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Phenolic contents, antioxidant activity and spectroscopic characteristics of *Pterocarpus angolensis* DC. stem bark fractions

Anokwuru CP[1], Sigidi MT[2], Zininga T[3], Tshisikhawe MP[4], Shonhai A[3], Ramaite IDI[1], TraoréAN[2] & Potgieter N[2,5]

[1]Chemistry Department, [2]Microbiology Department, [3]Biochemistry Department, [4]Botany Department, [5]Dean, School of Mathematical and Natural Sciences, University of Venda; Private Bag X5050; 0950 Thohoyandou, South Africa
E-mail:afsatou.traore@univen.ac.za

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Oxidative stress has been implicated in the damage of biological molecules resulting in aging and diseases such as Alzheimer, cancer, diabetes, cardiovascular disorders. The study aimed at determining the phenolic contents and antioxidant activities of *Pterocarpus angolensis* crude extract and fractions. The crude extract and fractions of *P. angolensis* were evaluated for their phenolic contents using Follin-Ciocalteu reagent. The antioxidant activities were evaluated using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and reducing power assays. Ultraviolet-Visible and Fourier transform infrared spectroscopy were used to assess the spectroscopic characteristics. We obtained 7 fractions from the crude extracts after column chromatography on silica gel 60. The results showed that fraction two (PaF2a) displayed the highest DPPH radical scavenging activity (18.7 µg/ml) but had the lowest phenolic content while fraction three (PaF2b) exhibited the highest reducing power activity (44.28 µg/ml) with high phenolic content. Spectroscopic details showed that PaF2a had maximum absorbance at 287.1 nm while PaF2b displayed maximum absorbance at 288.2 nm. The infra-red spectroscopy presented four main characteristic fingerprinting at 1606, 1518, 1444 and 1064 cm^{-1} as fingerprints for *P. angolensis*. There was a difference in the wave number at C==C and C-O vibrations between PaF2a and PaF2b. In conclusion, this study has shown that PaF2a and PaF2b are the antioxidant rich fractions of *P. angolensis* stem bark and exhibited different spectroscopic characteristics.

Keywords: Antioxidant activity, *P. angolensis* DC., Fractions, Spectroscopic characteristics, Reducing power
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Oxidative stress occurs when there is an imbalance between the generation of reactive oxygen species (hydroxyl radicals, singlet oxygens, hydrogen peroxides, superoxide anions) and the antioxidant defense system in favor of the former during metabolic processes[1]. The accumulation of the oxidative species lead todamage of biological molecules resulting in aging and diseases such as Alzheimer, cancer, diabetes, cardiovascular disorders[2-4]. Plant offers a wide variety of natural antioxidants which can protect either biomolecules from degradation or the endogenous antioxidants from depletion through the quenching of reactive oxygen species[5]. Phenolics are important secondary metabolites that are wide spread in plant kingdom[6]. They are good antioxidants and are able to counteract oxidative stress by acting as reducing agents, hydrogen donators, singlet oxygen quenchers and metal chelators[7-9].

*Corresponding author

Pterocarpus angolensis DC. belongs to the family of Fabaceae and is commonly knownas blood wood in English and *mutondo* in TshiVenda[10]. It is commonly used by traditional healers in the Venda region to treat malaria, venereal disease, pile, amenorrhoea, haematuria, bilharzia, headache, stomachaches, diarrhea, mouth sores and rashes[10-12]. Epicatechin and its derivatives isolated from ethanol crude extract of the bark have been reported to be active against *Staphylococcus aureus*[10]. Hydrodihydrochalcones, α-methyldeoxybenzoins and isoflavonoids have also been identified in the heart wood[13]. The aim of our study was to explore the antioxidant capacity and spectroscopic characteristics of *P. angolensis.*

Methodology

Materials

All materials and chemicals used for extraction, elution, chromatography and analyses were purchased from Merck (Darmstadt; Germany) or Sigma Aldrich (St Louis, MI; USA) or unless specified.

Description of the plant material

Stem Bark of *Pterocarpus angolensis* (MPT00118) was collected in 2014 at the Vhembe District of the Limpopo Province (South Africa). The collected plant was identified using its vernacular name and later confirmed by the taxonomic rank at the Department of Botany, University of Venda using its IPNI (*Pterocarpus angolensis* DC.).

