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Total Phenolic Content and Antioxidant and Antibacterial Activities of hydroethanolic extract from *Phragmanthera regularis* leaves

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Abstract
The species *Phragmanthera regulars* is a shrub that belongs to the family Loranthaceae and to the genus *Phragmanthera*. In Mozambique, this plant is used to treat respiratory and infectious diseases, however, scientific information regarding its phytochemical composition and pharmacological activity is scarce. In the present research, antioxidant capacity of hydroethanolic extract of the leaves of the *Phragmanthera regularis* was studied. The total phenolic and total tannins were quantified by the spectrophotometric methods of Folin-Ciocalteu. The total flavonoid content (TFC) were determined using aluminum chloride colorimetric method. The antioxidant capacity of the extracts was evaluated using reducing methods and free radical scavenging methods. The antibacterial activity was measured using the agar diffusion method against Gram-positive and Gram-negative bacteria. The extract showed relatively higher content of total phenols, tannins, and flavonoids. The antioxidant potential found for the extract was relatively higher than quercetin in the DPPH and phosphomolybdenum method and no significant differences were found for the FRAP ($p<0.05$). Regarding the antibacterial activity, the extract inhibited the growth of all test microorganisms with minimal inhibitory concentrations in the order of: *S. diarizonae* (30 µg/disc), *K. pneumoniae* and *S. epidermidis* (50 µg/disc)> *P. mirabilis*, *S. boydii*, *S. typhimurium* and *S. pyogenes* (375 µg/disc). The present study demonstrated that *P. regularis* leaves can be a good source of polyphenols, which can be useful in preventing diseases where free radicals are involved. In addition, the results demonstrate that the leaves can be a good antibacterial agent responsible for gastrointestinal and respiratory infections in humans.

Keywords: *Phragmanthera regularis*, total phenolics, total flavonoids, antimicrobial activity, antioxidant activity.

Introduction
Mozambican flora, as is the case in most tropical countries, is immensely rich in plant diversity, including medicinal plants. Some of these plants are known since ancient times and their active components have already been isolated and studied to determine their therapeutic efficacy (Cioffi et al., 2004; Tikhomiroff and Jolicoeu, 2002).
Study of medicinal plants for treatment of various diseases is still a priority in Mozambique, and research in this area is growing continuously, particularly in last years, as recognition of their potential in the delivery of solutions for health maintenance or disease treatment, especially in rural areas.
Various plants have been used to treat or reduce the impact of diseases like diarrhea, malaria, respiratory infections, erectile dysfunction, anemia, mental disorders and rheumatism. Bandeira et al. (2011) refer particularly to native species like *Momordica balsamina*, used to treat nausea and vomiting; *Rauvolfia caffra*, used in the treatment of malaria and opportunistic infections in HIV infected people, and also as antihypertensive agent due to the presence of reserpine; *Prunus africana* e *Hypoxis hemerocallidea*, used in treatment of prostate hypertrophy.
Research focuses especially on the study of phytochemical and bioactivity of plant extracts and pure substances isolated from plants, aiming to find alternative solutions for various health problems. Degenerative diseases caused by oxidative stress (Alzheimer's disease,

Parkinson's disease, cancer and other neurodegenerative diseases) and other infectious diseases caused by bacteria fungi and viruses (respiratory and gastric diseases) are receiving hereby a special attention. Carpinella et al. (2010) studied 73 native plants from Argentina and found that organic extracts of *Achyrocline tomentosa*, *Eupatorium viscidum*, *Ruprechtia apetala*, *Trichocline reptans* and *Zanthoxylum coco* present a significant inhibition of acetylcholinesterase. Mathew and Subramanian (2014) studied methanolic extracts of 20 plants used in the Indian ayuverdic medicine system, with a potential benefic effect on cognitive functions and found that *Emblica officinalis*, *Nardostachys jatamansi*, *Nelumbo nucifera*, *Punica granatum* and *Raulfia serpentina* show a significant inhibition of acetylcholinesterase. Evaluation of the antioxidant activity of these species by the DPPH method showed better efficacy for *Terminalia chebula* and *Emblica officinalis*, with EC_{50} values of 10 mg/mL. Results of the evaluation of the antimicrobial activity of extracts of these plants show a potential in promotion of cell wall disruption, induction of the production of reactive oxygen species, inhibition of biofilm formation, inhibition of microbial DNA replication and release of bacterial toxins to the host (Mickymaray, 2019). Authors describe further a proven antibiotic efficacy of significant part of studied plant extracts.

