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) solution (0.1 mM). The solution then was vortexed and incubated in dark environment at room temperature for 30 minutes. Methanol was used as a control and the antioxidant references were 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and butylated hydroxytoluene (BHT). The DPPH free radical scavenging activity was measured through the absorbance at the wavelength of 517 nm using a spectrophotometer (Thermo Fisher Scientific, USA) and according to Equation 2.

Antioxidant activity as free radical scavenging is expressed as IC_{50}, showing the concentration of the extract which can provide inhibition of free radicals by 50%. IC_{50} is expressed in µg/mL.

Ferric Reducing Antioxidant Power (FRAP)

The FRAP analysis was referred to the method reported by Di Mattia et al. [16], with several modifications. Briefly, 10 µL of extract (0.2 mg/mL) was added to a 96-well plate containing 240 µL of FRAP reagent and then incubated for 5 min at room temperature. FRAP reagent was prepared by mixing 300 mM of acetate buffer (pH 3.6), 10 mM of 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM of HCl, and 20 mM of $FeCl_3.6H_2O$ in 10:1:1 ratio. The sample absorbance was determined at the wavelength 593 nm using a microplate reader (Thermo, United State). Antioxidant activity was calculated using standard curve of $FeSO_4$. $7H_2O$ and expressed as mM of ferrous equivalent Fe(II) per gram of dry sample.

Statistical Analysis

The obtained results are reported as mean ± standard deviation of triplicate measurements. IBM SPSS Statistics version 26.0 was used to calculate the significance difference for multiple comparisons. The differences were considered significant at $p<0.05$.

$$\text{Radical Scavenging activity (\%)} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \times 100 \qquad \text{Equation 2}$$

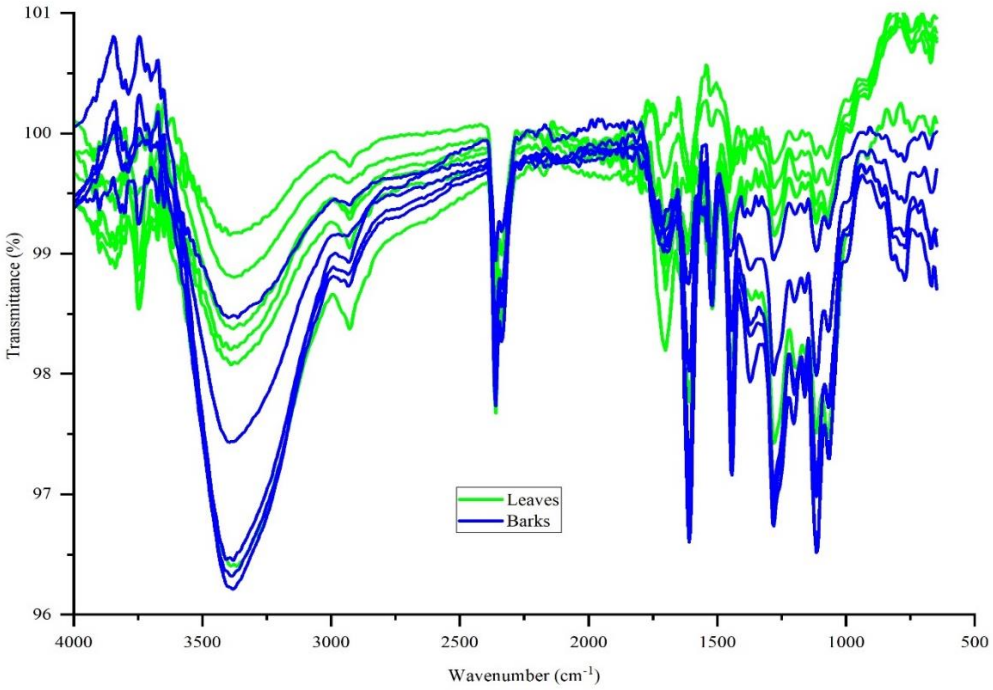

Figure 1. FTIR spectra obtained from leaf and bark extracts of *Neolamarckia cadamba.*

RESULTS AND DISCUSSION

Polyphenol Content in Leaf and Bark Extracts of *Neolamarckia cadamba* Assessed with FTIR Spectroscopy and Nuclear Magnetic Resonance

FTIR spectroscopy is regarded as the most effective method for identifying the functional groups characteristic of polyphenol compounds in plant extracts, and it offers several advantages over conventional techniques used in such types of chemical analysis [17]. The leaf and bark extracts of *N. cadamba* were analyzed by using ATR-FTIR in triplicate and the spectra obtained are shown in Figure 1, with the wavenumbers between 4000 and 600 cm^{-1}. The spectra obtained in this research are comparable with the spectra described in a previous study done by Tahir et al. [18]. The spectra in Figure 1 are combined for the sake of comparison, which would reflect their close similarity to each other and for the identification of peak purposes. The wavenumber and intensity of peaks give off the functional groups present in the analyzed samples.

Referring to Tahir et al. [18], the absorption bands between 3700 and 3000 cm^{-1} showed broad and intense peaks which are linked to the stretching vibrations of the functional groups -OH from carbohydrates, phenolics, water, and organic acids present in the extracts. The stretching vibrations of C-H bonds that comprise of the chemical skeletons of sugars and alcohols correspond to the absorption bands in the range of 3000-2700 cm^{-1}. The band at 1710 cm^{-1} was caused by the presence of polar group stretching vibration and is attributed to the associated C=O of the carboxylic acid groups. The bands in the range of 1700-1600 cm^{-1} are typical of conjugate C=O and deconjugate C=O vibrations with aromatic or C=C (the typical C=C stretch appearing around 1635 cm^{-1} shifted towards lower wavenumbers in conjugated aromatic systems between 1600 and 1520 cm^{-1}).