Sample preparation

About 500 gm of dried samples of *P. angolensis* bark was soaked in 10 L dichloromethane: methanol (1:1) at room temperature for 24 hrs. Then, the extract was filtered and evaporated with a rotarvapor (Buchi, Switzerland) at 40 ºC to obtain 102 gm of crude extract. The crude extract (95 gm) was subjected to silica gel 60 column chromatography and was eluted with hexane and increasing polarity with ethyl acetate and finally methanol to obtain 7 fractions. Fraction 1 (*P. angolensis* Fraction 1; PaF1) was obtained from hexane: ethyl acetate (1:1), fraction 2 and 3 (PaF2a and 2b) were obtained from ethyl acetate: methanol (9:1), fractions 4-6 (PaF3a-c) were obtained from ethyl acetate: methanol (7:3) while fraction 7 (PaF4)was obtained from ethyl acetate: methanol (1:9).

Determination of total phenolic content (TPC)

The total phenolic content of the crude extract was estimated using a previously described method by Anokwuru *et al.*[14] with a slight modification. Briefly, the crude extract and fractions (1 mg/ml; 20 µl) were added to a 96 well microplatecontaining 80 µl of distilled water. Follin Ciocalteu reagent (10 %; 20 µl) was added to the diluted samples and was allowed to stand at room temperature for 1 min before $NaCO_3$ (7 %; 60 µl) was added to the mixture to stop the reaction. Distilled water (120 µl) was added for further dilution and samples were read in a microplate reader (Versa max, China) at 760 nm after 30 min of incubation at room temperature. Gallic acid was used as a standard for the calibration curve and the total phenolic content was expressed in mg Gallic Acid Equivalent (GAE)/ gm of the extract.

Antioxidant assays

Antioxidant activity was assessed using the Free radical scavenging and the Reducing power assays described below:

Free radical scavenging assay

Free radical scavenging activity was evaluated for the crude extract and fractions using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay using a previously described method by Anokwuru *et al.*[14]. Different concentrations (0.03-0.5 mg/ml; 100 µl) of *P. angolenesis* crude extract and fractions were exposed to DPPH radicals (0.3mM; 200 µl) in a 96-well plate. The mixture was left at room temperature for 30 min and absorbance was read at 517 nm using a microplate reader (Versa max, China). Gallic acid was used as a standard. The radical scavenging activity (RSA) of the crude extract and fractions was calculated using the following equation:

$$\% \text{ RSA} = [(A_{DPPH}-A_S)/A_{DPPH}] \times 100$$

Where, A_{DPPH} is the absorbance of the DPPH solution and A_S is the absorbance of the samples and DPPH. The ability of the extracts to inhibit 50 % of the free radical (IC_{50}) was extrapolated from a graph of % RSA against concentration.

Reducing power assay

The capacity of the crude extract and fractions of *P. angolensis* to reduce Fe^{3+} to Fe^{2+} was estimated by a slight modification of the method reported by Pereira *et al.*[8]. Different concentrations (0.03-0.5 mg/ml; 50 µl) of *P. angolensis* crude extract and fractions were mixed with sodium phosphate buffer (0.2M, *p*H 6.2; 50 µl) and 50 µl 1 % Potassium hexacyanoferrate [$K_3Fe(CN)_6$] aqueous solution. After 20 min of incubation at 45 °C, 50 µl of 10 % trichloro acetic acid (TCA) was added and 80 µl of the mixture was transferred into another 96-well plate containing deionized water (80 µl) and $FeCl_3$ (0.1 %, w/v; 16 µl) . Absorbance was measured at 690 nm using a microplate reader (Versa max, China). Gallic acid was used as a standard. The Effective Concentration ($EC_{0.5}$) value was obtained from a linear regression analysis of absorbance value plotted against concentration. The $EC_{0.5}$ value is the effective concentration of the extracts to give an absorbance of 0.5[14]. All extracts were testedin triplicate.

Ultraviolet-visible (UV-VIS) Spectroscopy

The UV-VIS spectra of the fractions were obtained using SP-8001 UV-VIS spectrophotometer (MetertechInc, Taiwan). The samples (1mg/ml) were dissolved in methanol then transferred to quartz cuvettes of 1cm path length and scanned (range of 190 – 500 nm).

Fourier transform infrared (FT-IR) Spectroscopy

Infrared spectra were collected after 32 scans on Alpha FT-IR spectrometer (Bruker Optics; Germany)

equipped with Alpha Platinum ATR (attenuated total reflection) single reflection diamond ATR module with spectra range: 4000 – 375 cm^{-1}.

Statistical analysis

Data were expressed as mean ± standard error (S.E). One way ANOVA test was used to analyse the difference between groups while Pearson correlation test was used for correlation analysis. All data analysis were performed using SPSS version 23.0.

Results

Fraction yield

The results in Fig. 1 shows the fraction yield after column chromatography on silica gel 60. The column chromatography of *P. angolensis* yielded 7 fractions (PaF1-4). PaF3b obtained from 70 % ethyl acetate yielded the highest followed by PaF2a obtained from 90 % ethyl acetate.