Results obtained inspire new studies with the aim of searching for new active components against clinical important diseases, especially in the context of resistance of pathogens to conventional antibiotics. In this context we carried out present research, aiming to study the antioxidant and antibacterial activities of hydro-ethanolic extracts from *Phragmanthera regularis* leaves. Species of the *Phragmanthera* genus are widely used for the treatment of diseases of the respiratory system, gastrointestinal diseases, hypertension, diabetes and inflammatory diseases (Ameer et al., 2010; Islam et al., 1994).

Phragmanthera regularis is used in traditional medicine in Mozambique in the treatment of respiratory and inflammatory diseases. It is basically administered as an infusion of leaves in boiling water. Doses recommended by practitioners of traditional medicine is a ¼ of a 250 mL glass two times a day for adults, while children take a tea spoon two times a day. Research carried out included a preliminary and quantitative phytochemical analysis (total phenols, total flavonoids and total tannins) and the antioxidant and antibacterial activity of hydroethanolic extracts of the leaves.

Materials and methods

Sample preparation and extraction

Fresh leaves of *Phragmanthera regularis* were collected in Manhiça district, Maputo Province in May 2019. The samples were authenticated with botanist of Instituto Nacional de Investigação Agronómica (IIAM), Maputo, Mozambique. The Crude extract was prepared using aqueous ethanol (80%) by maceration. The solvent was removed under reduced pressure and the dry extract was kept at 5º Celsius until phytochemical and biological tests were carried out.

Phytochemical contents

Preliminary phytochemical analysis. Preliminary phytochemical screening involving chemical tests to determine the presence of alkaloids (Dragondroff and Mayer's test), flavonoids (Shinoda, Alkaline reagent test and Lead acetate test), tannins (Lead acetate test and Gelatin test), phenols (Ferric chloride test), steroids and triterpenes (Salkowski's test), anthraquinones (Borntrager's test and Ammonium hydroxide test) and coumarins (NaOH paper test) were carried out using the methods described by Shaikh and Patil (2020).

Total phenols and total tannins. The content of total phenolics (TPC) and total tannins in the extract was determined using the Folin-Ciocalteu procedure adopted by Jimoh et al. (2019) and Amorim et al. (2012). The extract sample was evaluated at a final concentration of 50 µg/mL. The equation obtained from the calibration curve of gallic acid or tannic acid was used to extrapolate the content of phenolics and total tannins and expressed in mg/g of equivalent gallic acid and mg/g equivalent tannic acid respectively.

Total flavonoids. The total flavonoids content (TFC) was determined using the aluminum chloride complexion method adopted by Phuyal et al. (2020). Extract sample was evaluated at final concentration of 100 µg/mL. Total flavonoid content was expressed as quercetin equivalent (mg/g) using the equation obtained from the calibration curve.

Antioxidant assay

To determine antioxidant activity, different concentrations of hydro-ethanolic extract were prepared in methanol. Six common tests for measuring antioxidant activity were applied to measure the antioxidant capacity of hydroethanolic crude extract of *Phragmanthera regularis* leaves: (1) DPPH (2,2-diphenyl-1-picrylhydrazyl) Radical Scavenging Assay, (2) ABTS Radical Scavenging Assay, (3) Ferric Reducing Antioxidant Power (FRAP) assay, (4) Phosphomolybdenum Antioxidant Assay and (5) Reducing Power Assay. Estimations were made against standard antioxidant compounds such as quercetin and ascorbic acid.