In the wavelength range of 1700-1630 cm^{-1}, conjugate carboxylic acid and ketones may overlap and absorb in addition to the C=O stretching vibration of quinones. The fingerprint region has multiple absorbance bands, with the bands between 1470 and 700 cm^{-1} being caused by stretching vibrations of bonds C-O, C-C, and C-H, as well as bending vibrations of C-H in the chemical structure of carbohydrates [19, 20]. They may also be related to the presence of organic acids, carotenes, and polyphenols [19].

The bark extract has some differences in the intensity of the peaks especially in the fingerprint region (600-1500 cm^{-1}), where the bark extract showed higher intensity of the peaks compared to the leaf extract. The bands in the range of 1370-1380 cm^{-1} only appeared in the bark extract spectra, which indicated that CH_3 scissoring is absent in the leaf extract and proves that there are variations in their chemical compositions.

FTIR Metabolomics of *N. cadamba* Extracts

An MVDA was performed using the OPLS-DA model to assess the significant difference between the bark and leaf extracts. According to the OPLS-DA model, the bark extract is clearly segregated from the leaf

extract, indicating significant variation between the two. The presented OPLS-DA demonstrateed an excellent model with R^2X (cum) = 0.958, R^2Y (cum) = 1 and Q^2 (cum) = 0.964.

Figure 2(A) shows an OPLS-DA score plot with two clearly separated clusters detected and no notable outliers. Blue triangles represent the bark extract, while green squares represent the leaf extract. The scoring plot was used to assess variance within *N. cadamba* in two separate parts of the plant, whereas the loading plot highlighted metabolite signals that may contribute to cluster differentiation. According to the loading plot in Figure 2(B), almost all wavenumbers are prominent in the region belonging to the bark extract, indicating that the bark contains more polyphenols than the leaves. To summarize, the OPLS-DA score plot demonstrates differences between the bark and leaf extracts from *N. cadamba*, while the loading plot reveals that the bark extract contains more phenolic chemicals than the leaf extract.

The OPLS-DA model was further validated using permutation test conducted to evaluate the goodness of fit of models, in which, x-axis is the correlation coefficients between permutated and original response variables (denoted as "r"), which represents the degree of the randomization of response variable y. Based on the permutation test (Supplementary material Figure A.1), when r = 0, y variables are completely randomized; while at r = 1, there is no permutation of y variables. The Q^2 was seen to decrease along the decrease of r, suggesting the model did not overfit. R^2 remained nearly unchanged with r, which is a normal phenomenon when there are a large number of predictors and a small number of observations in the models in biological sciences. The CV-ANOVA outcomes indicate that the model is highly significant, with a p-value of 2.317×10^{-24}.

^{1}H-NMR Spectroscopic Analysis for *N. cadamba*

^{1}H-NMR spectra can provide substantial data on the major organic functional groups of the extracts by referring to specific chemical shifts (ppm), relative intensity of signals, and multiplicity from the ^{1}H-NMR spectra, which give off clues or hints regarding the position, type, and number of protons within the samples. The chemical structures in the *N. cadamba* leaf and bark extracts were determined through the comparison of the ^{1}H-NMR spectra (Figure 3) with the literature [17, 21–23], resulting in a list of identified compounds found in the extracts; with a total of number of 24 together with their ^{1}H-NMR characteristics (Table 1).

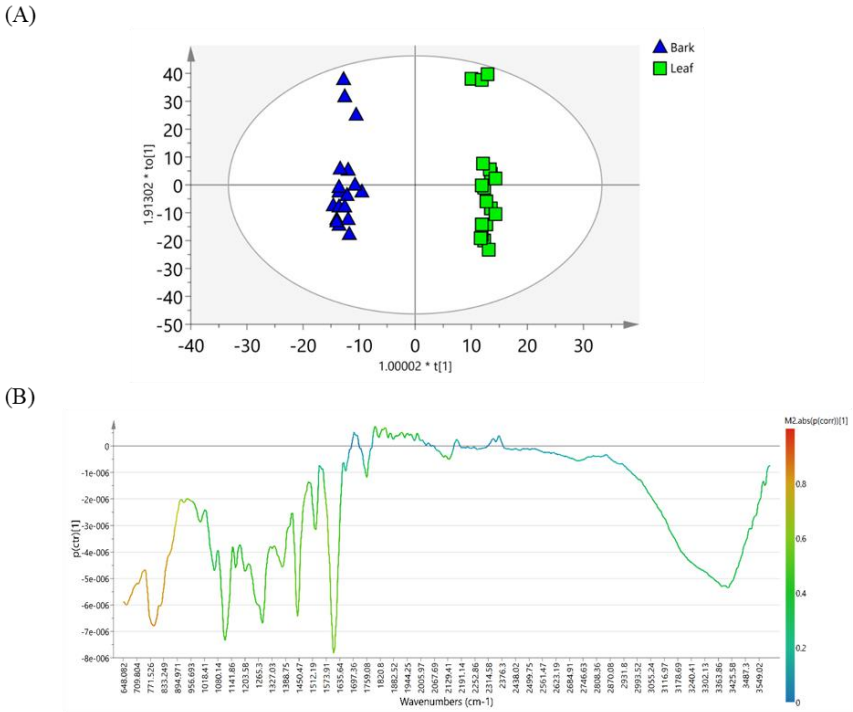

Figure 2. OPLS-DA (A) score and (B) loading plots of FTIR data of leaf and bark extracts of *Neolamarckia cadamba.*

