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Polyphenol Content and in Vitro Antioxidant Activity of Aqueous-Ethanol Extracts of *Pterocarpus soyauxii* and *Pterocarpus santalinoides*

By Emmanuela Nneoma Akaniro-Ejim, Marynn Ifunanya Ibe & Godwill Azeh Engwa
Godfrey Okoye University

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Keywords: antioxidants; Pterocarpus soyauxii; Pterocarpus santalinoides; flavonoids; flavonols, ferric reducing potential, hydrogen peroxide scavenging activity.

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Abstract- Recently, medicinal plants are gaining considerable attention for their therapeutic antioxidant activities. Though many studies have investigated the pharmacological and medicinal activities of *Pterocarpus soyauxii* and *Pterocarpus santalinoides,* there is limited knowledge of their antioxidant potential. Hence, this study aimed to assess the polyphenol content and investigate the in vitro antioxidant activity of these plants. Aqueous-ethanol extracts of the plants' leaves were obtained by maceration. The total flavonoid content (TFdC) and total flavonol content (TFlC) of the leaf extracts were determined by standard methods, while ferric reducing power and hydrogen peroxide scavenging assays were used to assess their *in vitro* antioxidant potentials. The mean TFdC of *P. santalinoides* (1083.33 ± 35.12 mg/g) was higher than that of *P. soyauxii* (730 ± 40 mg/g), while the mean TFlC was higher in *P. soyauxii* (390 ± 60.83 mg/g) than in *P. santalinoides* (260 ± 45.83 mg/g). The reducing potential of extracts of *P. santalinoides* was significantly higher ($p < 0.05$) than that of *P. soyauxii*, as well as the standard compound, at all concentrations tested. The hydrogen peroxide scavenging activity of *P. santalinoides* was superior to that of *P. soyauxii,* as well as ascorbic acid. The results of this study suggest that *P. soyauxii* and *P. santalinoides* are rich in flavonoids and flavonols and exhibit potent hydrogen peroxide scavenging activity and ferric reducing capacity, with the later showing greater activities. These properties may contribute to the therapeutic potential and medicinal applications of these plants and suggests a potential drug candidacy of flavonoid compounds of these species of *Pterocarpus*.

Keywords: antioxidants; Pterocarpus soyauxii; Pterocarpus santalinoides; flavonoids; flavonols, ferric reducing potential, hydrogen peroxide scavenging activity.

I. Introduction

Oxidative stress contributes to many pathological conditions and diseases including cancer, stroke, diabetes, inflammatory diseases such as arthritis, cardiovascular disorders, etc. [1-4]. It results from an overwhelming level of free radicals or reactive oxygen species (ROS) such as hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, peroxynitrite radical, etc. [5], which contain one or more unpaired electrons thus making them unstable and highly reactive [2]. Due to this high reactivity, these molecules rapidly attack adjacent molecules leading to disruption of membrane fluidity, lipid peroxidation, protein oxidation, alteration of platelet functions, DNA strand breaks, and modulation of gene expression [3, 4].

To circumvent the delirious and detrimental effects of free radicals, antioxidants are naturally present in living organisms and are capable of scavenging these free radicals, converting them to less reactive forms, thereby preventing or inhibiting cellular damage [6]. Antioxidant defense systems in humans include iron transport proteins such as transferrin, albumin, ferritin, and caeruloplasmin; metabolic products such as glutathione, ubiquinol and uric acid; and endogenous enzymes such as superoxide dismutase, catalase, various peroxidises etc. [2, 5, 7]. The antioxidant defense systems under normal physiological conditions are sufficient only to cope with the normal threshold of the physiological rate of free-radical generation. Therefore, any additional burden of free radicals, either from endogenous or exogenous sources on the human physiological system may lead to oxidative stress [2, 7]. Hence, supplementary sources of antioxidants are needed to prevent oxidative stress. Recently, medicinal and dietary plants are gaining considerable concern, as they are rich in micronutrients such as vitamin E (α-tocopherol), vitamin C (ascorbic acid) and β-carotene, as well as plants secondary metabolites such as phenolic compounds, flavonoids, saponins, etc. [4, 5, 8-11] which have been shown to exhibit promising therapeutic antioxidant properties. Though the activity of synthetic phenolic antioxidants is often observed to be higher than that of natural antioxidants, [12] there is evidence of increased predisposition to various fatal diseases following use of synthetic antioxidants [4, 8-10], hence the renewed interest in natural antioxidants.

