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Original Research Article

Macroporous Resin-assisted Enrichment of Total Flavonoids, Polyphenols and Antioxidants from *Newbouldia laevis* Leaf Extracts

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ABSTRACT

Polyphenols are plant-derived antioxidant compounds that have shown great prospects as natural alternatives to synthetic antioxidant compounds. As a result of the rising interest in natural antioxidants, this study investigated the potential use of different Macroporous Adsorptive Resin (MAR) including X5, ADS17, ADS7, AB8, S400 and D101 in the enrichment of total polyphenols from ethanol extracts of *Newbouldia laevis* leaf, an abundant West African medicinal plant. The efficiency of the MAR-assisted polyphenol enrichment process was investigated and optimized under varying conditions like adsorption time, adsorption pH, extract loading concentration, elution time, and eluent ethanol concentration. The total phenols, total flavonoids, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities, Ferric reducing ability of the plasma (FRAP) activities and inhibitory potentials against Pb-induced hemolysis and Fenton-chemistry mediated peroxidation of egg yolk homogenate were determined for the crude and polyphenol-rich extract of *Newbouldia laevis*. Using a batch system that comprises X5 MAR, extract loading concentration of 15 mg NEE/g MAR, adsorption time of 40 min, eluent ethanol concentration of 70 %, elution time of 30 min and under a neutral pH condition, the total flavonoids and phenol contents of *Newbouldia laevis* extract improved from 30 to 74 % and from 12 to 33 % respectively. The enrichment process improved the DPPH radical scavenging, FRAP, and the anti-peroxidative activity of *Newbouldia laevis* leaf extracts by 2.3, 3.2 and 2.5 folds respectively. Results from our study could aid the utilization of this abundant medicinal plant resource for functional food development, drug development and industrial applications.

***Keywords*:** Polyphenols, Flavonoids, Antioxidant, Macroporous adsorptive resin, *Newbouldia laevis,* DPPH

Introduction

Antioxidants are compounds that can slow down or inhibit oxidative processes.[1] Such oxidative processes generate free radicals which readily react with other biological molecules to trigger oxidative chain reactions.[1] Antioxidants such as ascorbic acid, butylated hydroxytoluene, beta hydroxy acid, carotenes and flavonoids are widely applied in food processing, pharmaceuticals, personal care products and industrial processes.[2,3] The natural antioxidant market was projected to increase by 542.7 million USD between 2022 and 2026. [3] In food processing for instance, antioxidants can inhibit the oxidation of lipids thereby slowing down rancidity and deterioration of processed foods.[2] Human studies have shown that the consumption of plant-derived antioxidants can improve the total antioxidant capacity of the plasma as well as prevent or ameliorate oxidative stress-associated disease like cardiovascular diseases, inflammatory diseases and cancer.[4,5]

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antioxidant over synthetic ones due to accumulating findings that associates high intakes of synthetic antioxidants with cellular toxicity like DNA damages.[2] Despite this preference, the natural antioxidant market suffers from high production cost often tied to its expensive raw materials and isolation methodologies. This limits its widespread application and so presents an existing challenge in the natural antioxidant industry.[3] Polyphenols are a class of plant-derived compounds that has been reported to exert potent antioxidant properties.[6] Polyphenols exerts antioxidant effects through mechanism that may involve inhibition of reactive oxygen species (ROS) generation,[7] direct scavenging of ROS, [8] chelation of transition metals [9] and enhancing the activities of endogenous antioxidants.[10] Polyphenols are finding recent application in food processing and pharmaceutical industries. For instance, polyphenol-rich extract from *Salvia Rosmarinus* leafis currently approved for use as a food grade preservative in Europe due to its high antioxidant and antimicrobial properties.[2,11] Similarly, grape seed extract is marketed as health supplements due to its rich polyphenols and antioxidant content.[12] Improvements in the polyphenol contents of plant extracts appear to be

a potential way of improving the antioxidant and bioactivities of such extracts. Several strategies including the use of Deep Eutectic Solvent extraction,[13] Aqueous Two-Phase System,[14] Preparative High Performance Liquid Chromatography (PHPLC),[15] Counter Current Chromatography [16] and Column Chromatography with Silica, Polyamide, Sephadex gel, Ion-exchange resin or macroporous adsorptive resins (MAR),[17–19] have been applied in the isolation and purification of polyphenols from plant extracts. Some of the existing methods are more suitable for purifying selected phenolic compounds and has low effectiveness in enriching total polyphenol from plant extracts while techniques such as counter current chromatography and HPLC requires expensive instrumentations, potentially toxic reagents as well as trained expertise. In recent time, MAR has gained considerable applications for total polyphenol enrichment from plant sources due to its low cost, ease, speed of use, reusability and high polyphenol enrichment efficiency.[18,20–22]

*Newbouldia laevis(N. laevis) (*commonly called Boundary tree) belongs to the Bignoniaceae family and is abundant in West Africa and other tropical regions of the world.[23] It is commonly referred to as Ogirisi or Akoko or Aduruku in the Igbo, Yoruba and Hausa native Nigerian Languages respectively. *N. laevis* is used in the traditional management of epilepsy, rheumatic swelling, pains, cough, sexually transmitted diseases, sickle cell disease and wounds.[23–27] Scientific evidences have demonstrated that extracts from *N. laevis* possesses antidiabetic, antipyretic activity, antioxidant, antimicrobial, antidepressant and uterine contraction properties.[24,25,28–30] A previous study has shown that the ethanol extract of *N. laevis* exerted antioxidant activities.[24] High performance liquid chromatography fingerprinting study on *N. laevis* leaf extract revealed the presence of different flavonoids and polyphenols.[31]

Many of the existing studies on *N. laevis* leaf dwelled on ascertaining the bioactivities and chemical constituents of its extracts. To the best of our knowledge, no study till date has attempted to isolate total polyphenols from leaf extract of *N. laevis* despite its tropical abundance and polyphenol compositions. This study was, therefore, aimed at evaluating the potential application of MAR in the isolation of total polyphenols from leaf extracts of *N. laevis*. Based on the known antioxidant potencies of polyphenols, we had reasoned that polyphenol-rich fractions of *N. laevis* leafextracts could possess improved *in vitro* antioxidant properties than its crude extract. We tested this hypothesis by comparing the DPPH radical scavenging activities, FRAP activities, anti-peroxidation and anti-hemolytic effects of both crude and polyphenol rich extracts of *N. laevis*. Our findings could enhance the utilization of this abundant tropical plant resource for pharmaceutical and industrial applications.