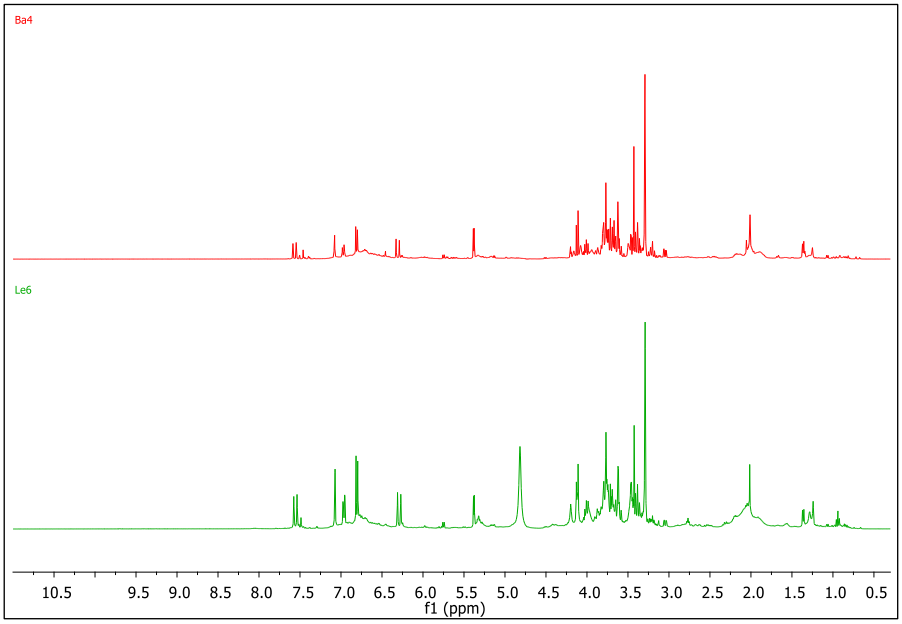

Figure 3. ^{1}H--NMR spectra of leaf and bark extracts of *Neolamarckia cadamba* (green = leaf; red = bark).

Table 1. Identification of compounds in *Neolamarckia cadamba* based on ^{1}H-NMR spectroscopy.

Compound	^{1}H-NMR characteristics
Chloropyhll a	9.40 (Hβ, s), 9.26 (Hα, s), 8.30 (Hδ, s)
Chloropyhll b	9.70 (Hα, s), 9.42 (Hβ, s), 8.15 (Hδ, s)
Kaempferol	8.06 (m), 6.94 (m), 6.37 (d, J= 2.0 Hz), 6.17 (d, J= 2.0 Hz)
Quercetin	7.68 (d, J= 2.0 Hz), 7.55 (dd, overlap), 6.90 (d, overlap), 6.46 (s), 6.25 (s)
Rutin	7.68 (d, J= 2.0 Hz), 7.63 (dd, J= 2.0, 8.4 Hz), 6.91 (d, J= 8.4), 5.08 (d, J= 7.6 Hz), 4.53 (br s), 1.09 (d, J= 6.8 Hz)
Catechin	6.73 (m), 5.80 (d, overlap)
Chlorogenic acid	7.58 (d, J= 16 Hz), 7.08 (d, J= 2.0 Hz), 6.98 (dd, J= 2.0, 8.0 Hz), 6.82 (d, J= 8.0 Hz), 6.31 (d, J= 16.0 Hz), 4.21 (m, overlap), 3.88 (dd, overlap), 2.21 (m), 2.06 (m)
Vanillic acid	7.52 (d, overlap), 7.46 (dd, J= 1.6, 8.4 Hz), 6.94 (d, overlap), 3.90 (s)
4-Hydroxybenzoic acid	7.76 (d, J= 8.4 Hz), 6.91 (d, overlap)
Syringic acid	7.38 (s), 3.92 (s)
Gallic acid	7.04 (s)
Gentisic acid	7.29 (d, J= 1.6 Hz), 7.01 (dd, J= 1.6, 8.4 Hz), 6.82 (d, overlap)
Maleic acid	6.01 (s)
UDP-glucose	5.98 (m), 5.59 (m), 4.35 (m), 4.27 (m)
Sucrose	5.38 (d, J= 4.0 Hz), 4.20 (d, overlap), 4.01 (t, J= 8.4 Hz)
Glucose	5.13 (d, J= 3.6 hz), 4.59 (d, J= 8.0 Hz), 3.24 (m)
Choline	3.19 (s)
Aspartic acid	2.64 (dd, J= 5.6, 16.8 Hz), 2.50 (dd, J= 7.0, 16.8 Hz)
Glutamic acid	2.34 (m), 2.12 (m, overlap)
Malic acid	4.31 (m), 2.69 (dd, overlap), 2.36 (dd, overlap)
Alanine	1.47 (d, J= 7.2 Hz)
Lactic acid	1.36 (d, J= 6.4 Hz)
Fatty acids	5.32 (m), 1.24-1.28 (m)
β-Sitosterol	5.35 (m), 3.53 (m), 1.01 (s), 0.90 (d, overlap), 0.84 (t, overlap), 0.79 (m), 0.66 (s)

