SHORT REPORT

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Chemical Composition and Antihypertensive Effect of *Phoenix roebelenii* Using Angiotensin Converting Enzyme Inhibition *in vitro* and *in vivo*

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Abstract: This study aimed to evaluate in vivo anti-hypertensive effect of *Phoenix roebelenii*. To access the chemical composition, the EtOH extract and CH_2Cl_2 fraction of *P. roebelenii* were analyzed using electrospray ionization (ESI) source combined with the Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) technique. The ACE inhibitory effect was evaluated in vivo by Ang I administration. The antihypertensive assay was performed in Spontaneously Hypertensive Rats (SHR) and Wistar rats that were treated with enalapril (10 mg/kg), CH_2Cl_2 fraction (80 mg/kg; twice a day) or vehicle for 30 days. ACE activity in vivo was measured by colorimetric assay. ESI(-)FT-ICR mass spectrum for EtOH extract identified the presence of rutin, quercitrin, kaempferol-7-O-glucoside and kaempferol-3-*O*-rutenoside, and in the CH_2Cl_2 fraction, paradol, gingerol, ursolic/betulinic acid and maslinic/corosolic acid. CH_2Cl_2 fraction exhibited anti-hypertensive effect in vivo by reducing blood pressure in the SHR models. It may be concluded that the presence pungent vanilloids compounds in CH_2Cl_2 fraction contributed to the ACE inhibition in vitro and in vivo and that action could be the mechanism of the anti-hypertensive effect, known for its medicinal value.

Keywords: *Phoenix roebelenii*, phoenix palm; angiotensin converting enzyme; gingerol.

1. Plant Source

Phoenix roebelenii O'Brien, Arecaceae, is popularly known as the dwarf palm and phoenix palm, being used in the decoration of vases, parks and gardens [1].

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The plant material was collected in private gardens in the municipality of Vila Velha, Espírito Santo, Brazil, in June 2012. A voucher specimen was prepared and identified by Profa. M.Sc. Solange Z. Schneider, botanic of the herbarium at the University Vila Velha (UVV), where a voucher specimen is deposited under the number UVVES 1809. The compound leaves were dried (40 °C), and separated in leaflet and petioles. The leaflets were ground.

2. Previous Studies

Data on the biological activity of *P. roebelenii* are scarce [2-4], being reported *in vitro* inhibitory activity of α-glucosidase and α-amilose [2] and angiotensin converting enzyme (ACE, 79,7 ± 7.4%) [4,5]. These data suggest that the assay described by Serra et al. [2] may be a useful instrument for selecting species for testing in vivo, being reasonable to select *P. roebelenii* to evaluate potential the antihypertensive effects. There is no previous works describing the ability of *P. roebelinii* to reduce in vivo blood pressure at experimental models. Therefore, ACE in vitro inhibition is not being confirmed biologically.

3. Present Study

Aliquot (240 g) of ground leaflet material were extracted by percolation with EtOH, followed by extract reduction under reduced pressure at 40ºC until a residue was formed (48 g). An aliquot of 15 g was suspended in water and then partitioned with pentane, CH_2Cl_2, EtOAc. The solvents were removed using rotary evaporator to give dark gummy crude extracts of pentane (1.1 g, 7.30%), CH_2Cl_2 fraction (2.9 g, 19.0%), EtOAc fraction (0.8 g, 5.30%) and aqueous fraction (6.4 g, 42.67%).

Total polyphenol content of the EtOH extract and CH_2Cl_2 fraction was quantificated by colorimetric Folin-Ciocalteu method [6]. The analytical curve ($y = 1.1429\ x + 0.007$, $r^2 = 0.9977$) was obtained with a solution by pyrogallol (Sigma-Aldrich, St. Louis, USA) (10- 350 mg/mL). The results were expressed as mg of pyrogallol equivalents (PE) per g of dry extract. All analyzes were performed in triplicate. As expected, the EtOH extract showed higher content of total polyphenols (72.3 ± 5.6 mg EP/g of dry extract) ($p<0.01$) than the CH_2Cl_2 fraction (27.3 ± 2.0 mg EP/g fraction dry), and higher of previous study [3].