Materials and Methods

Reagents

Quercetin, Gallic acid and Ascorbic acid were purchased Sigma (USA), Qualikems (India) and BDH (England) respectively while Lead (II) Acetate trihydrate (Pb Acetate) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from BDH (England) and Sigma (USA) respectively. The MARs including S400, X5, AB8, ADS17 and D101 were purchased from Anhui Sanxing resin technology (China) and ADS7 from Rixing Tianjin International Co. ltd (China). Other reagents were of analytical grades.

Ethical Approval

Ethical approval was obtained before the commencement of this study from the research ethics committee of the University of Port Harcourt, Rivers State, Nigeria (Approval number: UPH/CEREMAD/REC/MM72/091). The methodologies used in this study were done in line with relevant guidelines and regulations.

Plant collection and preparation

Fresh leaves were harvested on February 2022, from a *N. laevis* tree that is growing in Nanka community of Anambra State, Nigeria. The leaf was identified as *Newbouldia laevis* (P.Beauv.) Seem. ex Bureau by Dr Chimezie Ekeke of the Plant Science and Biotechnology Department of University of Port Harcourt, Rivers State, Nigeria. A voucher specimen was deposited in the University's Herbarium (Herbarium number: UPH/P/316). The leaves were washed, dried under shade, ground into powder and sieved before use.

Ethanol extract was obtained from *N. leavis* leaves (NEE) using the methods reported by Umeoguaju *et al*.[32]In brief, powdered *N. laevis* leaves (1 kg) was immersed into 80 % ethanol for 48 h at 25ºC. The resultant residue was refluxed at 90 ºC for 4 h using a glass reflux apparatus. Filtrates from both extractions were pooled and concentrated over a water bath at temperature that spanned between 70- 90ºC.

Determination of Total Flavonoid and Phenols

Total flavonoids were determined in the extracts of *N. laevis* leaves using the method reported by Tavassoli and Afshar [33] (See Supplementary S1). Total phenols were determined using Folin-Ciocalteu (FC) reagent as reported by Ainsworth and Gillespie[34] and Awah *et al*.[35] Folin-Ciocalteu reagent was prepared as reported by Singleton *et al*.[36] (See Supplementary S2). The flavonoid contents of the extract was reported as quercetin equivalent values while the polyphenol content of *N. laevis* extracts was reported as gallic acid equivalent values.

Enrichment of Total Polyphenols

The enrichment of total polyphenols from NEE was done using MAR as reported by Yang *et al*. [37] Zhang *et al*. [38] and Wu *et al*.[39] The different MAR including S400, X5, ADS17, AB8, ADS7 and D101 were pretreated according to the manufacturer's instruction prior to the enrichment process. This pretreatment involved shaking 500 g of each resin intermittently, in 1 L of 96 % ethanol for 12 h following which the resins were rinsed thoroughly with distilled water. The physical property of the MAR, as specified by the manufacturer is presented in supplementary table S1.

Optimization of the MAR-assisted Polyphenol Enrichment Process

The optimization of polyphenol enrichment from NEE by MAR was investigated in a static batch system using adaptations of the methods reported by Zhang *et al*.[38] and Umeoguaju *et al*.[32] The effects of the MAR type, extract loading capacity, adsorption pH, desorption ethanol concentration and adsorption time on the desorption or adsorption characteristics of polyphenols on the MAR were assessed.

Effect of MAR Type

The potentials of different MAR to adsorp or release adsorbed polyphenols from *N. laevis* leaf extract was investigated by mixing 1 ml of diluted NEE (2 mg/ml) with 0.5 g of each MAR including X5, S400, ADS17, D101, ADS7 and AB8.. Each mixture was shaken periodically for 30 min followed by filtration and subsequent recovery of the unbound MAR filtrate and polyphenol-bound MAR. The polyphenol-bound MAR was rinsed twice with distilled water to remove loosely adsorbed and water soluble substances. Each polyphenol-bound MAR was treated with ethanol (1 ml; 95 %), followed by intermittent agitation (30 min), and subsequent filtration to yield a polyphenol-rich MAR ethanol eluent for each experimental sample. Total flavonoid and phenols were subsequently determined in the diluted NEE, unbound MAR filtrates and MAR ethanol eluents. The percentage adsorption, desorption capabilities and recovery of flavonoids and phenols on MAR for each experimental samples was determined using the equations below.

Percentage Adsorption =

$$((Fg * Vg) - (Fs * Vs)) * 100/(Fg * Vg) \quad (1)$$

Where "Fg" is the concentration of flavonoid (or phenol) in NEE solution (in mg/ml), "Vg" is the volume of NEE solution (i.e. 1 ml), "Fs" is the concentration of flavonoid (or Phenol) in the unbound MAR filtrate (in mg/ml), "Vs" is the volume of the unbound filtrate (i.e. 1 ml).