Figure 3 depicts the ^{1}H-NMR spectra of the *N. cadamba* bark and leaf extracts, with the green spectrum representing the leaf extract and the red spectrum representing the bark extract. The majority of the 1H-NMR spectra appeared as continuous distributions of signals, implying the presence of a complex mixture of substances. Most organic hydrogen atoms can be classified into five types: (1) H-C, aliphatic protons in extended alkyl chains (0.8-2.0 ppm); (2) HC-C=, aliphatic protons attached to carbon atoms adjacent to a carbonyl or aromatic group (2.0-3.0 ppm); (3) H-C-O, protons attached to carbon atoms singly bonded to oxygen (3.3-5.0 ppm); (4) A_r-H, aromatic protons (6.5-8.5 ppm); (5) O–H, phenolic (4-10 ppm) [17]. The wide range of ^{1}H-NMR chemical shifts of aromatic protons suggest the appearance of aromatic rings substituted: alkylbenzenes and phenols (6.5-7 ppm); benzoic acid or esters and flavonoids (>7 ppm) [17].

Partial Least Square-Discriminant Analysis (PLS-DA) Model Validation

Partial least squares-discriminant analysis (PLS-DA) was generated for obtaining a better group classification between both extracts from *N. cadamba*. The results were displayed in the form of score plots, loading plots, and volcano plots. The PLS-DA model was shown to be a good model indicated by its R^2Y (cum) value of 0.968 and Q^2 (cum) value of 0.886. For a good model, Q^2 must be greater than 0.5, R^2 must always be greater than Q^2, and the difference between R^2 and Q^2 should be in the range of 0.2-0.3 [24, 25]. Based on the score plot of the leaf and bark extracts of *N. cadamba* (Figure 4(A)), two clusters that were significantly different could be found, indicating that the extracts have different compositions. Green dots represent the bark extract and blue squares represent the leaf extract.

The corresponding loading plots (Figures 4(B) and 4(C)) showed a discriminant metabolite responsible for the separation of the two clusters in the score plot. From the loadings, it could be deduced that the bark extract of *N. cadamba* contained higher levels of sitosterol, catechin, syringic acid, and vanillic acid. The leaf extract was marked by higher chlorogenic acids, quercetin, sucrose, and fatty acids.

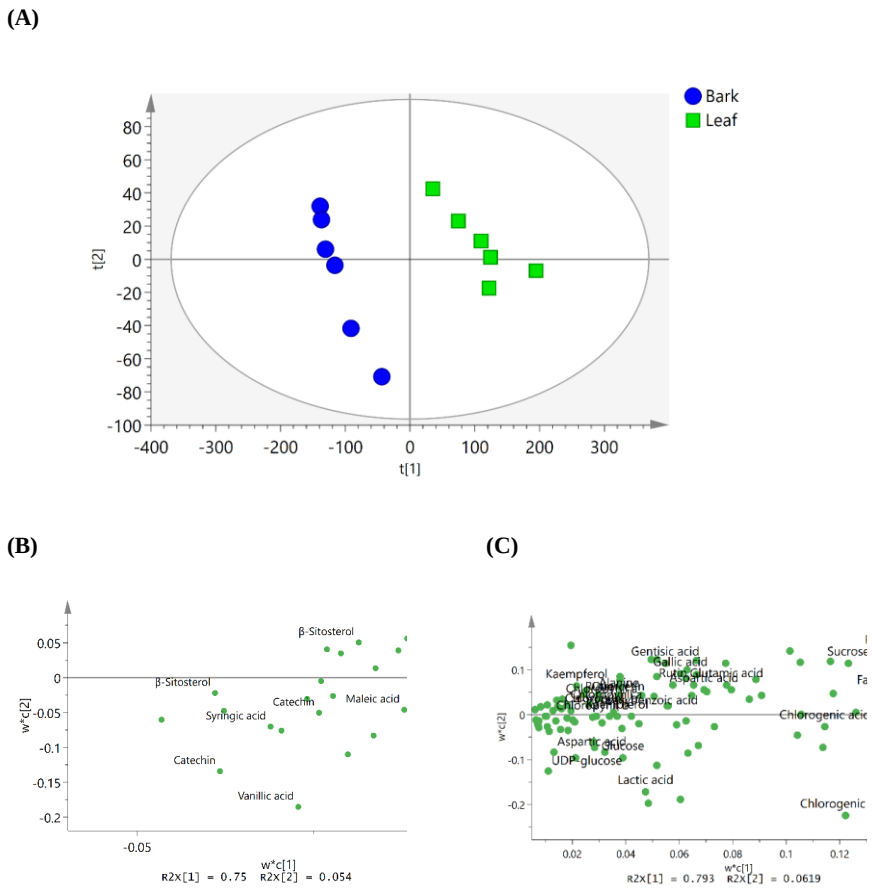

Figure 4. PLS-DA model of leaf and bark extracts of *Neolamarckia cadamba*. (A) Score plot of PLS-DA for leaf and bark extracts of *Neolamarckia cadamba*. PLS-DA loading plots of (B) leaf and (C) bark extracts of *Neolamarckia cadamba*.

The PLS-DA model's validity and degree of overfit were determined using the permutation test. The permutation plot plots the correlation coefficient on the X-axis between the original and permuted Y-variables versus the cumulative R^2 and Q^2 on the Y-axis. The intercepts of the regression lines indicate the degree of overfitting of the results (SIMCA-P software v. 13.0, Umetrics, Umea, Sweden). The model did not show over-fitting for both extracts, based on Y- axis intercept values of R^2= 0.529 and Q^2= -0.0141 for the lea extract and R^2= 0.521 and Q^2= -0.0201 for the bark extract, and the fact that the R^2 line for both samples were far from being horizontal.