Colorimetric method of aluminum chloride [7] was applied to determine the total amount of flavonoid. The analytical curve ($y= 0.0033\ x + 0.0121$, $r^2= 0.9991$) was achieved using quercetin solutions (Sigma-Aldrich, St. Louis, USA) (3.91 to 500.00 μg/mL). The quantification of flavonoid was determined using the analytical curve. The results were expressed as mg of quercetin equivalents (eq) per g of dry extract. The content of total flavonoid for the EtOH extract (41.3 ± 2.0 mg EQ/g dry extract) suggested a higher concentration of these constituents.

The EtOH extract and the CH_2Cl_2 fraction were diluted to ≈ 0.25 mg/mL in water: acetonitrile (1:1) which contained 0.1% m/v of NH_4OH for ESI in negative mode, ESI(-) and ESI(-)FT-ICR method was performed as previous described [8,9]. The mass spectra were acquired and processed using Data Analysis Software (Bruker Daltonics, Bremen, Germany). The MS data were processed, and the elemental compositions of the compounds were determined by measuring the m/z values. The proposed structures for each formula were assigned using the chemspider database (www.chemspider.com).

ESI(-)FT-ICR mass spectrum for EtOH extract (Tabel 1) identified mainly the presence of flavonoids (rutin, quercitrin, kaempferol-7-O-glucoside and kaempferol-3-O-rutenoside), in which, their chemical structure, molecular formula, measured and theoretical m/z values, mass error and DBE are shown in Table 1.

Table 1. Chemical composition assigned to signals detected in the ESI(-)FT-ICR mass spectrum of ethanolic extract from leaves of *P. roebelenii*

	$m/z_{Measured}$	$m/z_{Theoretical}$	[M-H] $^-$	Error (ppm)	DBE	Proposed compound	Reference
1	255.23303	255.23295	$[C_{16}H_{32}O_2\text{-}H]^-$	0.31	1	Palmitic acid	[10]
2	277.18103	277.18092	$[C_{17}H_{26}O_3\text{-}H]$	0.40	5	p-decyloxybenzoic acid	
3	335.07745	335.0772	$[C_{16}H_{16}O_8\text{-}H]^-$	0.63	9	5-O-Caffeoylshikimic acid	[3,11]
4	431.09869	431.09837	$[C_{21}H_{20}O_{10}\text{-}H]^-$	0.74	12	Apigetrin	[12]
5	447.09362	447.09329	$[C_{21}H_{20}O_{11}\text{-}H]$	0.74	12	Quercitrin	[12,13]
6	471.3483	471.34798	$[C_{30}H_{48}O_4\text{-}H]$	0.59	7	Maslinic acid; Hederagenin	
7	593.15157	593.15119	$[C_{27}H_{30}O_{15}\text{-}H]$	0.64	13	Vicenin-2	[14]
8	609.1467	609.14611	$[C_{27}H_{30}O_{16}\text{-}H]^-$	1.03	13	Rutin	[13, 15]

For CH_2Cl_2 fraction, the ESI(-)FT-ICR mass spectrum (Table 2) showed the presence of paradol, gingerol, ursolic acid (or betulinic acid) and maslinic acid (or corosolic acid).

Table 2. Chemical composition assigned to signals detected in the ESI(-)FT-ICR mass spectrum of dichlorometanic fraction from of leaves of *P. roebelenii*

	$m/z_{Measured}$	$m/z_{Theoretical}$	[M-H] $^-$	Error (ppm)	DBE	Proposed compound	Reference
9	255.23298	255.23295	$[C_{16}H_{32}O_3\text{-}H]^-$	0.12	1	Ac. Palmitic	[3,10]
10	277.18093	277.18092	$[C_{17}H_{26}O_3\text{-}H]^-$	0.04	5	p-decyloxybenzoic acid	
11	293.17588	293.17583	$[C_{17}H_{26}O_4\text{-}H]^-$	0.17	5	Unidentified	
12	441,3740	441,37480	$[C_{30}H_{50}O_2\text{-}H]^-$	-1.79	6	Betulin	[13]
13	455,3533	455,3531	$[C_{30}H_{48}O_3\text{-}H]^-$	0.51	7	Betulinic acid	[13]
14	471,3482	471.34798	$[C_{30}H_{48}O_4\text{-}H]^-$	0.47	7	Maslinic acid; Hederagenin	

