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Analysis of phenolic compounds and evaluation of antioxidant activity of aqueous and hydromethanolic extracts of the leaves and bark of *Alstonia boonei* De wild

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Abstract
Oxidative stress is one of the precursors of a number of diseases. It can be eliminated by an external supply of antioxidants, which are sometimes difficult to obtain. We assessed the antioxidant power of extracts of *Alstonia boonei* De Wild using the DPPH method, and determined and characterised the phenolic compounds using UV/visible spectrophotometry and TLC. The aqueous and hydromethanolic extracts of the plant contain polyphenols with contents varying from 1163.87±5.12 to 2577.50±7.06 mg GAE/100 g DM, flavonoids: 142.72±8.19 to 1472.10±5.34 mg QE/100 g DM, condensed tannins: 4.507±0.041 to 161.963±0.35 mg CE/100 g DM and hydrolysable tannins: 22.07±1.60 to 305.95±0.91 mg GAE/100 g DM. TLC yielded compounds that could be flavonoid glycosides and proanthocyanidins. The antioxidant activity of these extracts gave 50% CIs ranging from 7.53 to 19.15 µg/mL. Extracts of the leaves and bark of *Alstonia boonei* De Wild are rich in phenolic compounds and have good antioxidant activity.

Keywords: *Alstonia boonei* De Wild, aqueous extract, hydromethanolic extract, phenolic compounds, antioxidant activity

Introduction
Oxidative stress, also known as oxidative pressure, is defined as the body's inability to defend itself against reactive oxygen species (ROS). It is due to an endogenous imbalance between these reactive oxygen species (oxidising molecules) and antioxidants (antioxidant molecules) [1]. In humans, oxidative stress is generated by internal factors such as mitochondrial damage, endothelial dysfunction, chronic inflammation, iron overload, etc.; external factors such as atmospheric pollution and irradiation; and lifestyle factors such as smoking, sun exposure, intense physical exercise and a diet low in fruit and vegetables [2-3]. Although less widely considered, overexposure to oxidative stress is the cause of numerous consequences for the body, including Alzheimer's disease, Parkinson's disease, congestive heart failure, kidney failure, cell ageing and damage to the immune system [4-5]. To combat this oxidation phenomenon, the body uses two defence systems: the endogenous system made up of enzymes, proteins and others; and the exogenous system, which consists of providing antioxidant compounds such as vitamins (C and E), flavonoids and polyphenols through food or medication [3-5, 7]. However, external antioxidant treatments are generally expensive and inaccessible to remote, low-income populations. Studies into the antioxidant activity of plants, which are readily available resources, could therefore facilitate access to antioxidants [8]. *Alstonia boonei* De Wild, an accessible plant widely used in Africa in the traditional treatment of a number of illnesses, could well be an alternative to the problems of inaccessibility to antioxidants for people in difficulty [9-11].

Materials and Methods
Materials
In addition to glassware and other equipment, we worked with the leaves and bark of *Alstonia boonei* De Wild, which were collected in MANIANGA, Arrondissement 7, Talangaï (Brazzaville - Congo) and then dried in the shade for one month. A specimen of this species is

registered in the National Herbarium of the Natural Sciences Research Institute (IRSEN) under reference number 15786.

Preparation of solutions

The decoctate, macerate and methanol-water extract (80:20) of the leaves and bark of *Alstonia boonei* De Wild were prepared by mixing 5 g of plant material in 100 mL of solvent, then the mixtures were heated separately for 15 minutes for the decocts and subjected to magnetic stirring for 30 minutes for the macerates and methanol-water extracts, then filtered.

Qualitative study of *Alstonia boonei* De Wild extracts

Extracts from the leaves and bark of *Alstonia boonei* De Wild were also separated by thin layer chromatography (TLC) in this study [12]. The various extracts prepared were deposited in spots on a silica plate, introduced into the chromatographic tank, dried after migration on a solvent system composed of ethyl acetate, methanol and water (10/15/5), a specific system for the separation of flavonoid glycosides and proanthocyanidins then developed with Diphenylborate Aminoethanol-PEG 400 (DPBA-PEG 400), a flavonoid reagent, and finally observed under ultra-violet/visible radiation at 365 nm [13].

Determination of phenolic compound

The phenolic compounds in the various extracts were assayed by the colorimetric method using a JENWAY-7205, UV-Visible spectrophotometer, and the operations were carried out in triplicate to ensure reproducibility and accuracy of the values.