Percentage Desorption $= Fm * Ve * 100/((Fg * Vg) - (Fs * Vs))$(2)

Where "Fm" is the flavonoid (or phenol) content of ethanol eluent (mg/ml), "Ve" is the volume of the ethanol eluent (i.e. 1 ml), "Fg" is the concentration of flavonoid (or phenol) concentration in NEE solution (in mg/ml), "Vg" is the volume of the NEE solution (i.e. 1 ml), "Fs" is the concentration of flavonoid (or phenol) in the unbound MAR filtrate (in mg/ml) and "Vs" is the volume of the unbound MAR filtrate (i.e. 1 ml)

Flavonoid Recovery (%) = $(Fm * Ve * 100)/(Fg * Vg)$(3)

Where "Fm" is the flavonoid content of MAR ethanol eluent (in mg/ml), "Ve" is the volume of MAR ethanol eluent (i.e. 1 ml), "Fg" is the concentration of flavonoid in NEE solution (in mg/ml), "Vg" is the volume of NEE solution (i.e. 1 ml)

Effect of NEE Concentration on the Adsorption and DesorptionCharacteristics of N. laevis Flavonoids on ADS17 and X5 MAR

One ml of varying concentrations (i.e. 50, 20, 10, 3 and 0.5 mg/ml) of NEE was added to 0.2 g of either X5 or ADS17 resin. The mixtures were shaken periodically for 30 min following which the unbound MAR filtrate and MAR ethanol eluent were recovered and assayed for flavonoids. The percentage adsorption and recovery of flavonoids were subsequently calculated using equation 1 and 2.

Effect of Adsorption Time on the Static Adsorption Characteristics of N. laevis Polyphenols on X5 MAR

In this procedure, NEE solution (1 ml; 2 mg/ml) was added to 0.2 g each, of the X5 MAR (in 6 replicate containers). Unbound MAR filtrates were recovered from each of the replicate 1, 2, 3, 4, 5 and 6 after 10, 20, 30, 60, 120 and 1440 min of the experiment respectively. Total flavonoids and phenols in the unbound MAR filtrate were determined and substituted into equation 1 to compute the percentage adsorption of flavonoid and phenol unto X5 MAR.

Effect of pH on the Static Adsorption Characteristics of N. laevis Polyphenols on X5 MAR

Replicate tubes containing 0.2 g of X5 MAR were saturated with different buffers (5 ml; 0.05 M), including Sodium Acetate buffer (pH 3.8, 4.5 and 5.5), Sodium Phosphate buffer (pH 6.5 and 7.9) and Sodium Carbonate buffer (pH 10.4) for 5 min following which the buffer-washed resins were recovered. To this was added NEE solutions (1 ml; 2.7 mg/ml) which was previously buffered with each test buffer (to final buffer strength of 0.05 M). The mixtures were allowed to stand for 30 min with periodic shaking after which the percentage adsorption of flavonoids and phenols were determined using equation 1.

Effect of Eluent ethanol concentration and Elution Time on the Static Desorption Characteristics of N. laevis Polyphenols from X5 MAR

Phenolics from NEE solution (1 ml; 2 mg/ml) were adsorbed unto X5 (0.2 g) in multiple replicates by incubating both NEE solutions with X5 MAR for 30 min. The resultant polyphenol-bound resins were washed thrice with distilled water following which the polyphenols were eluted under two experimental conditions consisting of a 30 min incubation with varying ethanol concentration (i.e. 25, 40, 50, 60, 70, 80 and 95 %) and incubation with 95 % ethanol at varying incubation period (i.e. 10, 20, 30, 60 and 120 min). Percentage desorption of total flavonoids and phenols from the MAR were then calculated using equation 2.

Preparation of N. laevis Polyphenol-Rich Extract (NPE)

The mass enrichment of total polyphenols from NEE was done in a static batch system [40]using information derived from the MAR-assisted polyphenol enrichment optimization. In each batches of the experiment, 6 g of NEE was dissolved in 150 ml of absolute ethanol following which the resultant volume was made up to 2 L with distilled water. The NEE solution was filtered and mixed with 400 g of X5 MAR. The resultant mixture was agitated periodically for 40 min under neutral pH, following which the unbound MAR filtrate was recovered by filtration. The polyphenol-bound X5 MAR was washed with tap water and was briefly drained. Polyphenols were eluted from the polyphenol-bound resins by agitating the resins in 1 L of ethanol (70 %) for 30 min. The ethanol treatment stage was done twice following which the resultant MAR ethanol eluents were pooled and filtered. The polyphenol-rich ethanol eluent was concentrated to dryness by heating over a water bath (70 - 90ºC) in order to obtain the *N. laevis* Polyphenol-rich Extract (NPE).

Assessment of the DPPH Radical Scavenging Potentials of NEE and NPE

The DPPH radical scavenging potentials of NEE and NPE was investigated using the methods reported by Souza and De Giovani. [41] The percentage reductions of DPPH radicals and the IC_{50} of DPPH radicals were determined for each test extract (See Supplementary S3). Also calculated was the DPPH radical scavenging potentials of the test extracts relative to ascorbic acid (obtained by dividing the inverse transformation of the IC_{50} of the test extract with the inverse transformation of the IC_{50} of standard ascorbic acid.) It is our opinion that the unitless quantity so obtained enabled a more standardized comparison of the DPPH scavenging potentials of different plant extract across different experimental setups or studies, relative to ascorbic acid standards subjected to the same test condition.

Assessment of the Ferric Reducing Ability of the Plasma (FRAP) activity of NEE and NPE

The Ferric Reducing Ability of the Plasma (FRAP) assay was done according to the method described by Benzie and Strain [42] and Reitz *et al*.[43](See Supplementary S4). The results were presented as concentration of Fe^{2+} (µM) produced per µg of test or standard sample. For ease of standardized comparison with data from other studies, the result was normalized by expressing it as a ratio of FRAP activity of test extract to FRAP activity of ascorbic acid under same test conditions.

