Revista Virtual de Química
ISSN 1984-6835

Artigo

Evaluation of Phenolic Compounds in Rhizomes of Three *Renealmia* L. f. Species: Quantification, Antioxidant Activity and Histolocalization

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Rev. Virtual Quim., **2019**, *11* (5), 1625-1634. Data de publicação na Web: 31 de outubro de 2019

http://rvq.sbq.org.br

Avaliação das Substâncias Fenólicas em Rizomas de Três Espécies de *Renealmia* L. f.: Quantificação, Atividade Antioxidante e Histolocalização

Resumo: A histolocalização das substâncias fenólicas foi feita por técnicas usuais em Anatomia Vegetal. A determinação do teor de fenóis totais e do teor de flavonoides foram realizadas por métodos espectrofotométricos. A atividade antioxidante foi avaliada pelos métodos DPPH, ABTS e FRAP. Todos os extratos avaliados apresentaram capacidade antioxidante; entretanto, os melhores resultados foram para os extratos de *R. nicolaioides*. Correlação positiva foi encontrada entre o conteúdo total de fenólicos e os ensaios de capacidade antioxidante. Os resultados são inéditos para o gênero em questão e contribuem para novas pesquisas sobre substâncias fenólicas e suas atividades antioxidantes.

Palavras-chave: Fénois totais; flavonoides; radicais livres; histoquímica; órgãos subterrâneos; Zingiberaceae.

Abstract

The histolocalization of phenolic compounds in *Renealmia* was performed by usual techniques in plant anatomy. Determination of total phenol content and flavonoid content were performed by spectrophotometric methods. Antioxidant activity was evaluated by DPPH, ABTS and FRAP methods. All the evaluated extracts had antioxidant capacity; however, the best results were for extracts of *R. nicolaioides*. We found a positive correlation between total phenolic content and antioxidant capacity assays. The results are unprecedented for the genus focused and contribute to further research on phenolic compounds and their antioxidant activities.

Keywords: Flavonoids; free radicals; histochemistry; total phenols; underground organs; Zingiberaceae.

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DOI: 10.21577/1984-6835.20190114

Volume 11, Número 5 Setembro-Outubro 2019

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ISSN 1984-6835

Avaliação das Substâncias Fenólicas em Rizomas de Três Espécies de *Renealmia* L. f.: Quantificação, Atividade Antioxidante e Histolocalização

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Recebido em 30 de setembro de 2019. Aceito para publicação em 30 de setembro de 2019

1. Introduction

Phenolic compounds are constituted by one or more aromatic nuclei containing hydroxylated substituents and/or their

functional derivatives, and the flavonoids belong to this group.[1] Plant polyphenols are multifunctional and can act as antioxidants by removing or inactivating free radicals. This activity consists of the donation of hydrogen atoms, present in the phenolic hydroxyls, or the performance of these substances as chelating agents/metal ion hijackers, inhibiting lipid oxidation.[2-4]

The side effects produced by synthetic antioxidants have generated a search for natural and less toxic compounds, especially those of derived from plants. Protecting cells against oxidative damage may prevent chronic diseases, such as cancer and cardiovascular diseases.[5]

Renealmia L.f. belongs to the family Zingiberaceae, subfamily Alpinioideae and Alpinieae tribe, and presents approximately 75 species distributed through tropical regions of the Americas and Africa.[6,7] *Renealmia* is the only native genus in Brazil, and is represented by 21 species distributed throughout all phytogeographic domains, and most of these species are found in Amazonia.[8] Most of these species are used as ornamental, medicinal and/or food.[9]

Anatomical and histochemical studies detected phenolic compounds were in the cortical region and central cylinder in rhizomes of ten species of Zingiberaceae.[20]

Some studies involving the identification of phenolic compounds in the rhizomes and the antioxidant activity of different extracts have been performed for the Zingiberaceae family, but more has included *Renealmia* species.[11-20] The total phenolic content and antioxidant capacity of leaf methanolic extracts belonging to five species of *Etlingera* were quantified, and a positive correlation was found between these variables.[12] The same was observed for leaf extracts and rhizomes of *Alpinia, Boesenbergia, Curcuma, Kaempferia* and *Zingiber*.[15,17,18,20] Phytochemical analysis and antioxidant activity of aqueous and ethanolic extracts of leaves detected flavonoids, saponins, tannins and steroids from *Amomum muricarpum* Elmer, *Etlingera philippinensis* (Ridl.) R. M. Sm. And *Hornstedtia conoidea* Ridl.[19] The antioxidant activity, evaluated by the DPPH method, was slightly better for leaf extracts (EC_{50} 0.25 - 0.93 $mg.mL^{-1}$) when compared to rhizome extracts (EC_{50} 0.26 - 1.82 $mg.mL^{-1}$).[19]

This work aims to determine total phenolic and flavonoid content and assess the antioxidant capacity of methanolic extracts of rhizomes from three species of *Renealmia*, as well as the histolocalization of the phenolic compounds in this organ.