The determination of the antioxidant CH_2Cl_2 fraction was performed by the method of capturing radicals 2,2-diphenyl-1-picryl hydrazyl (DPPH), as previously described [16]. The percentage of inhibition of DPPH was calculated from the equation: I%= [(Abs0 – Abs1) / Abs0] x 100. With the absorbance obtained Abs0 white and Abs1 the sample absorbance. Increasing concentrations of the sample (0.2 to 0.8 mg/mL) were used. The reading was performed in a spectrophotometer at a wavelength of 517 nm. The concentration needed to provide 50% inhibition of the radical (IC_{50}) was calculated by the equation of the straight-line calibration curve (y= 3.426 x – 0.5071, r^2= 0.9924). The antioxidant was expressed by the degree of antioxidant activity (IAA), which was calculated from the equation: IAA = final concentration of DPPH / IC_{50}, and IAA < 0.5 low antioxidant effect and IAA 0.5, 1.0 antioxidant effect moderate (IAA) between 1.0 and 2.0 strong antioxidant effect and IAA > 2.0 very strong antioxidant effect [17]. Analyses were performed in triplicate. The CH_2Cl_2 fraction showed a marked antioxidant activity (AAI= 2.21 and IC_{50}= 18.9 µg/mL).

The inhibitory activity of extracts and fractions of *P. roebelenii* on ACE were evaluated through cleavage of the substrate Hip-Gly-Gly ECA as described by Endringer et. al. [18]. All samples from *P. roebelenii* inhibited ACE *in vitro*: leaflet EtOH extract (80.2 ± 13.8%), leaflet aqueous fraction (56.0 ± 2.3%), leaflet CH_2Cl_2 fraction (88.2 ± 18.3%), pentane fraction (33.33 ± 2.4%) and EtOAc fraction (26.11 ± 0.8%). However, the values considered promising for evaluation of *in vivo* activity, were only that ones with mean inhibition greater than 80% [18]. Therefore, the CH_2Cl_2 fraction was selected for *in vivo* evaluation.

The *in vivo* antihypertensive evaluation followed the ethics national and international recommendations for animal experiments and approved by the Ethics Committee, Bioethics and Welfare Animal the UVV (CEUA – UVV; protocol 120/2010). We used spontaneously hypertensive

rats (SHR) and their normotensive controls, Wistar-Kyoto (WKY). The animals were about three months old, weighing between 280-350 g. SHR and WKY rats were randomly divided into four groups (n = 5 each). The animals received a daily treatment of CH_2Cl_2 fraction (SHRP and WKYP) solution. The animals in the control groups (SHR and WKY) received the vehicle (sunflower oil). The treatment was performed for 30 days using a dose of 40 mg/kg CH_2Cl_2 fraction solution twice a day intraperitoneally with a 12 hours interval between them (80 mg/kg/day). The animals were kept in a vivarium of UVV, at a temperature of 22 ± 3ºC, in a cycle of 12 h light / 12 h dark with free access to standard pellet diet (diet Probiotério, Mill Primor SA) and tap water.

The animals submitted to chronic anti-hypertensive treatment were weighed at the beginning of the experiment (initial body weight - IBW) and distributed according to the body weight evenly between the various groups: SHRP, WKYP, SHR and WKY in individual cages. The animals were weighed daily to calculate the dose (volume) of the solution being administered. On the day of the experimental protocol animals were weighed for the last time, obtaining the final body weight (CCW) of these animals.

At the end of the experimental protocol, the animals submitted to chronic anti-hypertensive treatment were euthanized by decapitation. The hearts were removed to obtain the estimated weight and cardiac hypertrophy. They were isolated, washed with saline solution and excess liquid was removed with filter paper and then weighed. The ratio of heart weight (mg) final body weight (g) (HEART / BW) was used as an index of cardiac hypertrophy. In the antihypertensive evaluation, the administration of CH_2Cl_2 fraction inhibited did not alter MAP in WKY animals (Table 3), but the treatment decreased MAP in the SHRP group when compared with the SHRP ($p<0.01$) (Table 3). The MAP reduction after chronic administration of CH_2Cl_2 fraction inhibited was similar to those elicit by the enalapril treated SHR (Table 3).