The genus *Pterocarpus* is a tropical and sub-tropical plant with about 60 species of which 20 of these are found in Africa, particularly in Nigeria, Cameroon,

Author α σ: Department of Biotechnology and Applied Biology, Godfrey Okoye University, P.M.B 01014 Thinkers Corner Enugu Nigeria.
e-mail: nakaniro@yahoo.com
Author ρ: Biochemistry, Chemical Sciences Department, Godfrey Okoye University, P.M.B 01014 Thinkers Corner Enugu Nigeria.

Sierra Leone and Equatorial Guinea [13, 14]. *Pterocarpus soyauxii* and *Pterocarpus santalinoides*, locally known as "oha" and "uturukpa" respectively in Igbo, are abundant and widely consumed as vegetables in South-Eastern Nigeria [13, 14]. They are traditionally used in the treatment of headaches, pains, fever, convulsions, skin rashes and respiratory disorders, and as antiabortive, antidiabetic, hepatoprotective and antimicrobial agents [13-15]. Though many studies have investigated the pharmacological and medicinal activities of these species [16-18], little is known about their antioxidant potential. Hence, this study was aimed to determine the polyphenol content of these plants and investigate the in vitro antioxidant activity of *Pterocarpus soyauxii* and *Pterocarpus santalinoides*.

II. Materials and Methods

a) Plant materials

Fresh leaves of *Pterocarpus soyauxii* and *Pterocarpus santalinoides* were purchased from Ahia Abapka in Enugu, Enugu State of Nigeria. The plants were identified taxonomically by Prof C. U. Okeke (Department of Botany, Nnamdi Azikiwe University, Akwa) as *Pterocarpus soyauxii* (*P. soyauxii*) and *Pterocarpus santalinoides* (*P. santalinoides*). The leaves were air-dried at room temperature (28 ± 2°C) in the Biotechnology Laboratory of Godfrey Okoye University Enugu for seven days and thereafter pulverized before further processing.

b) Chemicals and reagents

Ethanol and ascorbic acid were purchased from JHD, Guangdong Guanghua Sci-Tech Co., Ltd., Guangdong, China. Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) and disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) for phosphate buffer were purchased from Merck KGaA, Darmstadt, Germany. Potassium ferricyanide and trichloroacetic acid were obtained from Vicker Laboratories Ltd, West Yorkshire, England. Ferrous chloride was obtained from Griffin and George, Wembley, England. Hydrogen peroxide and aluminium chloride were obtained from BDH Laboratory Supplies, Poole, England. Sodium acetate was obtained from Burgoyne Burbidges and Co., Mumbai, India. Rutin was obtained from Sigma-Aldrich, St. Louis, MO, USA. All solvents and reagents used in the study were of analytical grade.

c) Maceration and extraction of plant materials

Extraction was carried out according to the methods of Bothon *et al*. [15] with slight modifications. Hundred gram (g) of the pulverized leaves of *P. soyauxii* and *P. santalinoides* were separately macerated in 500 ml of aqueous-ethanol for 48hours. The aqueous-ethanol extracts were prepared by adding 500 ml of an ethanol-water mixture (70:30) to 100g plant powder and mechanically stirred for 48 hours. The resulting solutions were filtered through Whatman No. 1 filter paper and the extracts obtained were then concentrated and finally dried to a constant weight. The extraction yields of the samples were calculated using the following equation:

$$\text{Total extraction yield, } Y_t\ (\%) = \frac{\text{Mass of extract, } M_t}{\text{Mass of sample, } M_s} \times 100\%$$

Extracts were stored in sterile containers at 4 °C until further use.