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. The antioxidant activity of the extract of *Phragmanthera regularis* leaves was determined by DPPH methods described by Alimi and Ashafa (2017), with some modifications. In brief, 1.8 mL of a 120 µM DPPH (dissolved in methanol) was added to 200 µL of different concentrations (50– 150 µg/mL) of the extract or quercetin (*A*) at room temperature for 30 minutes. The standard solution (*Ao*) was obtained by the 1.8 mL mixture of DPPH with 200 µL of methanol. The absorbance of solutions resulting was measured at 516 nm using a UV-vis spectrophotometer (Shimadzu 1601) at 695 nm. The percentage of DPPH radical elimination was calculated using equation 1.

$$I\ (\%) = \frac{A_o - A}{A_o} x100 \quad (1).$$

2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS radical scavenging ability. This test was conducted using the procedure described by Jimoh et al. (2019). The ABTS radical was produced by reaction of equal volumes of 2.45 mM of potassium persulfate and 7 mM ABTS (dissolved in methanol). The resulting solution was incubated at room temperature for 16 hours in the absence of light. The resulting solution was diluted with methanol until an absorbance of 0.700 ± 0.005 was attained at 734 nm. Exactly 200 µL of the various dilutions of extract or

quercetin (50–150 µg/mL) was added to 1.8 mL $ABTS^+$ solution at room temperature. After 15 min, the absorbance of solutions resulting was measured at 734 nm using a UV-vis spectrophotometer (Shimadzu 1601) at 695 nm. The percentage of $ABTS^+$ radical elimination was calculated using equation 1.

Phosphomolybdenum inhibition or total antioxidant capacity (TAC) assay. The total antioxidant activity (TAA) of the extract or quercetin was determined by the methodology described by Prieto et al. (1999), with some modification. In briefing, equal volumes (1000 µL) of extract or quercetin solutions (50-150 µg/mL) was combined in a test tube with 1000 µL of phosphomolybdic acid solution. The reaction was carried out at 95 ° C for 90 minutes in water bath. The tubes were cooled and the volumes were adjusted to 10 mL with distilled water. The absorbance of the solutions (*As*) was read in the UV-vis spectrophotometer at 695 nm. The ascorbic acid solution (*Ao*) (150 µg / mL) was used as standard. The percentage inhibition of the tested samples was thus calculated as:

$$TAA\ (\%) = \left(1 - \frac{A_0 - A_s}{A_0}\right) x100 \quad (2)$$

Antioxidant capacity by Ferric reducing power assay. The FRAP assay was determined using the methodology described by Benzie and Strain (1996) and the Ferricyanide complex reducing (FCR) of samples was investigated by method described by Jemli et al. (2016) with some modification. In briefing, for FRAP assay, equal volumes (200 µL) of plant extract or quercetin standard drugs of different concentrations (50-150 µg/mL) or 200 µL of ascorbic acid solution (150 µg/mL) were reacted with 1.8 mL of fresh FRAP reagent. After incubation at 37º C (water bath) for 30 min, the absorbance of the resulting solutions was read at 593 nm. For FCR, 1 mL of each sample (85-255 µg/mL) were mixed with 2.5 mL of 200 mM Na_3PO4 buffer solution (pH 6.6) and 2.5 mL of potassium ferricyanide (10 g/L). The reaction was finished mixing 2.5 mL of 10% (w/v) $Cl_3CHCOOH$ followed by centrifugation at 1000 rpm for 10 min. After that, 2.5 mL of supernatant (10-30 µg/mL) was mixed with 2 mL deionized water and 500 µL of 0.1% of ferric chloride. The absorbance of the resulting solution was read at 700 nm. Ascorbic acid solution was used with reference and the ferric reducing (in percentage) of the tested samples was thus calculated using equation 2.

Antimicrobial activity

The Antibiotic activity was assessed against Gram-negative bacteria: *Proteus mirabilis* (ATCC 25933), *Klebsiella pneumoniae* (ATCC 700603), Shigella boydii (ATCC 9207), *Salmonella diarizonae* (ATCC 12325), Salmonella typhimurium (ATCC 14028) and Gram-positive: *Staphylococcus epidermidis* (ATCC 12228) and *Staphylococcus pyogenes* (ATCC 19615) following the methodology described by Cumbane and François (2017), with some modifications. These standard strains were obtained from the Laboratory of Microbiology of Centro de Investigação de Saúde de Manhiça (CISM)-Mozambique. All tests were made in three repetions and strains in which the mean diameter of the inhibition zone was equal to or greater than 8 mm were considered sensitive.

Statistical analysis

All data were analyzed using ANOVA test and means of significant differences were separated using Student t test at the 0.05 level of probability. Linear regression was performed using GraphPad Prism® (Version 7.0) to find dose-response curves in antioxidant activity.