Effect of NPE and NEE on Pb-induced Hemolysis of Isolated Human RBC

The method used for this assay was adapted from the works of Soni *et al*.,[44] Casado *et al*.[45] and Mrugesh *et al*. [46] Venous blood was collected from a healthy male volunteer and transferred into a lithium heparin container. The blood was centrifuged to remove blood plasma and Buffy coats as well as isolate the red blood cells (RBC). The isolated RBC was washed with Phosphate Buffered Saline (PBS; pH 7.4) and later constituted into a 2 % RBC suspension in PBS (pH 7.4). The test extracts (NEE and NPE) were formulated into a 60 µg/ml solution shortly before analysis, by mixing 0.006 ml of a 5 mg/ml ethanolic preparation of each test sample with 0.494 ml of PBS (pH 7.4).

Experimental design

The study was conducted using 6 treatment groups. The treatment group 1 (i.e. control group) consisted of a mixture of RBC (0.5 ml; 2 %) and PBS (0.485 ml: pH 7.4). The mixture was incubated at 37ºC for 15 min after which an additional 0.015 ml of PBS (pH 7.4) was added to it. In group 2 and 3, NEE (0.05 ml; 60 µg/ml) and NPE (0.05 ml; 60 µg/ml) respectively, were individually incubated with RBC (0.5 ml; 2 %) and PBS (0.435 ml; pH 7.4) at 37ºC (for 15 min) following

which additional 0.015 ml of PBS (pH 7.4) was added to each mixture. Group 4 consisted of a mixture of RBC (0.5 ml; 2 %) and PBS (0.485 ml; pH 7.4) incubated at 37ºC for 15 min, and eventually mixed with 0.015 ml of Pb Acetate (100 mM). Groups 5 and 6 consisted of individual mixtures of NEE (0.05 ml; 60 µg/ml) and NPE (0.05 ml; 60 µg/ml) respectively, with RBC (0.5 ml; 2%) and PBS (0.435 ml; pH 7.4). The mixtures were incubated at 37ºC for 15 min followed by the addition of Pb Acetate solution (0.015 ml; 100 mM).

Each treatment group was incubated for 4 h in a water bath set at 37ºC after which they were centrifuged. The absorbances of the RBC-free supernatant were recorded at 540 nm using a spectrophotometer. Percentage hemolysis of each treatment group was calculated relative to the spectrophotometric reading of a 100 % hemolyzed RBC sample using the equation below:

Percentage hemolysis = $(At * 100)/Ac$ (4)

Where "At" is the absorbance reading of the test sample at 540 nm and "Ac" is the mean absorbance value obtained from the 100 % hemolyzed RBC.

Effect of NPE and NEE on Fenton-chemistry mediated peroxidation of Egg Yolk Homogenate

A slight modification of the method reported by Dairam *et al*.[47] was employed in evaluating the inhibitory potentials of NPE and NEE against the peroxidation of egg-yolk homogenate induced by exposure to Fenton-chemistry reagents. The formation of thiobarbituric acid reactive substances (TBARS) in each experimental mixture was estimated by the method reported by Ohkawa *et al*.[48] (See Supplementary S6) and expressed as the amount of Malondialdehyde (MDA) (in nmoles) produced per ml of egg yolk.

Statistical Analysis

Data were presented in tabular or bar chart forms as mean ± standard deviation (exceptions are stated where applicable). Statistical differences between treatments groups were evaluated using the one way analysis of variance (ANOVA) statistics, performed with the Statistical Package for the Social Sciences (SPSS; version 16.0). Treatment groups having $p \leq 0.05$ were considered to have a significant statistical difference.

Results and Discussion

Total polyphenols and flavonoids content

Polyphenols are considered the most abundant plant-derived antioxidants which has diverse structural compositions.[49] Flavonoids are polyphenolic compounds with over 8000 structurally distinct members and whose function in plants involves enhancing plant survival against stressors such as ultraviolet (UV), microbial or herbivore attacks.[7] Results from this study revealed that 100 g of *N. laevis* leaves contained 4.19 ± 1.2 quercetin-equivalent g of flavonoids and 2.64 ± 0.3 gallic acid-equivalent g of phenols (Table 1). The total phenolics and flavonoids observed in crude extract of *N. laevis* (i.e. 12 ± 0.71 g/100 g extract and 30 ± 4.24 g/100 g extract respectively), is higher than the 91.49 mg and 22.42 mg of total phenols and flavonoids respectively, reported by Tuo *et al*.[50] per g of aqueous *N. laevis* leaf extract but lower than the total phenolics (222 mg/g) in methanol leaf extract of *Alafia barteri*.[51]

Optimization of MAR-assisted enrichment of polyphenols from N. laevis

Macroporous adsorptive resins are porous synthetic materials made of styrene or acrylate esters and having pore size of above 50 nm. [52,53] Findings from the effect of different MAR types (i.e. X5, S400, ADS17, ADS7, AB8 and D101) on the adsorption and subsequent recovery of polyphenols from NEE showed that X5 and ADS17 had good adsorption and desorption characteristics for NEE flavonoids while X5, ADS17, AB8 and D101 had good adsorption and desorption potentials for NEE total phenols (Figure 1). We observed that an extract loading concentration ranging between 15 to 50 mg NEE/g resin is suitable for optimal flavonoid adsorption and desorption on X5 and ADS17 MAR (Figure 2). An obvious reduction in the adsorption and recovery efficiency of flavonoids on X5 and ADS17 MAR was observed when the extract concentration is greater or equal to 100 mg NEE/g resin (Figure 2). Our finding revealed that the majority of the phenolic content of NEE adsorbed unto X5 resin within 20 min of contact (Figure 3a). The observed optimal pH for the adsorption of NEE phenolics unto X5 was pH 6.53 (Figure 3b) while NEE flavonoids had optimal adsorption at pH range of 5.49- 6.53. The adsorption of phenols and flavonoids of NEE unto X5 MAR reduced steadily at pH values above 6.53 Adsorbed flavonoids were readily eluted from X5 MAR within 10 min of contact with 95% ethanol while majority of the phenolic contents of NEE were eluted within 20 min of contact with ethanol (Figure 4a). Hydroethanol mixtures ranging between 50-95 % were effective at eluting majority of the phenols and flavonoids adsorbed unto X5 MAR (Figure 4b).