d) Estimation of polyphenol compounds

i. Total flavonoids content

Total flavonoids content of the plant extracts was determined based on the formation of an aluminium-flavonoids complex, using the methods described by Ordon Ez *et al*. [19]. A volume of 0.5 ml (2 %) aluminium chloride-ethanol solution was mixed with 0.5 ml of plant extracts (100 mg/l). The mixture was incubated at room temperature for 1 hr and the absorbance measured at 420 nm. All determinations were carried out in triplicates. The same procedure was repeated for the various concentrations (6.25 - 100 mg/l) of a standard solution of rutin, and the rutin calibration curve was constructed. The concentration of flavonoids was expressed as rutin (mg/l) equivalent from the calibration curve of rutin (Figure 1) using the equation: $Y = 0.001X - 0.003$, $R^2 = 0.991$

$$X = \frac{Y + 0.003}{0.001}$$ where, Y was absorbance and X was concentration of rutin (mg/l).

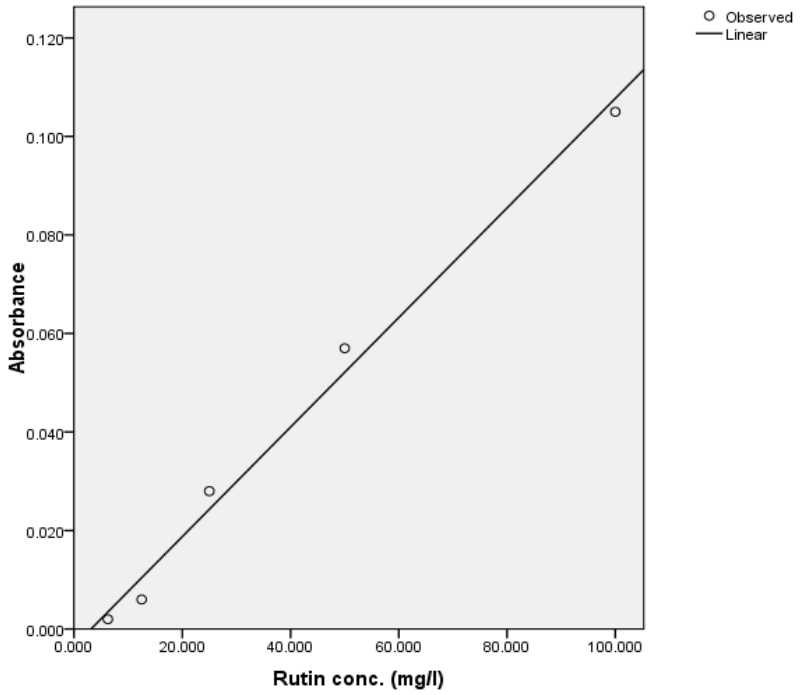

Figure 1: Calibration curve of total flavonoids of rutin

The total flavonoids content (TFdC) of extracts was calculated in terms of rutin equivalent (mg of RU/g of dry weight extract) using the following formula:

$$\text{TFdC (mg RU/g)} = \frac{\text{Concentration of rutin (mg/l)} \times \text{[Total volume of extract solution (ml)} \times 10^{-3}\text{ (l/ml)]}}{\text{Weight of extract (mg)} \times 10^{-3}\text{ (g/mg)}}$$

ii. *Total flavonols content*

The total flavonols content was estimated based on the method of Kumaran and Karunakaran [20], using rutin as a reference compound. Two milliliters of the extracts (100 mg/l) were separately mixed with 2 ml of 2% aluminium chloride-ethanol solution and 3 ml of sodium acetate solution (50 mg/ml). The resulting solution was incubated at room temperature for two and half hours, and the absorbance was read at 440 nm. All determinations were carried out in triplicates. The same procedure was repeated for the various concentrations (6.25 - 100 mg/l) of standard solution of rutin and the rutin calibration curve was constructed. The concentration of flavonols was expressed as rutin (mg/L) equivalent from the calibration curve of rutin (Figure 2) using the equation: $Y = 0.001X + 0.008$, $R^2 = 0.990$

$X = \frac{Y-0.008}{0.001}$ where, Y was absorbance and X was concentration of rutin (mg/l).