Results and Discussion

The preliminary phytochemical essay shows the presence of tannins, steroids, alkaloids and flavonoids (table 1), while quantification of total phenols, total flavonoids and total tannins show the results presented in table 2. Results of the antioxidant activity obtained with different methods are presented in tables 3 to 7.

Table 1: Preliminary phytochemical screening

Phytochemical	Extract
Tannins	+
Steroids and triterpenes	+
Alkaloids	+
Flavonoids	+
Coumarins	-
Anthraquinones	-
Phenols	+
(+) Present e (-) Absent	
(mg AGE/g DE) – mg of gallic acid equivalents per gram of dry weight; (mg QE/g DE) - mg of quercetin equivalents per gram of dry weight; (mg TAE/g DW) - mg of tannic acid equivalents per gram of dry weight.	

Table 2: Total phenolic compound content

Total phenols	Total Flavonoids	Total tannins
(mg of AGE/g DW)	(mg of QE/g DW)	(mg of TAE/g DW)
72.310 ± 2.610	48.110 ± 3.00	46.990 ± 0.97
(mg AGE/g DE) – mg of gallic acid equivalents per gram of dry weight; (mg QE/g DE) - mg of quercetin equivalents per gram of dry weight; (mg TAE/g DW) - mg of tannic acid equivalents per gram of dry weight.		

Antioxidant activity shows an increasing tendency within concentration range studied in this research. Extracts present at lower concentrations better values of the antioxidant activity, when compared to quercetin, but at higher concentrations (normally between 10.0 and 15.0 µg/mL) better results were obtained for quercetin.

Following behavior observed in Cumbane et al. (2020), results of the antioxidant activity obtained by the DPPH, FRAP and Phosphomolybdenum methods, tested at same concentrations (5.0-15.0 µg/mL), where used to test the eventual correlation between results from different methods. Results show a good correlation, with some small deviations that can be attributed to the experimental error of measured values.

Although widely accepted we noticed some discrepancies when analyzing correlations between total phenolic content, on one side, and total flavonoid, on the other side, with antioxidant activity. Total phenolic content show in general a strong correlation and some authors see total phenolic content as the main contributor for the antioxidant activity (Ahmed et al., 2019; Fu-Xiang et al., 2019; Mwamatope et al., 2019; Pandey et al., 2018). Correlation between total flavonoid and antioxidant activity show a stronger deviation from the expected linear relation between both quantities, as seen on the attempt to correlate results obtained by Mahboubi et al. (2013) and Aryal et al. (2019).

Table 3: DPPH radical elimination (%)

Conc. (µg/mL)	Extract	Quercetin
5.00	54.780 ± 0.590	46.070 ± 1.100
7.50	63.030 ± 0.920	59.990 ± 0.560
10.0	66.080 ± 0.410	72.160 ± 0.830
12.5	72.160 ± 0.830	82.610 ± 0.390
15.0	76.950 ± 0.530	91.310 ± 0.570

Table 4: $ABTS^+$ radical elimination (%)

Conc. (µg/mL)	Extract	Quercetin
5.00	54.320 ± 0.460	30.340 ± 1.160
7.50	59.080 ± 0.120	51.610 ± 0.970
10.0	67.630 ± 0.520	68.210 ± 0.780
12.5	72.050 ± 1.200	85.120 ± 0.170
15.0	80.140 ± 0.390	99.830 ± 0.530

Table 5: The total Antioxidant activity (TAA) (%)

Conc. (µg/mL)	Extract	Quercetin
5.00	8.720 ± 0.250	8.610 ± 0.180
7.50	13.070 ± 0.090	14.000 ± 0.277
10.0	16.940 ± 0.640	17.400 ± 0.250
12.5	21.280 ± 0.540	20.690 ± 0.110
15.0	27.160 ± 0.480	23.450 ± 0.170

Alam et al. (2021) and Ahmed et al. (2019) accept that the large variety of phenolic compounds present, with different structures and functional groups, and their complex chemical composition may affect correlation. Additionally, different reaction of the reagents used in the different methods with the compounds responsible for antioxidant activity may affect specificity of the method and the correlation, since each component may have a specific contribution (Alam et al., 2021).