Determination of total polyphenols

Total polyphenols were determined using a reagent that is reduced to a mixture of blue tungsten and molybdenum oxides during the oxidation of phenols: the Folin-Ciocalteu reagent. The method applied, has been used by several authors [14-16]. 500 µL of diluted solution of the various extracts of *Alstonia boonei* De Wild are mixed with 500 µL of Folin-Ciocalteu reagent and 400 µL of a 7.5% sodium carbonate solution. The mixtures were stirred and incubated in the dark at room temperature for 1 hour, followed by measurement of absorbance at 760 nm. Concentrations were determined from the calibration curve y=0.009555x+0.03426 with R^2=0.9959, plotted using gallic acid and then expressed as milligram equivalent of gallic acid per gram of dry plant matter [17].

Flavonoid assay

Flavonoids were assayed using the following method: 500 µL of each diluted extract was mixed with 500 µL of 10% $AlCl_3$ and incubated for 15 minutes at room temperature. The absorbances of the samples were measured at 430 nm. This method is used by several authors [12-14]. Flavonoid contents were determined using the calibration curve y=0.02121x+0.01128 with correlation R^2=0.9954, plotted with quercetin and then expressed in milligram equivalents of quercetin per gram of dry plant matter [17].

Determination of tannins

Condensed tannins were determined in the presence of vanillin in an acid medium, using the method proposed by Price in 1978, which is specific for flavon-3-ols, and hydrolysable tannins, by reaction with Ferric Chloride ($FeCl_3$), using the method described by Mole in 1987 [16;18-20].

Condensed tannins

500 µL of each dilute solution of *Alstonia boonei* De Wild extracts are mixed with 1000 µL of 1% vanillin solution prepared in methanol and 500 µL of concentrated hydrochloric acid (33%). The mixtures were placed in test tubes and heated in a water bath at 80 °C for 15 minutes, and the absorbances were measured at 500 nm. The condensed tannin content was calculated as a percentage using the formula: TC (%) = $(5.2.10^{-2}.A.V)/P$ and then reformulated as milligram cyanidin equivalent per gram of dry matter [21]. With TC: condensed tannins; $5.2.10^{-2}$: constant expressed as cyanidin equivalent; A: absorbance; V: volume of extract used; P: sample weight.

Hydrolysable tannins

500 µL of the various diluted solutions were added to 1500 µL of a solution prepared from 0.01 M ferric trichloride ($FeCl_3$) in methanol and 500 µL of concentrated hydrochloric acid (33%). After 15 seconds, the absorbances of the mixtures were measured at 660 nm. The hydrolysable tannin content was expressed as a percentage using the following formula: TH (%) = (A.M.V) / (E mole. P) then converted into milligram equivalent of gallic acid per gram of dry matter [22]. With TH: hydrolysable tannins; A: absorbance; E mole = 2169 of gallic acid (constant expressed in moles); M: mass = 300; V: volume of extract used; P: weight of sample.

Antioxidant activity of *Alstonia boonei* De Wild extracts

The antioxidant activity of the various extracts of *Alstonia boonei* De Wild was assessed qualitatively and quantitatively using the DPPH method. The principle of this method is to measure the scavenging of free radicals in a DPPH solution, which changes colour from violet to yellow in the presence of antioxidant compounds. This method was used by Takao in 1994 and by several authors [23-28].

Qualitative analysis by Thin Layer Chromatography

The decoctates, macerates and hydro-methanolic extracts of the leaves and bark are deposited in a spot on a TLC plate (silica gel 60 F 254), then eluted by a solvent system composed of ethyl acetate, methanol and water (10/15/5) and finally revealed by a DPPH solution of concentration 480 µg/mL prepared in methanol.

Kinetic study of DPPH reduction

Following the qualitative analysis, we monitored the rate of DPPH reduction using different concentrations (14, 28, 42, 56 and 70 µg/mL) of extracts from the leaves and bark of *Alstonia boonei* De Wild. The absorbance of the different solutions were recorded at 517 nm until the appearance of a plateau indicating the end of the reduction, in order to define the incubation time and determine the reactivity of the extracts as a function of time.

Quantitative analysis by spectrophotometry

Five (5) solutions of concentrations 14; 28; 42; 56 and 70 µg/mL of each extract were placed in triplicate in tubes with a DPPH solution of concentration 1300 µg/mL. After 30 minutes of incubation at room temperature and protected from light, we measured the absorbance at 517 nm and then determined the percentages of DPPH inhibition using the following relationship: %I=100 [1-(Abs (test) / Abs (control)]. The concentrations capable of inhibiting 50% of DPPH were determined from the regression lines.