2. Materials and Methods

2.1. Plant material

Renealmia species were collected at the Southeastern and Northern regions of Brazil. *Renealmia chrysotricha* Petersen were collected at the Parque Nacional de Itatiaia (22º27'54"S 44º46'11"W), Rio de Janeiro. *Renealmia breviscapa* and *R. nicolaioides* were collected at the Acre State, in the following areas: Fazenda Experimental Catuaba (10°4'40"S 67°37'35"W) and Reserva Florestal Humaitá (9°45'17"S 67°40'15"W). The material was identified and deposited in the Herbarium of the Universidade Federal Rural do Rio de Janeiro (RBR) voucher 33416 for *R. chrysotricha*, and in the Herbarium of the Universidade Federal do Acre (UFACPZ) vouchers 6645 and 6646 for *R. breviscapa* and *R. nicolaioides*, respectively.

2.2. Histochemical tests

Histochemical tests were performed on freehand sections of rhizomes to detect phenolic compounds. The reagents used were 10 % potassium dichromate and Hoephner-Vorsatz.[21,22] Freehand sections not exposed to specific reagents were used as controls. The tests were performed in triplicate.

2.3. Preparation of extracts

Air-dried powdered rhizomes were exhaustively extracted with CH_3OH (PA, Vetec) at room temperature. The periodicity of solvent exchange was on average 48 hours, and each solution was concentrated on a rotary evaporator under vacuum. This process yielded three extracts which were subjected to liquid-liquid partitioning by organic solvents of increasing polarity (dichloromethane, and ethyl acetate, both PA, Vetec). The aqueous fractions (RBM-Aq, RCM-Aq and RNM-Aq) of three species, dichloromethane fraction of *R. nicolaioides* (RNM-D), and ethyl acetate fraction of *R. chrysotrycha* (RCM-Ac) were evaluated.

2.4. Determination of total phenolic content

Total phenolic content of rhizome extracts was determined using the spectrophotometric-based modified Folin-Denis method.[23,24]

Solutions of 1 $mg.mL^{-1}$ rhizome extracts in spectroscopic methanol were prepared. A 0.5 mL aliquot of this solution was mixed with 2.5 mL of Folin-Denis reagent. After 5 min, 2.0 mL of the previous solution was added to 14 % fresh aqueous sodium carbonate solution. After 2 h the color of the green solution turned to blue, with the absorbance at 760 nm in quartz cuvettes of 1 cm optical path with ultrapure water as white. The calibration curve was prepared with gallic acid (25-1000 $\mu g.mL^{-1}$; Y = 0.12951 X + 0.12497; R = 0.999). Total phenolic content was calculated and expressed as milligrams of gallic acid equivalents per 100 mg of extract (mg E_{AG} 100 mg^{-1}).

2.5. Determination of total flavonoid content

Total flavonoid content was determined using by adapting a methodology using aluminum chloride as reagent.[25,26] This method is specific for flavones and flavonols. Spectroscopic methanol (Sigma Aldrich) was used to prepare solutions of 5 $mg\ mL^{-1}$ of rhizome extracts. The calibration curve was prepared with quercetin (1 – 50 $\mu g.mL^{-1}$, Y = 0.06553 + 0.04078 X, R = 0.999). The results were expressed as mg of quercetin equivalents per 100 mg extract (mg EQ 100 mg^{-1}).

2.6. Free-radical scavenging assay (DPPH)

Seventy-one microliters of rhizome extract solution (25-150 $\mu g.mL^{-1}$) were mixed with 29 µL of 0.3 $mmol.L^{-1}$ DPPH methanol solution. The absorbance was measured after 30 min at 517 nm using an ELISA 680 microplate reader (Bio-Rad, Brazil). The results were expressed as EC_{50} $\mu g.mL^{-1}$, that is, the amount of antioxidant needed to decrease the initial DPPH concentration by 50 %.