When r = 0, y variables are completely randomized; while at r = 1, there is no permutation of y variables (Supplementary material Figure B.1). The Q^2 was seen to decrease along the decrease of r, suggesting the model did not overfit. In addition, the model is highly significant with p value lower than 0.05 based on CV-ANOVA outputs [24].

According to Kumar et al. [26], four types of statistical procedures have been used primarily to identify differential metabolites: classical volcano approaches such as volcano plot, classical non-parametric approaches, Bayesian parametric approaches, and Bayesian non-parametric approaches. The volcano plot was used in this study to identify the different metabolites. The classical volcano plot is a suitable differential technique because it can control the false discovery rate [27]. The volcano figure is based on p-values from a t-test and fold-change (FC) values, both of which are affected by classical location and scatter [26].

The volcano plot, as shown in Figure 5, detected 18 compounds that are noteworthy and function as differential metabolites, causing variance in the extracts. The volcano plot demonstrated that rutin, aspartic acid, glutamic acid, chlorogenic acid, and kaempferol are upregulated in the bark extract, whereas b-sitosterol, syringic acid, and catechin are downregulated. When FC>0, the compounds are elevated in the bark extract against the leaf extract, and when FC<0, the compounds are downregulated in the bark extract versus the leaf extract. The p-value (y-axis) represents the level of statistical significance, with lower p-values indicating higher significance.

Further inquiry was carried out to validate the outcome of the volcano plot, in which the relative quantification of each compound based on ^{1}H-NMR signals was carried out, as shown in Figure 6. The compounds found in the extracts are represented as box plots. It may be inferred that 18 important components, including chlorogenic acid, quercetin, 4-hydroxybenzoic acid, and syringic acid, are responsible for distinguishing the extracts from *N. cadamba*. Furthermore, catechin, b-sitosterol, and syringic acid levels were higher in the bark extract than in the leaf extracts. As a result, the discovery supports the results of the volcano plot.