The present evidence from the optimization studies supports previous findings that MAR type, pH, adsorption time, elution time and eluent ethanol concentration are important determinants of the efficiency of the MAR-assisted polyphenol enrichment process.[18,54,55] Appropriate MAR readily adsorbs the polyphenol content of aqueous solution of plant extract in a process that has been described with Freudlich, Langmiur and Pseudo-second order kinetics models.[38,56,57] Following such adsorption, unadsorbed components of the extract (such as protein, carbohydrates and other water soluble substances) are removed by rinsing the resin with water. The phenol contents are subsequently recovered from MAR using hydroethanol mixture of appropriate concentration.

Evidence from the present study showed obvious increase in the total flavonoid (i.e. from 30% to 74%) and the total phenolic contents of NPE (i.e. from 12% to 33 %) after the enrichment process (Table 1). Similar improvements had been reported following MAR-assisted enrichments of polyphenols from other plant extracts. For instance in an experimental system that involved AB8 MAR, *Sophora tonkinensis* extract (7.7 mg/gram resin), pH 4 and 60 % eluent ethanol, Hou *et al*.[18]obtained a 4.76 fold improvement in the total flavonoid content of *Sophora tonkinensis*. The MAR-assisted procedure described in this paper therefore offers a simple, effective and environment-friendly way to enrich phenolics and flavonoids from ethanol extract of *N. laevis* leaf.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and Ferric Reducing Ability of the Plasma (FRAP) activities.

The DPPH assay tests the potential of test extracts to reduce or donate electrons to the relatively stable DPPH radicals with consequent formation of the more stable DPPH hydrazine derivatives. [58] The data presented in Figure 5 revealed that ascorbic acid and quercetin inhibited DPPH radical at much lower concentrations than either NPE or NEE. This finding was reflected in the concentrations of the test extract that inhibited 50 % of DPPH radicals (IC_{50}). The IC_{50} value of 0.034mg/ml observed for ascorbic acid revealed that ascorbic acid is a better scavenger of DPPH radicals than either NEE (i.e. IC_{50}= 0.38 mg/ml) or NPE (IC_{50}= 0.16 mg/ml) (Figure 6). Nevertheless, a 2.3 fold improvement was observed in the DPPH radical scavenging potentials of NEE after the polyphenol enrichment process. Following the normalization of the DPPH IC_{50} of the test extract with the DPPH IC_{50} of ascorbic acid (obtained under similar experimental conditions), the data from the present study revealed that NEE and NPE are 0.19 and 0.46 times respectively, as potent DPPH radical scavenger as ascorbic acid (Table 2). The normalized values indicates that NPE have lower DPPH radical scavenging potentials than *Zingiber officinale* and *Curcuma longa* which are 0.77 and 0.81 times the DPPH scavenging potentials of ascorbic acid respectively[59,60] but higher DPPH radical scavenging potentials than some Nigerian medicinal plants like *Piper guineense*, *Bridelia ferruginea*, *Nauclea diderrichii*, and

Acanthospermum hispidum which are 0.094, 0.123, 0.078 and 0.049 times respectively, the DPPH scavenging potentials of ascorbic acid.[61]

Table 1: Outcome obtained from the optimization of polyphenol enrichment of NEE using X5 MAR.

	Percentage content in dry leaf powder (%)	Percentage content in NEE (%)	Percentage Adsorption unto MAR (%)	Percentage content in NPE (%)	Fold Improvement	Percentage Recovery (%)
Flavonoid [#]	4.19 ± 1.2	30 ± 4.24	79 ± 1.76	74 ± 10.6	2.5 ± 0.013[a]	49 ± 7.77
Phenol [#]	2.64 ± 0.3	12 ± 0.71	76 ± 1.41	33 ± 1.41	2.8 ± 0.300[b]	57 ± 1.40

Values are presented as mean± standard deviation (n = 2)
[#] Flavonoids and Phenols are calculated based on quercetin and gallic acid equivalent values respectively.
[a] Determined by dividing the percentage content of flavonoids in NPE by the percentage content of flavonoids in NEE.
[b] Determined by dividing the percentage content of phenols in NPE by the percentage content of phenols in NEE.
NEE: *N. laevis* leaf ethanol extract; NPE*: N. laevis* leaf Polyphenol-rich extract; MAR: Macroporous Adsorptive Resin

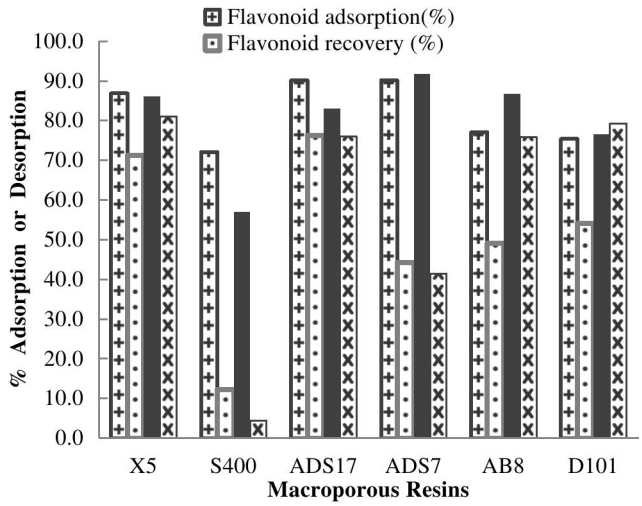

Figure 1: Static Adsorption and Desorption properties of selected MARs for *N. laevis* polyphenols. Each bar represents individual determinations.