Total phenolic contents

The phenolic contents of the crude extract and fractions are shown in Table 1. The total phenolic content of the crude extract (PaCE) was comparable to those of the fractions except PaF2a which was significantly ($p < 0.05$) lower than the other fractions.

Antioxidant activity

The result of the DPPH radical scavenging activity (Table 2) showed that PaF2a exhibited the highest activity while PaF4 displayed the lowest activity. In the reducing power assay, PaF2b exhibited the highest reducing power while PaF2a exhibited the lowest reducing power.

UV-Visible spectra

The UV-Visible spectra of the fractions of *P. angolensis* (Table 3) showed that fractions PaF2a, 3a, 3b, 3c and 4 displayed maximum absorption at the wavelength of 287.1nm. Fraction PaF1 showed maximum absorption at 289.2 nm while fraction PaF2b had its maximum absorption at 288.2 nm. The spectra also showed that all the fractions with absorptions at 287.1 also had intensities of about 0.5 with fraction PaF2a exhibiting the least intensity. However, all the fractions with absorptions greater than 287.1 showed maximum absorption above 0.7.

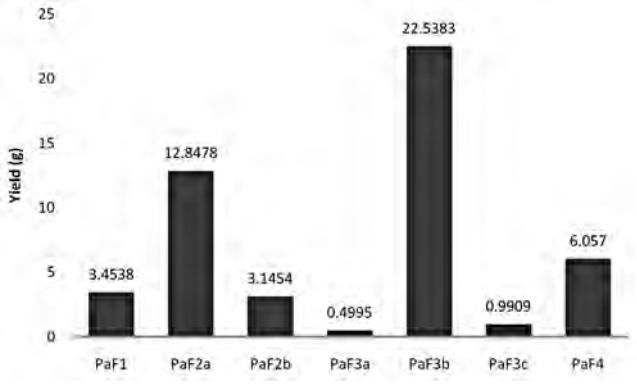

Fig. 1—Yield of *P. angol*ensis stem bark fractions (expressed as grams obtained)

Correlation analysis

The result of the Pearson correlation analysis is shown in Table 4. There was a positive correlation (r = 0.5) between TPC and DPPH activity and a

Table 1—Total phenolic content (mgGAE/gm) of the crude extract and fractions of *P. angolensis* stem bark

Extract/Fraction	TPC
PaCE*	6.74 ± 0.06^{a}
PaF1	6.53 ± 0.03^{a}
PaF2a	2.64 ± 0.1^{b}
PaF2b	6.64 ± 0.01^{a}
PaF3a	6.4 ± 0.04^{c}
PaF3b	6.64 ± 0.1^{a}
PaF3c	6.53 ± 0.02^{a}
PaF4	6.66 ± 0.03^{a}

*PaCE: *P. angolensis* crude extract. Data with different lower case letters in each column are significantly different ($p < 0.05$).

Table 2—Antioxidant activity (µg/ml) of *P. angolensis* fractions

Sample	Radical scavenging activity	Reducing power
PaCE	29.82 ± 0.63^{a}	79.73 ± 2.59^{a}
PaF1	$>1000^{b}$	$>1000^{b}$
PaF2a	18.70 ± 0.87^{c}	94.43 ± 1.17^{c}
PaF2b	23.79 ± 1.14^{d}	44.28 ± 0.17^{d}
PaF3a	21.71 ± 0.46^{e}	72.01 ± 0.69^{e}
PaF3b	20.06 ± 0.36^{e}	84.47 ± 1.3^{f}
PaF3c	24.31 ± 0.44^{d}	80.5 ± 0.64^{af}
PaF4	32.86 ± 0.17^{f}	81.37 ± 2.8^{af}
Gallic acid	7.32 ± 0.01^{g}	15.52 ± 0.03^{g}

*PaCE: *P. angolensis* crude extract. Data with different lower case letters in each column are significantly different ($p < 0.05$).