Statistical analysis of results

The results of our study were analysed using GraphPad Prism 9.3.1, the latest version in 2022, with a precision of p-value < 0.0001.

Results and discussion

Extraction"

Extractions yielded 0.77 g of bark extract and 0.55 g of leaf decoctate extract. The leaf and bark macerates yielded 0.48 and 0.65 g extracts respectively. The hydromethanolic extracts of the leaves and bark of *Alstonia boonei* De Wild yielded 0.028 and 0.015 g. The bark decoctate and macerate had higher masses than the leaves, with the opposite result for the hydromethanolic extracts.

Analysis of aqueous and hydromethanolic extracts of *Alstonia boonei* De Wild

After several separation attempts with the system composed of ethyl acetate, methanol and water (10/15/5), the hydromethanolic extracts gave the best separation. TLC analysis of the extracts from the leaves and bark of *Alstonia boonei* De Wild gave the results shown in Figure 1. Figures 1.a and 1.b show the hydromethanolic extracts from the bark (1) and leaves (2), seen at 365 nm and then with the naked eye, after spraying with flavonoid reagent (DPBA-PEG 400).

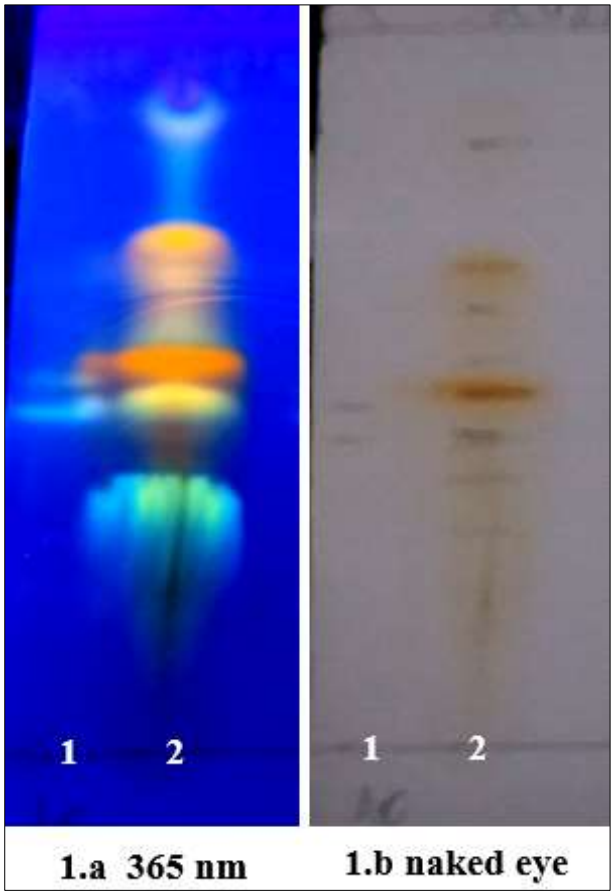

Fig 1: TLC profile of hydromethanolic extracts from the leaves and bark of *Alstonia boonei* De Wild

In Figure 1.a, positions 1 and 2, bearing the hydromethanolic extracts of the bark and leaves, show respectively three (3) spots for the bark with Rf values of: 0.20; 0.39; 0.43 and eight (8) spots for the leaves with Rf values of: 0.28; 0.34; 0.41; 0.46; 0.52; 0.60; 0.65 and 0.83 (Table 1). Figure 1.b shows two yellow spots in position 2 with frontal ratios of 0.52 and 0.65 and no spots visible in position 1. All these spots observed would be indicative of the presence of flavonoid glycosides and proanthocyanidins in the hydromethanolic extracts of the leaves and bark of *Alstonia boonei* De Wild [13]. The spots observed on the two extracts under UV/visible light show the difference in chemical composition and support the difference in yield between the hydromethanolic extracts of this plant.

Table 1: Frontal ratios of stains observed in hydromethanolic extracts of leaves and bark of *Alstonia boonei* De Wild

Organ	Solvent	RF	Observed colour
Bark	MeOH-H_2O	0.20;0.39;0.43	Blue
Leaves	MeOH-H_2O	0.28;0.41;0.46;0.52;0.65	Yellow and light yellow
		0.34;0.60	Brown
		0.83	Yellowish blue

Determination of polyphenolic compounds in extracts of *Alstonia boonei* De Wild: The contents of total polyphenols, flavonoids and tannins obtained after assay are given in Table 2.