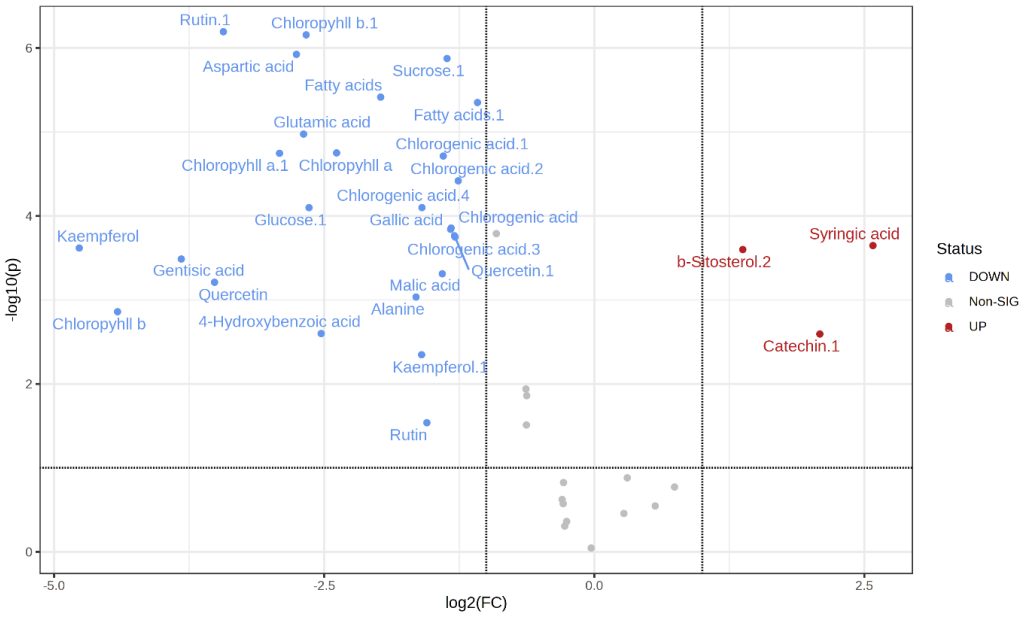

Figure 5. Volcano plot of bark and leaf extracts of *Neolamarckia cadamba.*

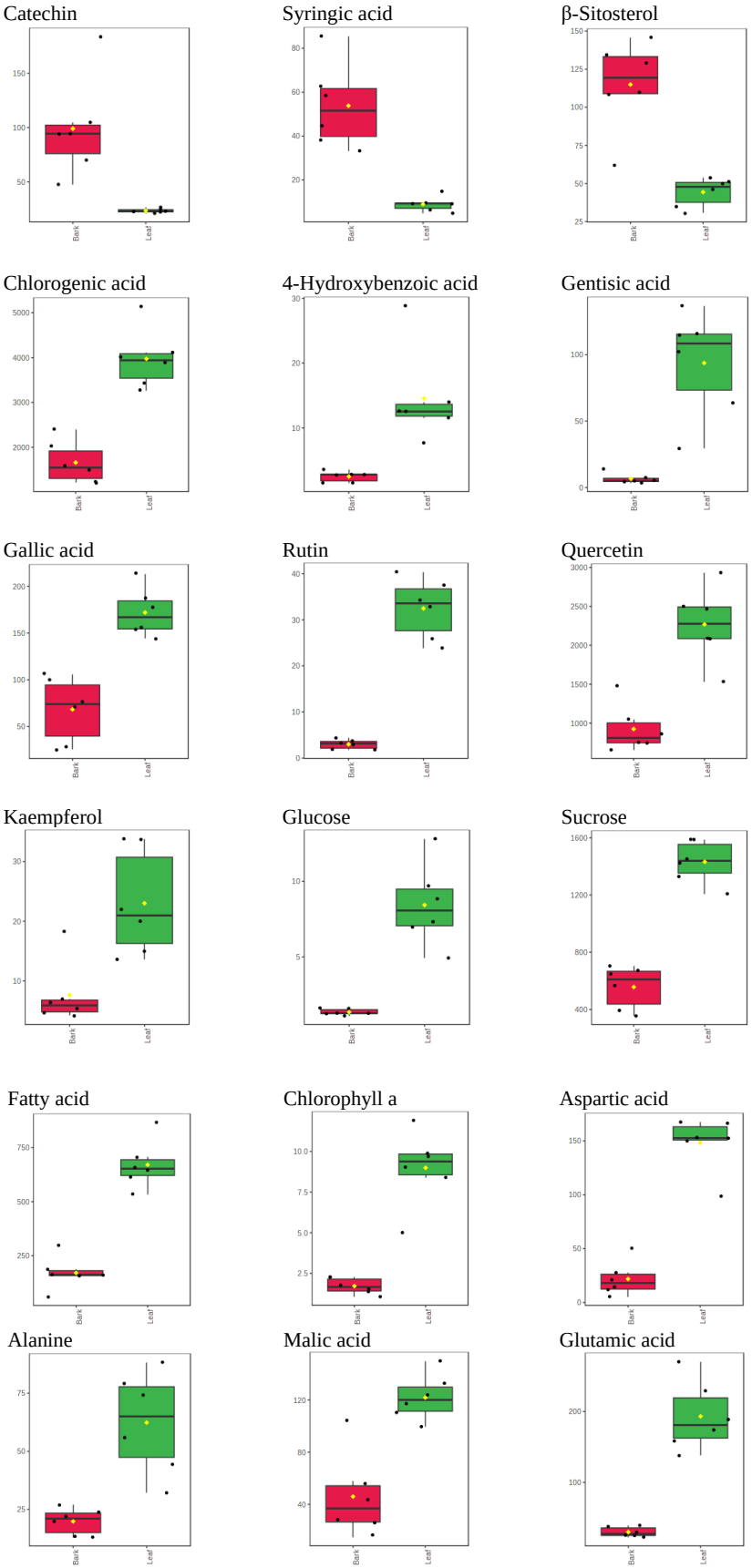

Figure 6. Relative quantification of compounds based on ^{1}H-NMR signals

The significance of chlorogenic acid in plants has been extensively reported due to its diverse bioactive properties [28, 29]. Chlorogenic acid is a phenolic compound found in many plants, most notably coffee, and it has antioxidant, anti-inflammatory, and antimicrobial properties [30, 31]. Chlorogenic acid is also reported to have high bioavailability due to the observation of catabolites in the circulatory system [32].

Flavonoids, such as quercetin, were also present in both extracts. Based on Bahramsoltani et al. [33], quercetin is one of the most popular flavonoids in human diet. Rutin and quercitrin, which are the derivatives from quercetin, are widely distributed in human dietary fruits and vegetables like apples, onions, cherries, and grapes [34]. D'Andrea [34] also stated that quercetin has been reported to contribute to several therapeutic activities such as anticancer, antioxidant, and anti-inflammatory, as well as its positive effects in obesity and diabetes.

Syringic acid is a phenolic acid compound which can be found in olives, dates, grapes, walnuts, and a few other plants [35]. It has various biological activities which include antioxidant, anti-inflammatory, hepatoprotective, cardioprotective, neuroprotective, antimicrobial, antidiabetic, and antiendotoxic properties [36].

Antioxidant Activity of *N. Cadamba*

Total Phenolic Content (TPC)

The total phenolic content (TPC) in the *N. cadamba* extracts is expressed as gallic acid (mg/g) equivalents is shown in Figure 7. The TPC of the methanolic extracts of *N. cadamba* leaves and bark were found to be 41.37 ± 3.77 mg GAE/g dry extract and 58.40 ± 2.47 mg GAE/g dry extract, respectively. The results obtained from this study show that the *N. cadamba* bark extract exhibited a significantly higher TPC than the leaf extract ($p<0.05$). The TPC obtained from both tested parts of the plant was compared with data from previous studies. Chandel et al. [37] found higher values which were 107.63 mg GAE/g in the leaves and 280.70 mg GAE/g in the bark. This report similarly shows a bigger TPC value in the bark than the leaves. Ganjewala et al. [3] reported a similar value of total phenolic content of the leaves, which was 47.97 ± 0.15 mg GAE/g. On the other hand, Batta & Rajput [4] reported a lower value which was 0.22 ± 0.01 mg GAE/g in the leaf sample and Tahia et al. [38] found that the bark contains 3.10 ± 0.23 mg GAE/g of total phenolic content.