The CH_2Cl_2 fraction did not change the HEART/BW in WKY animals (Table 3). However, in the SHR, the treatment with CH_2Cl_2 fraction reduced this ratio ($p<0.01$) with the same magnitude of the SHRE group. However, these parameters (MAP and HEART/BW) were not normalized by treatment with CH_2Cl_2 fraction, where as they maintained higher as compared with the groups of WKY animals ($p<0.01$) (Table 3).

ACE activity was lower in the serum of SHR treated groups (SHRP = 45.0 ± 5.0%, SHRE = 46.4 ± 3.0%) compared with negative SHR control group (82.0 ± 8.0%) ($p<0.05$). The same was observed in the WKY treated groups (WKYP = 54.0 ± 4.0%, WKYE = 47.8 ± 4.0%) in relation with the negative WKY control animal (64.0 ± 4.0%) ($p<0.05$). No difference was observed between the SHR treated with the normotensive animals.

Table 3. Hemodynamic parameters, heart weight to body weight ratio (HW/BW) of the experimental groups

Group	MAP (mmHg)	HR (bpm)	HEARTH/BW (mg/g)
WKY	109 ± 5	315 ± 10	2.71 ± 0.08
WKYP	108 ± 4	322 ± 11	2.69 ± 0.10
WKYE	109 ± 2	291 ±26	2.789 ± 0.22
SHR	184 ± 8**	333 ± 14	3.53 ± 0.02**
SHRE	152 ± 4$^{**\#\#}$	309±20	3.24 ± 0.19$^{**\#\#}$
SHRP	146 ± 9$^{**\#\#}$	338 ± 12	3.28 ± 0.03$^{**\#\#}$

WKY = Wistar Kyoto animals with no treatment; WKYP = Wistar Kyoto animals treated with dichloromethane fraction extract of *P. roebelenii* (DOSE); SHR = Spontaneously Hypertensive Rats with no treatment; SHRP = Spontaneously Hypertensive Rats treated with dichloromethane fraction extract of *P. roebelenii* . WKYE = Wistar Kyoto animals treated with enalapril (10 mg.kg^{-1}); SHR = Spontaneously Hypertensive Rats treated with enalapril (10 mg.kg^{-1}). Values are expressed as mean ± S.E.M. $^{**}p<0.05$ compared with normotensive animals. $^{\#\#}p<0.05$ compared to animals SHR group. MAP = Mean arterial pressure; HR= Heart rate.

There was an increase in the final body weight in WKY (IBW: 243 ± 47g vs. FBW: 316 ± 51g; $p<0.05$), WKYP (IBW: 245 ± 38g vs. FBW: 304 ± 22g; $p<0.05$) and WKYE (IBW: 247 ± 44g vs. FBW: 301 ± 13g; $p<0.05$) compared with its respective initial body weight. In the hypertensive groups

experimental groups there was no change in the FBW (SHR: 288 ± 13; SHRP: 287 ± 14; SHRE: 285 ± 11) compared with the IBW (SHR: 306 ± 19; SHRP: 302 ± 24; SHRE: 288 ± 16).

This study showed the antihypertensive effect of *P. roebelenii* in SHR rats. The chemical compounds of CH_2Cl_2 fraction (pungent vanilloids and triterpenes compounds) showed effect on the components on the renin-angiotensin system (RAS).

Altogether, these results indicate that CH_2Cl_2 fraction from the EtOH extract *P. roebelenii* has a marked concentration of gingerol-type compounds and significantly reduce blood pressure on the SHR model. One of the mechanisms of the antihypertensive effect is related to inhibition of ACE, since the dichloromethane fraction inhibit this enzyme in vitro and in vivo. These data suggest the potential anti-hypertensive activity of CH_2Cl_2 fraction of *P. roebelenii*.

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Supporting Information

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