Table 2: Phenolic compound content of *Alstonia boonei* De Wild extracts

Crude extracts	Plant part	Compound content			
		Total polyphenols (mg GAE/100 g DM)	Flavonoids (mg QE/100 g DM)	Condensed tannins (mg CE/100 g DM)	Hydrolysable tannins (mg GAE/100 g DM)
MeOH-Water	Leaves	2577.50±7.06	1472.10±5.34	9.98±0.65	305.95±0.91
	Bark	2524.05±5.92	869.10±6.40	4.51±0.04	264.09±1.92
Decocted	Leaves	2563.24±9.51	345.25±0.25	161.96±0.35	170.86±1.81
	Bark	3196.33±3.34	684.63±3.56	15.25±0.57	27.85±1.96
Macerated	Leaves	1163.87±5.12	142.72±8.19	61.35±6.23	22.07±1.60
	Bark	35.34±0.72	11.65±1.34	99.97±4.01	0.045±0.001

GAE: Gallic acid equivalent, **QE:** Quercetin equivalent, **CE:** Cyanidin equivalent and **DM:** Dry matter

This table shows that, of all the extracts assayed, it is the decocts and the hydromethanolic extracts of the leaves and bark of the plant that have the highest levels of total polyphenols, with contents of 2563.24±9.51 and 3196.33±3.34 mg GAE/100 g DM for the decocts, and 2524.05±5.92 and 2577.50±7.06 mg GAE/100 g DM for the hydromethanolic extracts. Similarly, flavonoids have contents of 345.25±0.25 and 684.63±3.56 mg QE/100 g DM for the decocts and 869.10±6.40 and 1472.10±5.34 mg QE/100 g DM for the hydromethanolic extracts. The levels of hydrolysable tannins were: 27.85±1.96 and 170.86±1.81 mg GAE/100 g DM in the decocted and 264.09±1.92 and 305.95±0.91 mg GAE/100 g DM in the hydromethanolic extracts. Unlike the first three chemical families, in terms of condensed tannins, decocted and macerated extracts dominated, with contents of: 15.25±0.57 and 161.96±0.35 mg CE/100 g DM in the decocts and:61.35±6.23 and 99.97±4.013 mg CE/100 g DM, for the macerates. The total polyphenol and flavonoid contents obtained with the hydromethanolic extracts of *Alstonia boonei* De Wild in this study are higher than 442.17±2.5 mg GAE/100 g DM and 783.23±3.26 mg QE/100 g DM, the respective contents of these families obtained by Obame in 2019 with the methanolic extract of the bark of this same species [29]. The bark decoctate showed a flavonoid content of 684.63±3.57 mg QE/100 g DM, higher than that of the same author, which is 462.40±6.78 mg QE/100 g DM [29]. The differences in content observed between the results of our study and those of the aforementioned author could be linked to the geographical location of the harvesting site and the harvesting periods, as the chemical composition of the plant may depend on this.

Antioxidant activity of extracts from the leaves and bark of *Alstonia boonei* De Wild

Qualitative analysis of antioxidant activity

Separation of the extracts of *Alstonia boonei* De Wild on plates with the ethyl acetate/methanol/water system and revelation with the DPPH solution (480 µg/mL) gave the results shown in Figure 2.