The differences of the TPC values in the *N. cadamba* samples could be due to different cultivated plants, different storage conditions, and loss of some phenolic compounds during sample preparation [39]. Higher TPC in the bark extract in comparison to the leaf extract can be attributed to the differential distribution of phenolic compounds, the protective role of the bark, and the distinct metabolic activities within plant tissues. The bark, which may serve as the plant's primary defence against environmental stressors such as UV radiation, pathogens, and herbivores, tends to accumulate more phenolic compounds, leading to its higher TPC [40]. Additionally, the bark, being older and more exposed than the leaves, synthesizes and stores these compounds as a protective mechanism [41]. Studies done by Gorinstein et al. [42] and Sellapan et al. [43] discovered that there are strong correlations between TPC and antioxidant activity in various kinds of fruits. The amount of TPC will affect the antioxidant capacity [39, 44]. Folin-Ciocalteu reagent is not specific and able to detect all phenolic groups found in extracts, including those found in extractable proteins. According to Rabeta & Faraniza [39], the method can cause interference of reducing substances, such as ascorbic acid, as well.

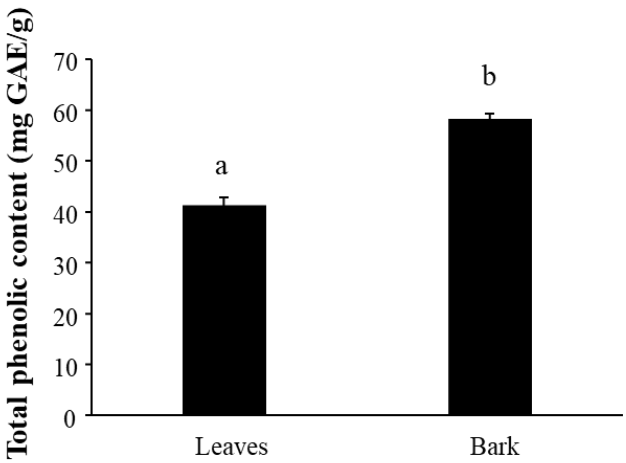

Figure 7. Total phenolic content in *Neolamarckia cadamba* leaf and bark extracts. Data are mean ± SD of triplicate samples (n=3). Different alphabets indicate significant difference at $p<0.05$.

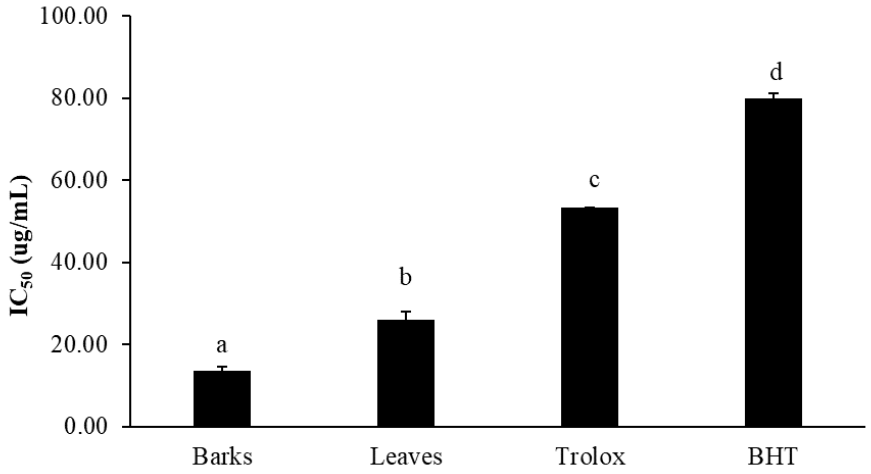

Figure 8. IC_{50} values of DPPH radical scavenging activity in *Neolamarckia cadamba* leaf and bark extracts, BHT, and Trolox. Data are mean ± SD of triplicate samples (n=3). Different alphabets indicate significant difference at $p<0.05$.

DPPH Free Radical Scavenging Activity

DPPH free radical scavenging assay is a standard method for investigating antioxidative activity, as its results are not affected by substrate polarity [45, 46]. The quality of antioxidants in an extract is determined by the IC_{50} value, where a lower IC_{50} value represents a better antioxidant activity in a sample [37]. The methanolic extracts of *N. cadamba* leaves and bark were inspected for potential DPPH free radical scavenging ability (Figure 8). The IC_{50} values of the leaf and bark extracts were recorded at 28.96 ± 1.74 µg/mL and 15.87 ± 1.08 µg/mL, respectively, while the values of the positive controls (BHT and Trolox) were 80.03 ± 1.27 µg/mL and 53.37 ± 0.09 µg/mL, respectively. The leaf and bark extracts were observed to have higher scavenging activity than the standard butylated-hydroxytoluene (BHT) and Trolox. A significantly higher radical scavenging activity (as characterized by the lowest IC_{50}) was observed in the bark of *N. cadamba* when compared to the leaf extract ($p<0.05$), which supports the observation in the TPC analysis.

The difference in antioxidant activity between the leaf and bark extracts of *N. cadamba* can be attributed to the variation in the concentration and types of phytochemicals present in each part of the plant. Bark often contains higher levels of certain phenolic compounds, flavonoids, and tannins compared to leaves, which may contribute to their superior antioxidant activity. These compounds are known for their ability to donate hydrogen atoms or electrons to neutralize free radicals, which is the mechanism by which DPPH free radicals are scavenged. This observation is similarly reported by Baidoo et al. [47], who observed that stem bark from *Entada africana* showed a more potent ability in neutralizing free radicals. Bark, being a protective tissue, may accumulate more potent antioxidants as a defense mechanism against environmental stressors, contributing to the observed higher antioxidant activity [48].