Table 3—UV characteristics of *P. angolensis* fractions (1mg/ml)

Fraction	Maximum wavelength (nm)	Intensity(A)
PaF1	289.2	0.708
PaF2a	287.1	0.505
PaF2b	288.2	0.766
PaF3a	287.1	0.583
PaF3b	287.1	0.575
PaF3c	287.1	0.509
PaF4	287.1	0.558

Table 4—Pearson correlation analysis of phenolic contents and antioxidant activity of *P. angolensis* fractions

Parameters	TPC	UV-VIS	DPPH	RP
TPC	1	0.39	0.5	-0.52
UV-VIS	0.39	1	0.06	-0.94**
DPPH	0.50	0.06	1	-0.15

** Correlation is significant at the 0.01 level

Table 5—Wavenumber of absorption bands (cm^{-1})

Fraction	C=O	C=C	C-O
PaF1	1711	1512, 1458	1379, 1246, 1078
PaF2a	-	1606, 1518, 1442	1379, 1280, 1064
PaF2b	-	1606, 1519, 1441	1374, 1281, 1057
PaF3a	-	1606, 1515, 1453	1394, 1250, 1066
PaF3b	-	1602, 1518, 1437	1374, 1250, 1044
PaF3c		1606, 1518, 1441	1378, 1282, 1065
PaF4	-	1601, 1517, 1437	1393, 1249, 1065

negative correlation (r = -0.52) between TPC and reducing power (RP). There was a significant ($p < 0.01$) negative correlation between UV-VIS intensity and reducing power. There was also a negative correlation (r = -0.15) between DPPH and RP.

FT-IR spectra

The FT-IR spectra of *P. angolensis* fractions (Table 5) showed four characteristic strong absorption bands at 1606, 1518, 1441 and 1065 cm^{-1}. Only PaF1 and PaF3a showed strong absorption bands between 2916 and 2848 cm^{-1}. PaF1 alone exhibited absorption peaks at 1711 cm^{-1}. There were differences between the wave numbers of PaF2a and PaF2b at the C=C and C-O vibrations.

Discussion

The protective effect of medicinal plants against diseases induced by reactive oxygen species has been attributed to the presence of polyphenols[15,16]. Our present study has investigated the antioxidant activities of the phenolic rich fractions of *P. angolensis* stem bark. The result of the fraction yield (Fig. 1) showed that PaF3b gave the highest yield. This showed that there were more compounds soluble in 70 % ethyl acetate 30 % methanol. The polarities of polyphenols range from non-polar to polar[17] due to the presence of different chemical characteristics[18]. Therefore, a particular solvent may not be efficient in extracting all types of phenolics[19]. Polar solvents like methanol are suitable for extracting polyphenols from plant matrix compared to ethyl acetate[15]. In our study, increasing the ratio of methanol to 30 % during the column chromatography increased the solubility of compounds in the solvent mixture and consequently increasing the yield of PaF3b. The high extraction yield of PaF3b did not translate to a high phenolic content compared to the other fractions (Table 1). It is possible that compounds in PaF3b are not predominantly phenolics. Plant antioxidants are beneficial to humans because they are useful in both medicine and food industry[20]. The antioxidant activity of *P. angolensis* stem bark was evaluated using DPPH free radical scavenging and reducing power assays. It is important to evaluate plant antioxidant activity using more than one assay because plants exhibit antioxidant activity through different mechanisms due to the variety of chemical compounds present[21]. The DPPH assay measures the ability of a sample to reduce the DPPH radical by donating the hydrogen atom which is monitored through the discolouration of the mixture from purple to yellow coloured diphenyl picrylhydrazine[22]. The result of our study demonstrated that PaF2a exhibited the highest free radical scavenging activity. This implied that compounds in PaF2a had higher ability to donate protons to the DPPH radicals. During the fractionation, PaF2a and PaF2b were obtained from 90 % ethyl acetate. However, the free radical scavenging activity of PaF2b was significantly lower ($p < 0.05$) than that of PaF2a.The ability of *P. angolensis* crude extract and fractions to reduce Fe^{3+}/ferricyanide complex to Fe^{2+} is shown in Table 2. In the assay, the change in the test solution from yellow to shades of green and blue indicates the reducing power of the compounds present[23]. Interestingly, the result of our study (Table 2) showed that PaF2b exhibited the highest activity. Surprisingly, PaF2a exhibited the lowest reducing power. Unlike the DPPH radical scavenging activity where all the fractions showed similar antioxidant capacity, PaF2b was the only fraction with a significant ($p < 0.05$) reducing power activity. To the best of our knowledge, this is the first report of the antioxidant activities of *P. angolensis.* The UV-Vis spectroscopy is a simple, cheap and easy-to use technique for identification and quantification of main phytochemicals[24]. In our study, this technique was used to determine the maximum absorption peaks of