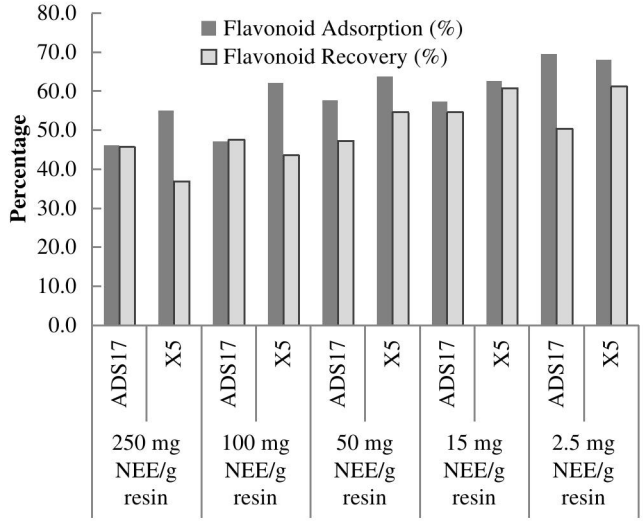

Figure 2: Static Adsorption and Desorption Characteristics of flavonoids on ADS17 and X5 MAR under Different Concentrations of NEE. Each bar represents individual determinations.

The FRAP activity gives a rough estimate of the sum effect of different antioxidants within the test extract by accessing the potential of the extract to reduce ferric ions into ferrous ions.[42] Higher FRAP values thus indicates a stronger reducing or antioxidant potential. There was a 3- fold improvement in the FRAP activity of *N.laevis* after the polyphenol enrichment process. *N laevis* Polyphenol-rich extract exhibited a lower FRAP activity (i.e. 4.45 µM Fe^{2+}/µg Sample) than ascorbic acid (i.e. 14.27 µM Fe^{2+}/µg Sample) or quercetin (i.e. 39.83 µM Fe^{2+}/µg Sample) (Figure 7). Normalization of the FRAP values of the test extracts with respect to the FRAP value of standard ascorbic acid (performed under the same experimental settings), showed that the FRAP activity of NPE is 0.311 times as much as that of ascorbic acid. This value is similar to that of *Ocimum gratissimum* (i.e. 0.33), [62] and higher than the value reported for *Zingiber officinale* (i.e. 0.27),[59] but less than values reported for *Bryophyllum pinnatum* (i.e. 0.63) [63]and *Curcuma longa (*i.e.0.83).[60] The corresponding improvement in the antioxidant capacity, phenolic contents and flavonoid composition of NPE suggests that phenolic compounds are important contributors to the DPPH and FRAP activities of *N. laevis* extract. Similar improvement in antioxidant activity had been previously reported following the treatments of some plant extracts with MAR.[64,65]

Effect on Pb-induced red blood cell (RBC) hemolysis

Exposure of RBC to oxidizing agents disrupts RBC membrane structure and functions subsequently causing hemolysis of RBC.[66] Casado *et al*. [45] observed that the exposure of RBC to high levels of Pb can trigger oxidative pre-hemolytic injury that can eventually result in hemolysis of RBC. Co-incubation of Pb-exposed RBC with antioxidants like as Trolox and Histidine as well as Deferoxamine (Fe-chelators), suppressed hemolytic breakdown of RBC. [45] The evidence from this study revealed that both NEE and NPE did not protect against Pb-induced hemolysis of RBC (Figure 8). The observed stimulation of hemolysis by NPE suggests possible pro-oxidant activities by constituents of NPE. Polyphenol like gallic acid and flavonoids such as apigenin and quercetin have been previously shown to exert pro-oxidant effect at certain experimental conditions.[67–69] The potential presence of a pro-oxidant-capable polyphenol in NPE, the abundance of hemoglobin-derived Fe^{3+}/Fe^{2+} ions and the potentially oxidized Pb-exposed RBC environment might have facilitated the observed stimulation of hemolysis.

Effect on Fenton reagent -induced peroxidation of egg yolk homogenate

From the present study, exposure of egg yolk homogenate to the Fenton reagent mixtures increased the levels of malondialdehyde from a mean value of 185 nmoles/ml of egg yolk (i.e. control group) to 493 nmoles /ml of egg yolk (i.e. Fenton-reagent exposed group) (Figure 9).