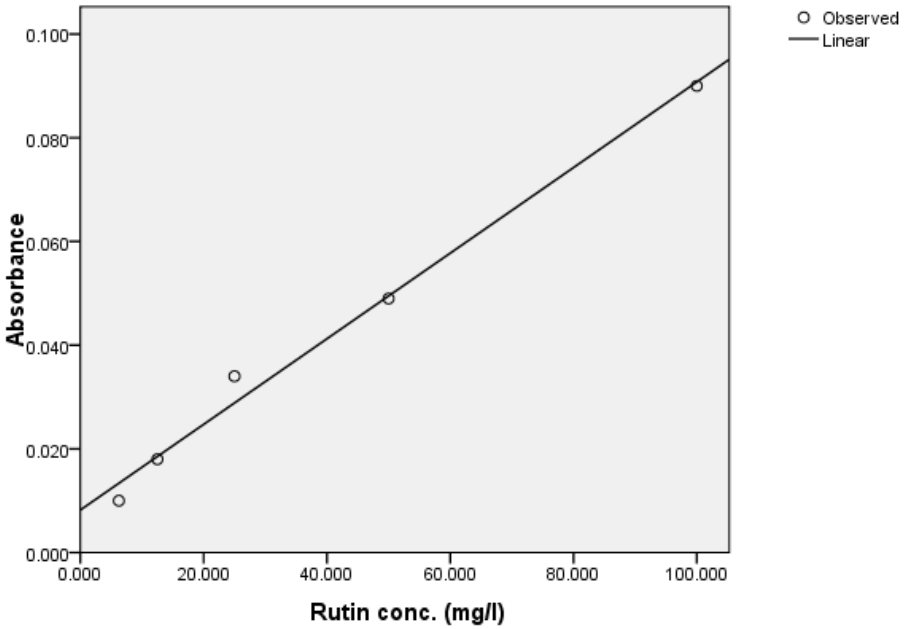

Figure 2: Calibration curve of total flavonols of rutin

The total flavonols content (TFlC) of extracts was calculated in terms of rutin equivalent (mg of RU/g of dry weight extract) using the following formula:

$$\text{TFlC (mg RU/g)} = \frac{\text{Concentration of rutin (mg/l)} \times [\text{Total volume of extract solution (ml)} \times 10^{-3}\ \text{(l/ml)}]}{\text{Weight of extract (mg)} \times 10^{-3}\ \text{(g/mg)}}$$

e) In vitro antioxidant activity

i. *Determination of reducing power*

The reducing power of the extracts was determined according to the methods of Yildirim *et al.* [21], with slight modifications. Extracts (1.25- 10.00 mg) in 1 ml of distilled water were separately mixed with 2.5 ml of phosphate buffer (0.2 M, pH 7.0) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe\ (CN)_6$]. The resulting solution was incubated at 50°C for 30 min, followed by addition of 2.5 ml of 10% trichloroacetic acid, and centrifugation of the resulting mixture at 3000 rpm for 10 min. Finally, 2.5 ml of the upper layer solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferrous chloride ($FeCl_3$) and the absorbance was measured at 700 nm against a blank sample using a UV-5800(PC) UV/VIS Spectrophotometer. Increased absorbance of the reaction mixture was indicative of high reducing power of the plant extracts. Ascorbic acid was used as standard.

ii. *Hydrogen peroxide scavenging activity*

The ability of the aqueous-ethanol extracts of *P. soyauxii* and *P. santalinoides* to scavenge hydrogen peroxide was determined using the methods of Yen and Chen [22]. A solution of hydrogen peroxide (4mM) was prepared in phosphate buffer (0.1 M, pH 7.0). The hydrogen peroxide solution (0.6 ml) was separately mixed with 4 ml of various concentrations of the extracts (1.25 - 10.00 mg/ml) and incubated at room temperature for 10 min. Absorbance of hydrogen peroxide at 230 nm was determined against a blank solution containing plant extracts without hydrogen peroxide. Percent scavenging activity of the plant extracts was determined by following formula:

$$H_2O_2\ \text{scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample}}{\text{absorbance of control}}\right) \times 100\%$$