Table 6: The Ferric Reducing Antioxidant Power (FRAP) (%)

Conc. (µg/mL)	Extract	Quercetin
5.00	36.140 ± 1.180	28.440 ± 0.780
7.50	39.910 ± 1.520	36.900 ± 2.150
10.0	47.860 ± 0.780	46.160 ± 1.280
12.5	51.710 ± 1.300	54.830 ± 1.440
15.0	60.130 ± 1.530	63.630 ±1.560

Table 7: Ferricyanide complex reducing power (FCRP) (%)

Conc. (µg/mL)	Extract	Quercetin
5.00	10.390 ± 0.130	24.550 ± 0.470
7.50	11.940 ± 0.360	38.370 ± 1.270
10.0	14.010 ± 0.230	47.070 ± 1.040
12.5	16.600 ± 0.33	56.540 ± 1.880
15.0	19.120 ± 0.260	64.970 ± 1.280

Table 8: Half maximal effective concentration (EC_{50}) in µg/mL

	DPPH	ABTS	TAC
Extract	2.24 ± 0.02^a	3.56 ± 0.02^a	28.28 ± 0.35^a
Quercetin	5.48 ± 0.08^b	7.53 ± 0.02^b	32.80 ± 0.14^b

Cont.	FRAP	FRCP
Extract	11.13 ± 0.16^a	50.22 ± 0.94^a
Quercetin	11.14 ± 0.12^a	10.93 ± 0.02^b

Each value in the table is represented as mean ± SE (n = 3). The values in the same column that do not share the same letter are statistically different ($p<0.05$).

Effective concentration, expressed as EC_{50} in µg/mL, show significant differences between extract and quercetin for the DPPH, Phosphomolybdenum, Ferric reducing power, while results from FRAP method show no significant differences between extract and quercetin (Table 8). Further research is needed to better understand this irregular trend observed.

Table 9: Antibacterial activities

Dose (µg) / Bacteria	30	50	375
	Inhibition zone diameter (mm)		
P. mirabilis	-	-	**11.20 ± 0.40^a**
K. pneumoniae	-	**9.20 ± 0.50^a**	16.30 ± 0.10^b
S. boydii	-	-	**12.80 ± 0.50^a**
S. diarizonae	**8.50 ± 0.40^a**	**10.40 ± 0.40^b**	18.40 ± 0.60^c
S. typhimurium	-	-	**12.50 ± 0.10^a**
S. epidermidis	-	**7.90 ± 0.50^a**	16.00 ± 0.60^b
S. pyogenes	-	-	**9.600 ± 0.40^a**

Cont.

Dose (µg) / Bactéria	500	1000
	Inhibition zone diameter (mm)	
P. mirabilis	-	-
K. pneumoniae	-	**9.20 ± 0.50^a**
S. boydii		-
S. diarizonae	**8.50 ± 0.40^a**	**10.40 ± 0.40^b**
S. typhimurium	-	-
S. epidermidis	-	**7.90 ± 0.50^a**
S. pyogenes	-	-

Each value in the table is represented as mean ± SE (n = 3). The values in the same line that do not share the same letter are statistically different ($p<0.05$). The values in bold are the inhibition diameters corresponding to the minimal inhibitory concentration (MIC) of the extract.

Results from the determination of antibacterial activity (Table 9) show a regular pattern. *P. mirabilis* ATCC 25933, *S. boydii* ATCC 9207, *S. typhimurium* ATCC 14028 and *S. pyogenes* ATCC 19615 show no significant differences while, on the other side, *K. pneumoniae* ATCC 700603 and *S. epidermidis* ATCC 12228 show similar results with no significant differences among them. This pattern has been observed for the different doses (375, 500 and 1000 µg). Although there is a significant difference between results of a certain microorganism at different doses, groups of microorganisms show a similar behavior within a certain dose. Statistical comparison of results has not been carried out by 30 and 50 µg due to a lack of results of antibacterial activity of some microorganisms at both doses.

Conclusions

The contents of total phenolics, tannins and flavonoids, antioxidants and the antibacterial activities of *P. regularis* leaves extract were estimated using well-established conventional methods. Significant amounts of phenolic, tannin and flavonoid content were found in the extract. Likewise, the results of antioxidant and bacterial activity

also showed significant activity.

Declaration of interest

The authors guarantee there are no conflicts of interest and therefore are the only ones responsible for the content of this article.

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