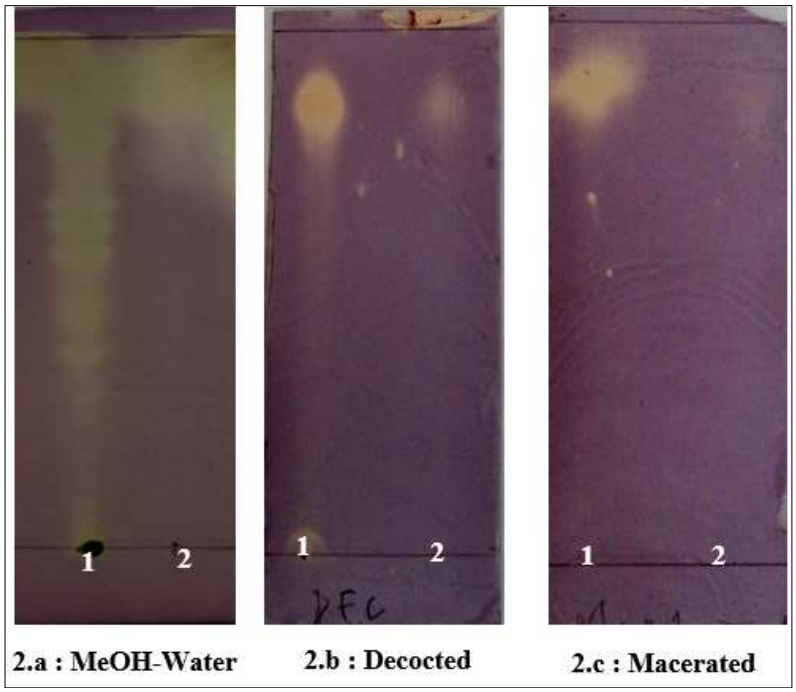

Fig 2: Antioxidant activity of *Alstonia boonei* De Wild extracts by TLC

Figure 2.a, showing the hydromethanolic extracts of leaves (1) and bark (2), gave eight (8) spots with the leaves and a single spot at the top for the bark. Figures 2.b and 2.c, showing the decocted and macerated leaves (1) and bark (2), showed one spot for each extract. The spots observed on these figures prove the presence of compounds or families of antioxidant molecules in the various extracts of *Alstonia boonei* De Wild. The number of spots observed on the hydromethanolic extract of the leaves would support the result of the TLC analysis of these extracts, which shows us approximately the same number of spots (8), likely to be flavonoid glycosides. This hydromethanolic extract of the leaves would contain more antioxidant compounds than the other plant extracts analysed. The stain observed with the

extracts that presented a single stain is indicative of one or more antioxidant compounds that were not separated by the solvent system used in the study.

DPPH reduction kinetics by extracts of *Alstonia boonei* De Wild

The kinetic study carried out on the different solutions, with a concentration of 28 µg/mL, of extracts of the leaves and bark of the plant with DPPH, gave the results shown in Figure 3. Observation of this figure shows that the curves evolve in three phases. From the first minute after mixing, there was a rapid and abrupt decrease in absorbance in all the extracts, but more significant with the hydromethanolic extracts of the leaves and bark. From the first minute to the seventh minute, the decrease becomes slight or even negligible, for all the solutions of the extracts studied. From the seventh to the fifteenth minute of the experiment, equilibrium levels were observed for all the curves. Apart from the curves for the decoctate and the bark macerate, which stabilised around absorbances of 0.2 and 0.3, all the curves for the remaining extracts stabilised around an absorbance of 0.1. This study enabled us to determine the duration of DPPH radical scavenging by the various extracts of *Alstonia boonei* De Wild and to support the efficacy of the hydromethanolic extract of the leaves, which is thought to be linked to the presence of more antioxidant compounds in this extract.