the fractions and also measure the intensity of the peaks. We also wanted to compare the intensities obtained from each fraction with the Follin Ciocalteu reagent assay for any correlation. The single absorption peaks observed in the spectra of the fractions (Table 3) showed that the compounds have symmetrical chemical structures[25]. The absorption bands obtained in our study correspond to the transfer of the π-π* electrons in the benzene ring[26]. The compounds present could be flavanones because of the absorptions between 280 nm and 290 nm[25]. It is interesting to note that PaF2a and PaF2b displayed different characteristics although they were eluted from the same solvent mixture. PaF2b showed the highest intensity and also exhibited the highest reducing power suggesting a strong relationship between the phenolics present in PaF2b and the reducing power activity of the plant. This data also showed that the compounds present in both PaF2a and PaF2b have slightly different absorption characteristics. The estimation of the total phenolic content (Table 1) showed that PaF2a contained the least phenolic content. This agrees with the result of the UV-Vis spectroscopy in which PaF2a showed the lowest intensity (Table 3). The results obtained here suggest that peak intensity obtained from a UV-Vis spectrophotometer corresponds to the concentration of phenolics in the plant. Comparing the DPPH free radical scavenging activity and reducing power, the later assay appears to be a suitable method to quantify antioxidant compounds in *P. angolensis*. This observation was further substantiated with the correlation analysis (Table 4). Both TPC and UV-vis showed better correlation with reducing power assay compared to DPPH assay. Since IC_{50} and $EC_{0.5}$ values were used for the correlation analysis, a negative value would indicate a good relationship between the parameters evaluated. Therefore, phenolic compounds present in *P. angolensis* exhibit antioxidant activity through a reductive process compared to the donation of protons. In a study by Gonçalves *et al.*[27], reducing power assay gave better antioxidant activity compared to DPPH assay.

In the FTIR analysis, the functional groups in the fingerprint region (1800 to 650 cm^{-1}) of the spectra were used to distinguish the differences in the fractions (Table 5). Since the O-H stretching between 3400 and 3200 cm^{-1} was common for all fractions, this part was left out when interpreting the results[28,29]. The bands at 2916 and 2848 cm^{-1} found at PaF1 suggest the presence of aliphatic groups. This is a possible explanation for the poor antioxidant activity even when the fraction had high phenolic content and good intensity on the UV-Vis spectrophotometer compared to the crude extract and other fractions.

The fractions of *P. angolensis* showed strong C=O, C=C and C-O vibration bands in the fingerprint region.

C=O vibration

A carbonyl stretching band was observed only for PaF1 at 1711 cm^{-1} typical of phenolic esters[30,31]. It is reported that Carbonyl stretching (C=O) shows intense peak observed in the range of 1600-1800 (cm^{-1})[32]. Carbonyl group vibrations in the ketone are the best characteristic bands in vibrational spectra and also expected in the region of 1680 to 1715 cm^{-1}. The increase in intensity of the bands is affected by conjugation[33]. Although PaF1 showed the presence of carbonyl group, the presence of the aliphatic region (Table 5) could be responsible for the weak antioxidant activity observed.

C=C vibration

The fractions of *P. angolensis* showed characteristic bands around 1606, 1518 and 1442 cm^{-1} except for PaF1 which did not show any band at 1600 and the usual band at 1442 was shifted to 1458 cm^{-1}. In literature, the C-C stretching vibration in aromatic compounds is known to give bands in the region 1650 to 1430 (cm^{-1})[34]. In addition, the benzene ring has six stretching vibrations and four of those, occur with the highest frequencies near 1600, 1580, 1490 and 1440 (cm^{-1})[29]. The difference in the position of the C=C absorption bands could be as a result of the presence and location of additional functional groups[26].

C-O vibration

The three ethereal (C-O) stretch vibration bands observed near 1374, 1250 and 1064 cm^{-1} in all the fractions of *P. angolensis* show the presence of dihydroxyl phenyl ring of flavonoids as previously reported[30,35]. The peak around 1064 was the strongest of the three C-O vibration for all the fractions. However, each fraction showed a slight difference in the wave number (Table 5). Considering the antioxidant rich PaF2a and PaF2b, the slight difference in the wave numbers of the characteristic peaks could be responsible for the difference in the antioxidant activity of the two fractions.

FT-IR spectra of *P. angolensis* fractions have shown four main characteristic fingerprints at 1606,

1518, 1444 and 1064 cm^{-1}. These vibration bands can serve as a unique fingerprint for *P. angolensis*.

Conclusion

The resultsfrom this study demonstrated that fractions PaF2a and PaF2b are the antioxidant rich fractions with different antioxidant capacities. This suggests that *P. angolensis* exerts its antioxidant activity through different mechanisms. Four main absorption bands at 1606, 1518, 1444 and 1064 cm^{-1} could be characteristic fingerprints for *P. angolensis* stem bark. Furthermore, phenolics were responsible for the reducing power activity of the plant.Further biological testing and compound identifications are currently in progress.

Competing interest

There are no competing interests

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