The IC_{50} values observed for DPPH radical scavenging activity in both *N. cadamba* leaves and bark in this study were found to be lower, indicating a higher antioxidant ability when compared to some previous reports. Ganjewala et al. [3] and Chandel et al. [37] found the IC_{50} values of DPPH scavenging ability in leaf samples to be at 69.8 µg/mL and 63.9 µg/mL, respectively. While for the bark of *N. cadamba*, Tahia et al. [38] and Chandel et al. [49] reported the IC_{50} values at 113.6 ± 0.4 µg/mL and 97.4 µg/mL, respectively.

These differences could be attributed to different drying methods. Chandel et al. [37] dried the bark of *N. cadamba* at 40ºC, whereas Batta & Rajput [4] prepared the leaf samples by drying them in the sun. This demonstrates how different drying methods can affect extract antioxidant activity [50]. Aside from that, differences in the antioxidant potential of extracts could be due to the levels of phytoconstituents which vary markedly in different species and cultivars, as well as the plant parts [51].

Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay is based on the ability of extracts containing antioxidants to reduce ferric iron to ferrous iron at acidic pH [52]. The ability to convert a compound from Fe^{3+}/ferricyanide complex to Fe^{2+}/ ferrous is an indicator of antioxidant activity. This process is related to the yellow color changing to green and blue, depending on the reduction power of the extracts' compounds [53]. The reaction is repeatable and linearly related to the antioxidant's molar concentration, but FRAP assay does not react quickly with some antioxidants, such as glutathione [54].

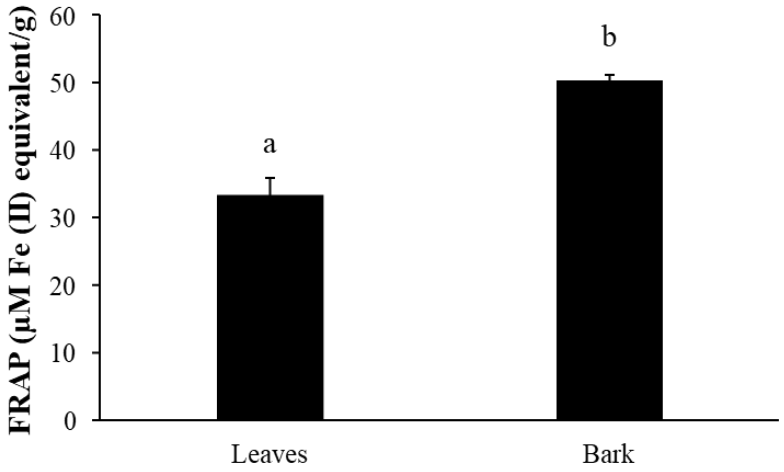

Figure 9. Ferric reducing antioxidant power (FRAP) in *Neolamarckia cadamba* leaf and bark extracts. Data are mean ± SD of triplicate samples (n=3). Different alphabets indicate significant difference at $p<0.05$.

Figure 9 displays the results of the FRAP assay in both leaf and bark samples of *N. cadamba*, expressed in µM Fe(II) equivalent/g dry sample. The FRAP values show that the bark extract had a significantly higher reducing power ($p<0.05$) (50.24 ± 6.14 µM Fe (II)/g) than the leaf extract (33.33 ±2.11 µM Fe (II) /g). This proves that the bark extract of *N. cadamba* has higher antioxidant property than the leaf extract, which supports the observation in the DDPH radical scavenging assay, as well as the data obtained in the TPC analysis.

The difference in the reducing power between the bark and leaf extracts of *N. cadamba*, as indicated by the FRAP assay, can be explained by the distinct phytochemical compositions of these plant parts. The bark generally contains higher concentrations of phenolic compounds, flavonoids, and tannins, which are known to have strong electron-donating abilities, contributing to their superior reducing power [55]. These compounds may be more abundant in the bark as it serves as a protective barrier against environmental stressors, thereby accumulating more potent antioxidants. The higher FRAP value in the bark extract, compared to the leaf extract, suggests a greater presence of these bioactive compounds, corroborating the results observed in the DPPH radical scavenging assay and TPC analysis [56].

The antioxidant activity in the *N. cadamba* leaf and bark extracts measured by the FRAP assay was compared to previous reports on other plant samples. Fe^{2+} reducing ability was found higher in other plants such as *Garcinia atrovirdis* leaves (325.85 µM/g) and *Strobilanthes crispus* leaves (267.5 µM Fe (II)/g) [38, 57]. Some factors influence both antioxidant compounds and extracted compounds from plant materials. According to Moure et al. [58], in addition to storage time, geographic origin, and harvesting date, environmental and technological factors also have an impact on the quality of natural extracts and antioxidant activities. Furthermore, because compounds with different polarities have different antioxidant potentials, the solvent used is an important factor in extracting antioxidant compounds from plant materials [42]. Plant antioxidant capacity may also vary depending on storage conditions [59].

CONCLUSION

Several phenolic compounds that were discovered in the methanolic extracts of *Neolamarckia cadamba* bark and leaves, were successfully determined and identified by ATR-FTIR and NMR spectroscopy. The DPPH and FRAP assays revealed that these extracts have valuable antioxidant capacity, and the total phenolic content and antioxidant activity of the bark extract were found significantly higher than the leaf extract. The findings support the use of *N. cadamba* bark and leaf extracts as antioxidant sources in pharmaceuticals and nutraceuticals. However, further investigation is required to identify other possible bioactive compounds which was not able to be identified by NMR and FTIR. Additionally, studies on antioxidant activity at the molecular level are needed to understand the mechanism of the anti-oxidant activity.

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