Where, Absorbance of control was the absorbance of hydrogen peroxide radical + solvent; Absorbance of sample was the absorbance of hydrogen peroxide radical + sample extract or standard. Ascorbic acid served as standard.

iii. *Statistical analysis*

Experimental results were reported as mean ± Standard deviation (SD) of three parallel measurements. Unpaired T-test was performed to compare the means of the total flavonoids and flavonols content of the plant extracts. For other analyses, significant differences were established by Two-way ANOVA, followed by Tukey's multiple comparisons test, using GraphPad Prism version 6.05 for Windows. A difference was considered significant at $p < 0.05$.

III. Results

a) Extraction yields

The percentage yield of *Pterocarpus soyauxii* and *Pterocarpus santalinoides* aqueous-ethanol extracts was 6.63% and 5.61% respectively.

b) Estimation of polyphenol compounds

The total flavonoids content (TFdC) and total flavonols content (TFlC) of aqueous-ethanol leaf extracts of *P. soyauxii* and *P. santalinoides* is summarised in Table 1. The mean TFdC level was higher in *P. santalinoides* (1083.33 ± 35.12 mg/g) than *P. soyauxii* (730 ± 40 mg/g), while the TFlC level was higher in *P. soyauxii* (390 ± 60.83 mg/g) *than P. santalinoides* (260 ± 45.83 mg/g).

Table 1: Total flavonoids content (TFdC) and total flavonols content (TFlC)

Compounds	Concentration of rutin (mg/g)	
	P. soyauxii (PSO)	*P. santalinoides* (PSU)
Flavonoids (mg of RU/g of extract)	770	1120
	730	1050
	690	1080
Mean TFdC	730 ± 40	1083.33 ± 35.12
Flavonols (mg of RU/g of extract)	350	220
	360	310
	460	250
Mean TFlC	390 ± 60.83	260 ± 45.83

c) Reducing power activity of P. soyauxii and P. santalinoides leaf extracts

The reducing power of leaf extracts of *Pterocarpus soyauxii* and *Pterocarpus santalinoides* exhibited different degrees of electron donating capabilities, all in a concentration-dependent manner (Figure 3).

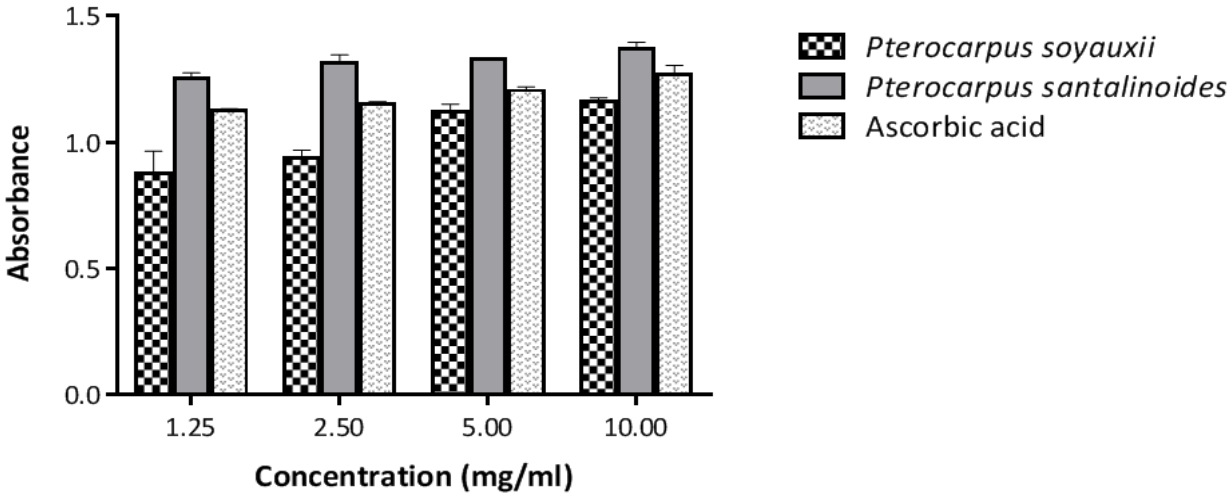

Figure 3: Reducing power of leaf extracts of *P. soyauxii* and *P. santalinoides* compared to ascorbic acid. The results are presented as mean ± SD