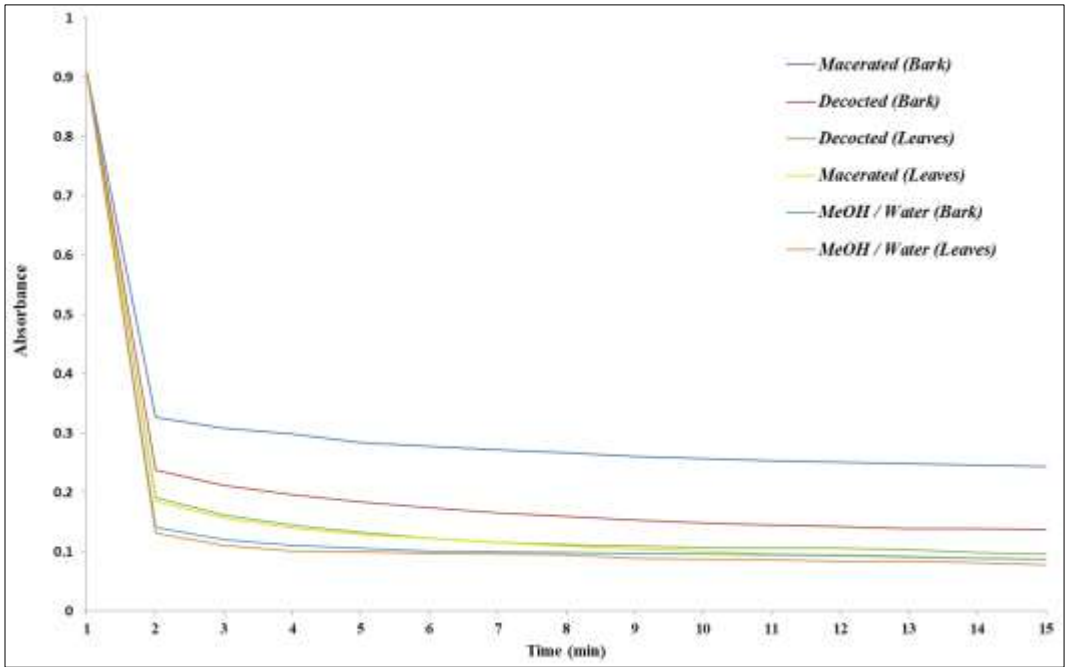

Fig 3: DPPH inhibition kinetics by extracts of *Alstonia boonei* De Wild

Trapping of the DPPH radical

Despite the observation of an equilibrium plateau after fifteen minutes following mixing of the extracts with the DPPH solution, we waited 30 minutes, as described in the work of other authors, to measure the absorbance and determine the inhibition percentages of our dilutions (Table 3) [25-28]. This table shows six (6) regression lines from the DPPH inhibition curves for macerated, decocted and hydromethanolic extracts of *Alstonia boonei* De Wild leaves and bark.

Table 3: Inhibitory concentrations of extracts of *Alstonia boonei* De Wild

Extract	Organ	Regression line	correlation	I.C.50 %
MeOH-Water	Leaves	Y = 0.73x + 44.54	0.9648	7.53 µg/mL
	Bark	Y = 0.72x + 44.10	0.9408	8.19 µg/mL
Decocted	Leaves	Y = 0.68x + 42.44	0.9701	11.14 µg/mL
	Bark	Y = 0.68x + 42.30	0.8898	11.24 µg/mL
Macerated	Leaves	Y = 0.62x + 43.51	0.9574	10.43 µg/mL
	Bark	Y = 0.73x + 35.94	0.9344	19.15 µg/mL

I.C50%:50% inhibitory concentration

The 50% inhibitory concentrations determined from the regression lines gave values of 19.15, 11.24 and 8.19 µg/mL respectively for the macerated, decocted and hydromethanolic extracts of the bark;10.43, 11.14 and 7.53 µg/mL for the macerated, decocted and hydromethanolic extracts of the leaves of *Alstonia boonei* De Wild. These contents allowed us to say that all these extracts gave low inhibitory concentrations (IC50%), evidence of very good antioxidant activities. The IC50% values for the various extracts are lower than those obtained by Obame for aqueous and methanolic extracts of *Alstonia boonei* De Wild, which are: 47.43±0.15 and 49.00±0.19 µg/mL [29]. Obiagwu's work on the methanolic extract of *Alstonia boonei* De Wild gave an antioxidant compound content higher than ours, at 141.67 μg/mL [30]. The low levels revealed by our study show a better antioxidant activity of the different plant extracts used. This study also proved that the hydromethanolic extract of the leaves remains the most active and could contain more antioxidant compounds than all the other extracts of *Alstonia boonei* De Wild analysed. The difference in IC50% between our study and those of the various authors could be linked to the difference in chemical composition of the extracts used in each case.

Conclusion

The antioxidant activity of *Alstonia boonei* De Wild was studied in relation to its use in the traditional treatment of several diseases in different African countries. This study enabled us to highlight the presence of phenolic compounds, such as:total polyphenols (1163.87±5.12 to 3196.33±3.34 mg GAE/100 g DM), flavonoids (142.72±8.19 to 1472.10±5.34 mg QE/100 g DM), condensed tannins (15.253±0.57 to 161.96±0.35 mg CE/100 g DM) and hydrolysable tannins

(22.07±1.60 to 305.95±0.91 mg GAE/100 g DM), in extracts from the leaves and bark of the plant. TLC analysis of the extracts of the leaves and bark of this plant led to the conclusion that the hydromethanolic extracts of these two parts of *Alstonia boonei* De Wild could contain flavonoid glycosides and proanthocyanidins. The plant extracts analysed showed very good antioxidant activity, with low 50% inhibitory concentrations ranging from 7.53 to 19.15 µg/mL. According to the results obtained from this study, *Alstonia boonei* De Wild is a potential source of polyphenols, flavonoids and tannins, some of which are antioxidants. The results of this study could provide a scientific basis for research into new phenolic compounds that could be used in pharmaceuticals, food, cosmetics, etc.

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