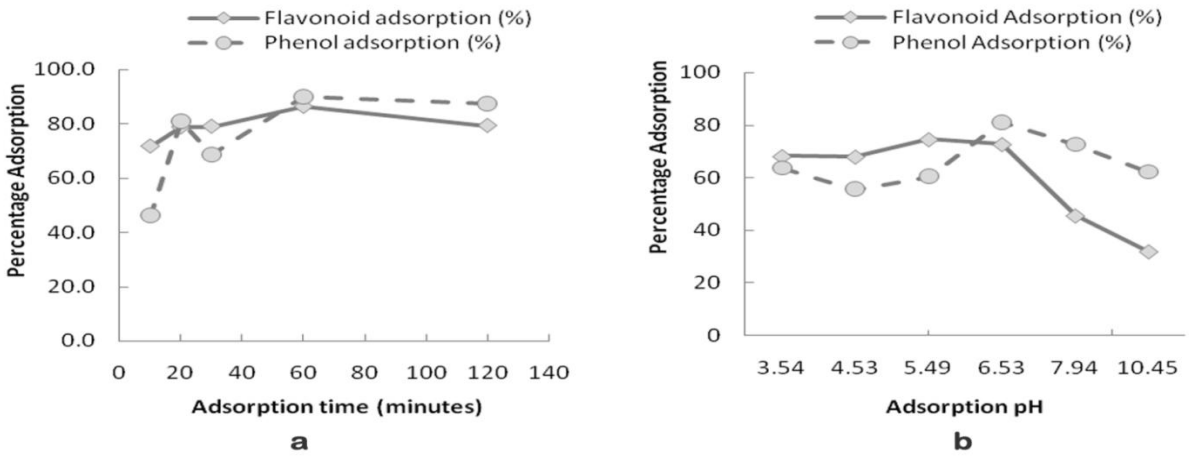

Figure 3: Static adsorption of NEE unto X5 MAR under conditions of varying adsorption time (a) and varying pH (b). Each data point represents individual determinations.

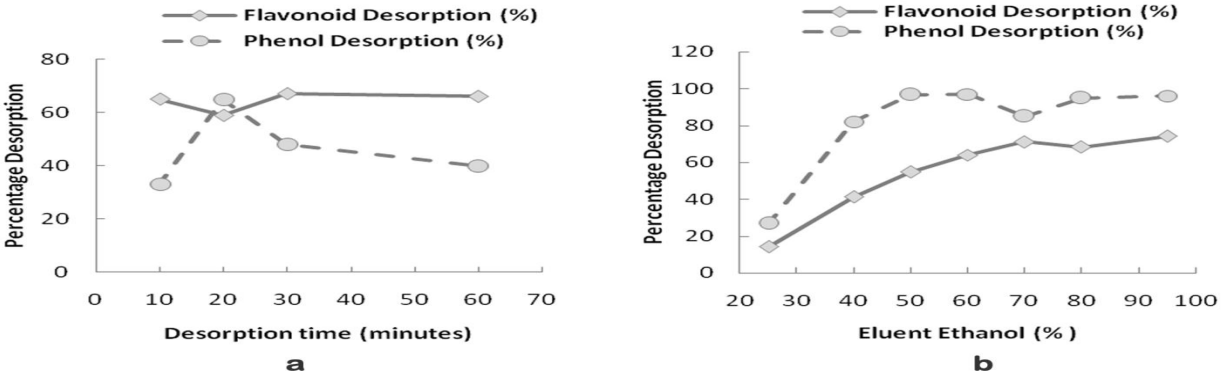

Figure 4: The effects of desorption time (a) and ethanol concentration (b) on the static desorption of NEE's flavonoids and polyphenols from X5 MAR. Each data point represents individual determinations.

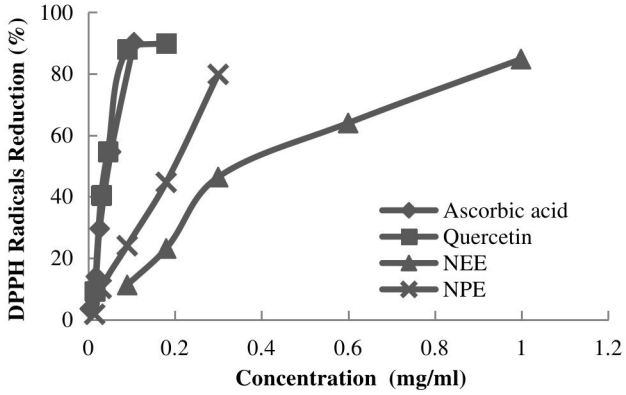

Figure 5: The percentage reduction of DPPH radicals by different test agents.
NEE: *N. laevis* leaf ethanol extract; NPE*: N. laevis* leaf Polyphenol-rich extract; DPPH: 2,2-diphenyl-1-picrylhydrazyl

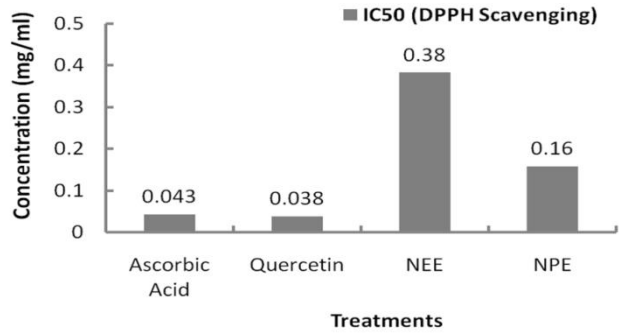

Figure 6: The DPPH radicals scavenging potentials of NEE and NPE.Each bar value was derived from 5 varied concentrations of the extract (or standard). NEE: *N. laevis* leaf ethanol extract; NPE*: N. laevis* leaf Polyphenol-rich extract; DPPH: 2,2-diphenyl-1-picrylhydrazyl.

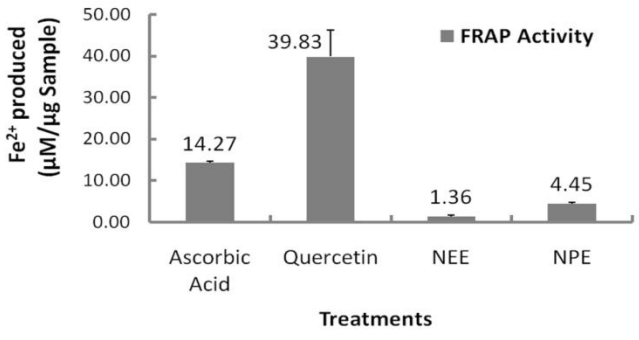

Figure 7: The FRAP activity of NEE and NPE. Each bar value represents the mean of 2 replicates with corresponding standard deviation appearing as error bars. NEE: *N. laevis* leaf ethanol extract; NPE*: N. laevis* leaf Polyphenol-rich extract; FRAP: Ferric Reducing Ability of the Plasma;