The reducing potential of extracts of *P. santalinoides* was significantly higher ($p < 0.05$) than that of *P. soyauxii*, as well as the standard compound (ascorbic acid) at all concentrations tested. The reducing potential of the tested compounds was greatest in *P. santalinoides,* followed by ascorbic acid and least in *P. soyauxii* (*P. santalinoides* > ascorbic acid > *P. soyauxii).*

d) Hydrogen peroxide scavenging activity of P. soyauxii and P. santalinoides leaf extracts

Hydrogen peroxide scavenging activity of aqueous-ethanol leaf extracts of *P. soyauxii* and *P. santalinoides* was observed to be concentration dependent (Figure 4). *P. soyauxii* exhibited the lowest scavenging activity at all concentrations tested, with an exception of the extracts at 10.00 mg/ml concentration, which had a higher percent inhibition of hydrogen peroxide (99.63 %) than the standard antioxidant compound at an equivalent concentration (99.23 %).

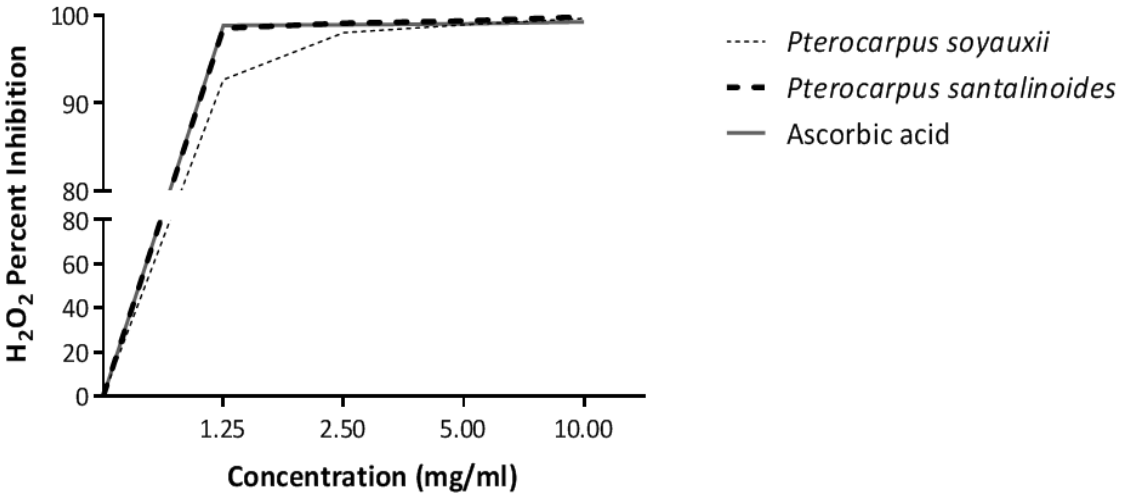

Figure 4: Hydrogen peroxide scavenging activity of *P. soyauxii* and *P. santalinoides* leaf extracts compared to ascorbic acid

Similar to its reducing power activity, the hydrogen peroxide scavenging activity of *P. santalinoides* was superior to that of *P. soyauxii,* as well as ascorbic acid, with percentage inhibitions of 98.50, 99.07, 99.33 and 99.80 at 1.25 mg/ml, 2.50 mg/ml, 5.00 mg/ml and 10.00 mg/ml respectively. The only exception of enhanced hydrogen peroxide scavenging activity of standard compound over *P. santalinoides* was for the starting concentration of 1.25 mg/ml, with a percent inhibition of 98.83 % as against *P. santalinoides* with percent inhibition of 98.50 % at similar concentration.