2.7. Evaluation of antioxidant activity by ferric-reducing antioxidant power assay (FRAP)

A 0.5-mL aliquot of rhizome extracts (1.00 $mg.mL^{-1}$) was mixed with 4.5 mL of FRAP reagent. The absorbance was read after 10 min of incubation at 37 °C at 593 nm using ultrapure water as white. The calibration curve was prepared with $FeSO_4.7H_2O$ (100-1000 µM, Y = 0.0018 X + 0.00107, R = 0.99961) and the results expressed as mmol Fe(II) 100 mg^{-1}.

2.8. Determination of antioxidant activity by radical-cation scavenging ($ABTS^{\cdot+}$)

A 50 µL aliquot of rhizome extracts (1.00 $mg.mL^{-1}$) was mixed with 5.0 mL of ABTS reagent. The absorbance was read after 6 min

at 734 nm using ethyl alcohol as white. The calibration curve was prepared with Trolox (0.00-2.40 mmol.L^{-1}; Y = -26.37778 X + 0.65164; R = -0.9997). The results were expressed in mmol TE 100 mg.

2.9. Statistical analyzes

The analyses were performed with three replicates and results were presented as mean ± standard deviation (SD). Pearson correlation, which indicates the positive or negative existence between two variables, was adopted in order to investigate correlation between total phenolic content and the extracts antioxidant activity. The statistical analyzes were performed using the R program.

3. Results and Discussion

3.1. Secondary metabolites in rhizomes

In cross section, *R. breviscapa* and *R. nicolaioides* showed an uniseriate epidermis, and *R. chrysotricha* presented a stratified suber. The phenolic compounds were concentrated at the subepidermal layer in *R. breviscapa* and *R. nicolaioides* (Figure 1A) and in the stratified suber of *R. chrysotricha* (Figure 1B), but were also scattered randomly throughout the cortex (Figure 1A-B and 1F) and the vascular cylinder (Figures 1C-D), as well as near the vascular bundle (Figures 1E).

We identified phenolic compounds in the rhizomes of three studied species. According to Tomlinson (1956), a pioneer in studies on morphology of Zingiberaceae, tannin was characteristic in all species of this family, and these compounds were very frequent associated with vascular bundles. Further anatomical and histochemical studies showed phenolic idioblasts in other species of Zingiberaceae.[10,27-30] Polyphenolic secondary metabolites were found in the underground organs and probably related to plant defense against microbial pathogens .[31]

3.2. Total phenolic and flavonoid content

Values of total phenolic (TP) and total flavonoid (TF) content of the samples are presented in Table 1. The values of TP varied between 32.56 and 33.66 mg E_{GA} 100 mg^{-1} in *R. nicolaioides*; 1.49 and 15.09 mg E_{GA} 100 mg^{-1} in *R. chrysotricha*; 3.20 and 5.53 mg E_{GA} 100 mg^{-1} in *R. breviscapa* (Table 1).

The TF results ranged between 2.3 to 5.12 mg EQ 100 mg^{-1} in *R. nicolaioides*, 0.07 to 0.74 mg E_Q 100 mg^{-1} in *R. chrysotricha,* and 0.10 to 0.46 mg E_Q 100 mg^{-1} in *R. breviscapa* (Table 1).

Following the histochemical tests, the phenolic compounds identified in the rhizomes of three studied species were quantified. The extracts from *R. nicolaioides* showed the highest total phenolic and flavonoid contents. Diarylheptanoids, 1,7-bis(4-hydroxyphenyl)-(1*E*)-1-hepten-3-one, 5R-1,7-bis(4-hydroxyphenyl)-1*E*-hepten-5-ol and (1R,2S,5S)-2-hydroxy-1,7(p-hydroxyphenyl)-centrolobine, as well as one flavonoid, 3-metoxi-quercetin, have been recently isolated from *R. nicolaioides* rhizome extracts.[32]

The flavonoid (flavones and flavonols) content was low in the three extracts and corresponded to 3.12-15.63 % of the TP. These results may be related to the diversity of flavonoid types (chalcones, flavanones, flavones, flavones, catechins) found in *Renealmia* species, as well as the tendency for rhizomes to accumulate diarylheptanoids, another class of phenolic compounds.[32-37]

3.3. Antioxidant capacity

Table 2 shows the results of the antioxidant activity of rhizome extracts from *Renealmia* species tested by three different methods, DPPH, FRAP and ABTS.