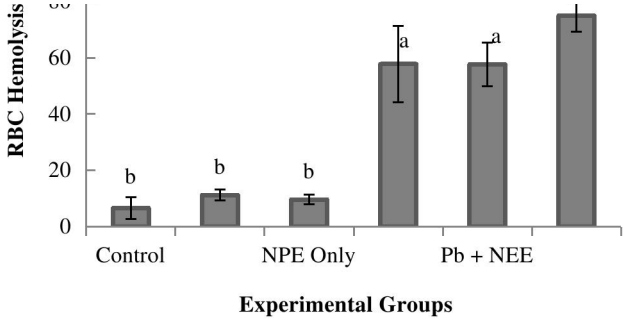

Figure 8: Effects of NEE and NPE on Pb- exposed RBC.
Each bar value represents the mean of 3 replicates with corresponding standard deviation appearing as error bars.
"a" is significantly different from control ($p < 0.05$).
"b" is significantly different from the Pb Only group ($p < 0.05$).NEE: *N. laevis* leaf ethanol extract; NPE*: N. laevis* leaf Polyphenol-rich extract; Pb: Lead; RBC: Red Blood Cell

Reactive oxygen species such as the hydroxyl radicals, are generated in a Fenton-reagent system containing both Fe^{2+} and H_2O_2.[67,70] The exposure of egg-yolk homogenates to such reagents predisposes the lipids of egg-yolk to peroxidation induced by the ROS generated under such experimental conditions.[35]
The treatment of Fenton-reagents exposed egg yolk homogenates with either NEE or NPE significantly inhibited malondialdehyde generation, with NPE having a 2.5 fold higher inhibition of malondialdehyde production than NEE. Similarly, quercetin was more effective in suppressing malondialdehyde generation than NEE but less effective than NPE (Figure 9). The suppressive tendency of *N. laevis* against Fenton-induced peroxidation may involve inhibition of radical formation or enhancement of radical scavenging. Both mechanisms are tenable since *N. laevis* can potentially interact with Fe ions, such as seen in its FRAP activity. There is also a possibility that the interactions between *N. laevis* extract and Fe ions or other constituents of the Fenton-reagent system might have interfered with the pathway of free radical generation thereby suppressing the peroxidation of egg yolk lipids. Additionally, the fact that NPE can scavenge DPPH radical implies that NPE possess the capability to donate electrons to free radicals thereby quenching their oxidative chain reactions. The improvement in the anti-peroxidative activities of NPE after the MAR-assisted polyphenol enrichment indicates that the constituent polyphenol of NPE has great potencies in suppressing free radical-mediated peroxidation of the lipids of egg yolk.

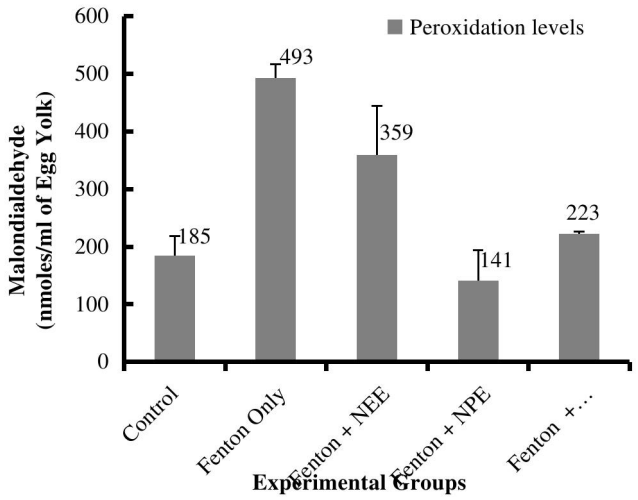

Figure 9: Effects of NEE and NPE on Fenton-chemistry mediated peroxidation of egg Yolk.
Each bar value represents the mean of 2 replicates with corresponding standard deviation appearing as error bars. NEE: *N. laevis* leaf ethanol extract; NPE*: N. laevis* leaf Polyphenol-rich extract

Table 2: Relative DPPH scavenging potentials with respect to standard ascorbic acid

Plant extracts	Relative DPPH scavenging potentials[#]	References
N. laevis (NEE)	0.19	Present study
N. laevis (NPE)	0.46	Present study
N laevis	0.431	[71]
Alangium salvifolium	0.149	[72]
Rosmarinus officinalis	0.142	[73]
Garcinia mangostana	0.278	[74]
Piper guineense	0.094	[61]
Bridelia ferruginea	0.123	[61]
Nauclea diderrichii	0.078	[61]
Acanthospermum hispidum	0.049	[61]
Zingiber officinale	0.77	[59]
Curcuma longa	0.81	[60]

[#] Values were computed by dividing the inverse transformation of the IC_{50} of the test extract by the inverse transformation of the IC_{50} of ascorbic acid. The ascorbic acid used for this normalization was subjected to the same experimental condition as the respective test extract. [*]Ascorbic acid has a value of 1; the higher the relative values, the higher the DPPH radical scavenging potentials.

Conclusion

The MAR-assisted enrichment procedure improved the total phenols, flavonoids, DPPH radical scavenging, FRAP and the anti-peroxidative activities of the *N. laevis* leaf extract. The crude extract and the polyphenol-enriched extract of *N. laevis* did not protect against Pb-induced hemolysis of RBC. The MAR-assisted polyphenol enrichment process therefore provided a simple strategy for purifying total

polyphenols and enriching antioxidant principles from *N. laevis* leaf extract. Our data could aid the utilization of this abundant medicinal plant resource for functional food development, drug development and industrial applications.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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