IV. Discussion

Plant secondary metabolites exert important functions in living plants. Flavonoids for instance, can protect against free radicals generated in plants [23]. High content of phenolics and flavonoids in medicinal plants have been associated with their antioxidant activities that play a role in preventing the development of chronic as well as age-related diseases, particularly caused by oxidative stress [6, 10, 24]. Preliminary phytochemical screening of *Pterocarpus soyauxii* and *Pterocarpus santalinoides* has revealed the presence of flavonoids in these plants [13, 14]. Estimation of polyphenols in this study revealed the presence of both flavonoids and flavonols in extracts of both *Pterocarpus* species. The total flavonoid content of aqueous-ethanol leaf extracts of *P. santalinoides* was significantly higher than that of *P. soyauxii* ($p = 0.0003$), while total flavonols concentration was higher in *P. soyauxii* than in *P. santalinoides*.

Flavonoids are well known for their antioxidant activity [8]. They are thought to exert their antioxidant activity by the mechanisms of radical scavenging and metal ion chelation to inhibit lipid peroxidation [4]. Several studies in recent years have shown that flavonoids, like other polyphenols in plants, scavenge reactive oxygen species and effectively prevent oxidative cell damage [1]. The activities of antioxidants have been ascribed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging [5, 21]. The reducing power of a compound may thus serve as an important marker of its possible antioxidant activity [21]. Reducing power of a plant extract correlates with phenolic constituents in the plant [10]. In this assay, the oxidation form of iron (Fe^{+3}) in ferric chloride is converted to ferrous (Fe^{+2}) through electron transfer ability by antioxidant compounds [10, 25]. The aqueous-ethanol extracts of *P. soyauxii* and *P. santalinoides* exhibited good reducing power activity at the different concentrations tested (Figure 3), however extracts of *P. santalinoides* showed a higher ferric reducing power than *P. soyauxii* and ascorbic acid at all concentrations tested. The observed higher ferric reducing activity of *P. santalinoides* over *P. soyauxii* may be attributed to its higher flavonoids content and possibly the presence of other bioactive compounds with antioxidant properties. Bothon *et al.* for instance, has reported the presence of coumarins in extracts of *Pterocarpus santalinoides* [15]. Coumarins are well established antioxidant compounds [26-28], hence their presence in *P. santalinoides* may potentiate the reducing power activity of these plants. The trend in the reducing power of extracts from *P. santalinoides* was similar to those of their hydrogen peroxide scavenging activities and the total flavonoids content, indicating that there is a correlation between the total flavonoids content and the antioxidant activities of plant extracts.

The ability of extracts of *P. soyauxii* and *P. santalinoides* to scavenge free radicals in vitro strongly suggests their antioxidant activity. Percentage inhibition of hydrogen peroxide (H_2O_2) by both extracts was comparable to that exhibited by ascorbic acid, a

standard antioxidant compound. In this study, this relationship was verified by the observation that both the total flavonoids composition and the H_2O_2 scavenging activity of species of *Pterocarpus* tested were in the order of *P. santalinoides* > *P. soyauxii*. Scavenging of H_2O_2 by plant extracts may be attributed to their phenolics and flavonoids which can donate electrons to H_2O_2, thus neutralizing it to water [29]. Although hydrogen peroxide is itself not very reactive, it is converted to highly reactive hydroxyl radicals by Cu^{2+} and Fe^{2+} ions, leading to lipid peroxidation, oxidative stress and cytotoxicity [30-32]. Thus, removing H_2O_2 throughout biological systems, particularly the human body, is very important.

V. Conclusion

Pterocarpus soyauxii and *Pterocarpus santalinoides* are shown to both be rich in flavanoid and flavonols compounds and exhibit potent hydrogen peroxide scavenging activity and ferric reducing capacity. This raises the possibility that phenolic-rich plants such as *Pterocarpus soyauxii* and *Pterocarpus santalinoides* could provide beneficial antioxidant effects in disease states characterized by oxidative stress conditions. Further in vitro and in vivo studies to validate the antioxidant potential of extracts of *Pterocarpus soyauxii* and *Pterocarpus santalinoides* are however suggested, to establish the potential drug candidacy of flavonoid and flavonols compounds from these plants.

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