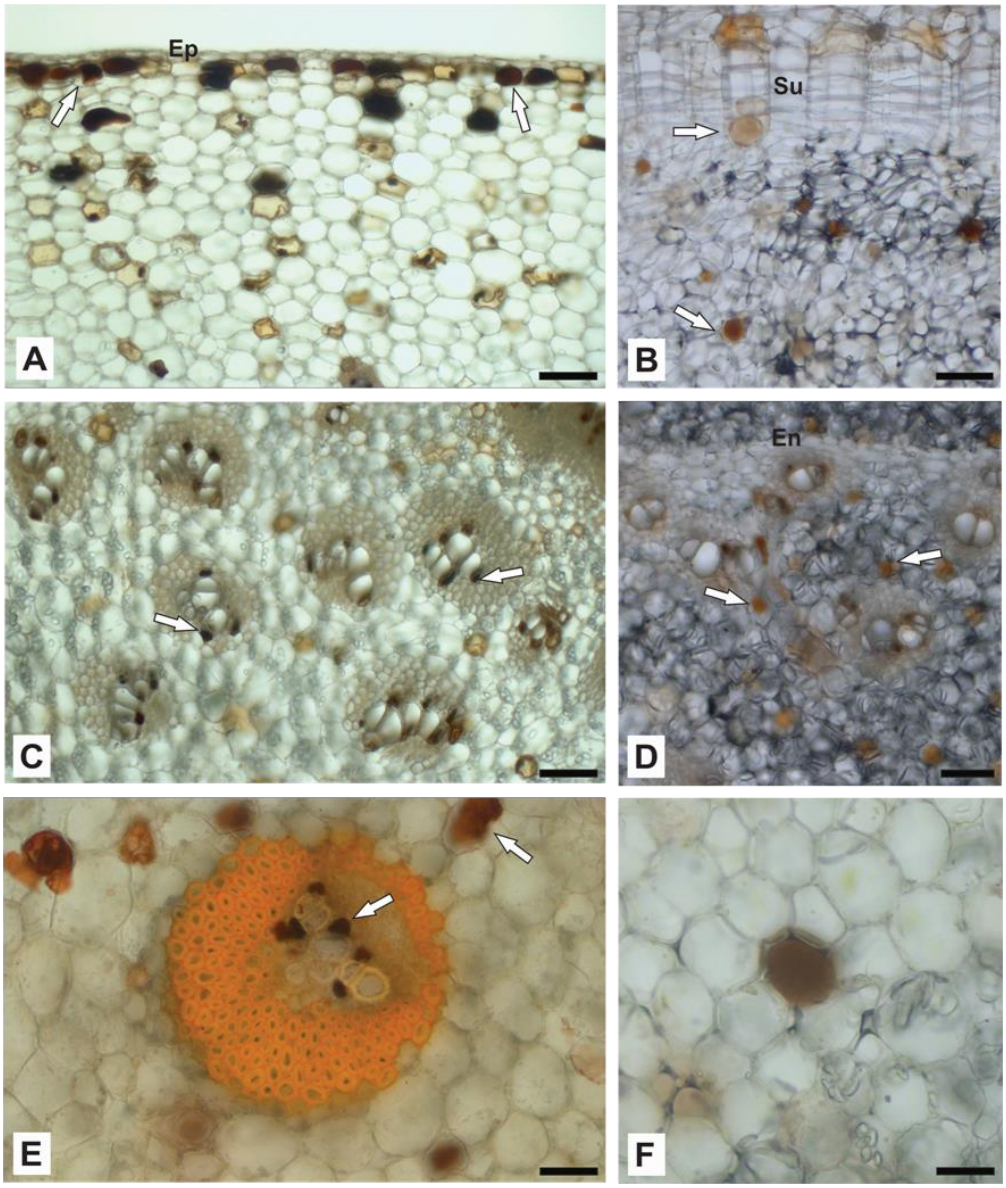

Figure 1. Phenolic compounds of *Renealmia* rhizome (TS). (A, C and E) *R. nicolaioides*. (B, D and F) *R. chrysotricha*. (A-D and F) Phenolic compounds stained by 10 % potassium dichromate. (A, B) Cortical region. (C, D) Central cylinder. (E) Phenolic compounds evidenced by Hoephner-Vorsatz test. Ep = epidermis, Su = stratified suber, En = endodermis. Bars = 100 µm (A-D); 50 µm (E, F)

Table 1. Total Phenolic (TP) and Total Flavonoid (TF) content for eight extracts of the studied rhizomes

Samples	Total Phenolic Content (mg E_{AG} 100 mg^{-1})[a]	Flavonoid Content (mg E_Q 100 mg^{-1})[b]
RNM-D	33.66 ± 0.072	2.30 ± 0.001
RNM-Aq	32.77 ± 0.024	5.12 ± 0.001
RNM	32.56 ± 0.026	2.85 ± 0.000
RCM-Ac	15.09 ± 0.012	0.74 ± 0.001
RBM	5.53 ± 0.005	0.46 ± 0.001
RBM-Aq	3.20 ± 0.007	0.10 ± 0.000
RCM	2.10 ± 0.031	0.07 ± 0.001
RCM-Aq	1.49 ± 0.004	0.14 ± 0.001

a- mg of gallic acid equivalents per 100 mg of extract; b- mg of quercetin equivalents per 100 mg of extract; RNM-D - dichloromethane fraction of *R. nicolaioides*; RNM-Aq - aqueous fraction of *R. nicolaioides*; RNM - methanolic extract of *R. nicolaioides*; RCM-Ac - ethyl acetate fraction of *R. chrysotrycha;* RBM - methanolic extract of *R. breviscapa*; RBM-Aq - aqueous fraction of *R. breviscapa*; RCM - methanolic extract of *R. chrysotrycha;* RCM-Aq - aqueous fraction of *R. chrysotrycha*

Table 2. Antioxidant activity of rhizome extracts

Samples	DPPH (CE_{50}) ($mg\ mL^{-1}$)	FRAP (µmol Fe(II) 100 mg^{-1})	ABTS (µmol TE 100 mg^{-1})
RNM	13.80±0.26	614.59±0.06	182.76±0.00
RNM-Aq	13.95±0.16	478.66±0.12	223.73±0.00
RNM-D	30.40±1.07	586.44±0.08	199.22±0.01
RBM	50.12±2.99	125.55±0.08	44.98±0.00
RCM-Ac	61.36±1.36	346.44±0.04	109.75±0.01
RCM-Aq	97.35±0.96	28.51±0.01	17.41±0.01
RBM-Aq	122.25±4.07	39.55±0.03	35.58±0.00
RCM	155.85±8.28	21.33±0.00	23.30±0.00

RNM - methanolic extract of *R. nicolaioides*; RNM-Aq - aqueous fraction of *R. nicolaioides*; RNM-D - dichloromethane fraction of *R. nicolaioides*; RBM - methanolic extract of *R. breviscapa*; RCM-Ac - ethyl acetate fraction of *R. chrysotrycha;* RCM-Aq - aqueous fraction of *R. chrysotrycha;* RBM-Aq - aqueous fraction of *R. breviscapa*; RCM - methanolic extract of *R. chrysotrycha*

We found a significant correlation between total phenolic content and the antioxidant activity in relation to FRAP and ABTS assays of the extracts (Table 3).

Table 3. Correlations between total phenolic content, total flavonoid content and antioxidant activity (DPPH, FRAP and ABTS) of rhizome extracts, by the Pearson method

	TP	TF	DPPH	FRAP	ABTS
TP	------	0.88	-0.84	0.98	0.99
TF	0.88	------	-0.78	0.79	0.92
DPPH	-0.84	-0.78	------	-0.86	-0.85
FRAP	0.98	0.79	-0.86	------	0.96
ABTS	0.99	0.92	-0.85	0.96	------

The methods used to test the antioxidant capacity are widely used due to their simplicity, fast acquisition data, sensitivity and reproducibility.[38] Despite all the extracts exhibited antioxidant capacity, the best results were obtained by *R. nicolaioides* extracts. The values of the DPPH assays, expressed in EC_{50}, were higher and less effective than those obtained from other Zingiberaceae species, such as *Amomum muricarpum* Elmer, *Etlingera philippinensis* (Ridl.) R. M. Sm. and *Hornstedtia conoidea* Ridl.[19]

We found significant correlation between total phenolic content and the antioxidant activity in relation to FRAP and ABTS assays of the extracts, as observed for extracts from other Zingiberaceae species.[12,15-18,20]

This study suggests *Renealmia* species are sources of phenolic compounds that may contribute to the treatment of diseases and degenerative processes associated with the overproduction of reactive oxygen species, an under exploited issue up to date.

Acknowledgments

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); the Fundação de Amparo à Pesquisa do Rio de Janeiro (FAPERJ). This study was part of the thesis of the first author at Programa de Pós-Graduação em Biociências e Biotecnologia/UENF.

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