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NOTE

Protective effects of flavonoids and extract from *Vellozia kolbekii* Alves against oxidative stress induced by hydrogen peroxide in yeast

Carmelita Gomes da Silva · Camilla Dayane F. Carvalho · Lidilhone Hamerski · Frederico A. V. Castro · Ruy José Válka Alves · Carlos Roland Kaiser · Elis Cristina Araújo Eleutherio · Cláudia Moraes de Rezende

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Abstract Two flavonoids 3,5,7,3′,4′-pentahydroxy-6-prenylflavonol (**1**) and 3,5,7,3′,4′-pentahydroxy-8-methyl-6-prenylflavonol (**2**) were isolated from the ethyl acetate extract of sheaths of *Vellozia kolbekii* Alves (Velloziaceae). This is the first time that compound **2** has been described. The crude extract and flavonoids were found to be active as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavengers and were able to the increase tolerance of the eukaryotic microorganism *Saccharomyces cerevisiae* to oxidative stress generated by H_2O_2. The protective effect was correlated with a reduction in the oxidation of proteins and lipids. In addition, flavonoids isolated from Velloziaceae showed an inhibitory effect on mutations in p53, which is mutated and nonfunctional in more than 50% of cases of human cancer.

Keywords *Vellozia kolbekii* (Velloziaceae) · Antioxidant activity · Antimutagenic activity · Flavonoids · *Saccharomyces cerevisiae* · p53 factor

Introduction

Vellozia kolbekii is a species typical of the Brazilian cerrado and was discovered in 1992 [1]. This plant belongs to the family of the Velloziaceae, which includes 270 species distributed mainly in tropical South America, with a few outliers in Africa and Arabia [2]. In Brazil, they are commonly known as canelas-de-ema.

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C. G. da Silva (✉) · C. D. F. Carvalho · L. Hamerski · C. M. de Rezende
Laboratory of the Analysis of Aromas, Department of Organic Chemistry, Institute of Chemistry, Federal University of Rio de Janeiro, Athos da Silveira Ramos, 149, University City, 21945-970 Rio de Janeiro, RJ, Brazil
e-mail: carme_gomes@yahoo.com.br

C. D. F. Carvalho
e-mail: camilla_dfc@hotmail.com

L. Hamerski
e-mail: lidihamerski@ig.com.br

C. M. de Rezende
e-mail: claudia.rezendeufrj@gmail.com

F. A. V. Castro · E. C. A. Eleutherio
Laboratory of the Investigation of Stress factors (LIFE), Department of Biochemistry, Institute of Chemistry, Federal University of Rio de Janeiro, 21949-900 Rio de Janeiro, RJ, Brazil

F. A. V. Castro
e-mail: frede.augusto@uol.com.br

E. C. A. Eleutherio
e-mail: eliscael@iq.ufrj.br

R. J. V. Alves
National Museum, Federal University of Rio de Janeiro, 20940-040 Rio de Janeiro, RJ, Brazil
e-mail: ruyvalka@yahoo.com

C. R. Kaiser
Nuclear Magnetic Resonance Laboratory, Department of Organic Chemistry, Institute of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil
e-mail: kaiser.ufrj@gmail.com

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Table 1 DPPH radical scavenging activity was expressed as IC_{50} and the total phenolic concentration was measured

Samples	IC_{50} (μg/ml)	Phenolic compound concentration (mg/ml)
A	108.4 ± 1.9	NP[a]
B	35.6 ± 1.2	81.5 ± 0.1
C	98.4 ± 1.5	56.3 ± 0.1
D	1727 ± 44.7	NP[a]
E	124.7 ± 2.4	NP[a]
F	74.7 ± 2.3	51.3 ± 2.6
1	8.4 ± 0.5	NP[a]
2	8.5 ± 0.2	NP[a]
G. biloba (GBE 761®)	26.6 ± 0	61.1 ± 0
Quercetin (**Q**)	5.0 ± 0.3	NP[a]

The experiments were performed as described in the "Materials and methods"

IC_{50}, concentration of sample required to scavenge 50% of DPPH radicals; **A**, hexane extract of sheaths of *V. kolbekii*; **B**, ethyl acetate extract of sheaths of *V. kolbekii*; **C**, ethanol extract of sheaths of *V. kolbekii*; **D**, hexane extract of leaves of *V. kolbekii*; **E** ethyl acetate extract of leaves of *V. kolbekii*; **F**, ethanol extract of leaves of *V. kolbekii*

[a] Tests were not performed

There are few records of biological activity of Velloziaceae in the scientific literature. Ethnobotanical reports indicate that some African tribes smoke the dried roots of certain species for the treatment of asthma [3]. In Brazil, many species of the genus *Vellozia* are used as fuel, because of the resin located at the base of the leaves. This resin is also used to treat skin cracks [4].

There are no reports about the popular use of *Vellozia kolbekii* in terms of its chemistry and biology. In this study we redress this omission and, moreover, report the antioxidant activity of Velloziaceae for the first time.

Results and discussion

Initially, the ability of the different extracts from leaves and sheaths of *V. kolbekii* to scavenge the free radical DPPH and the concentration of total polyphenols were measured (Table 1). Ethyl acetate and ethanol extracts of sheaths and the ethanol extract of leaves exhibited IC_{50} values lower than 100 μg/ml, 35.6, 98.4, and 74.7 μg/ml, respectively, suggesting the presence of polar substances with free radical scavenging activity. Ethyl acetate extracts of sheaths showed an IC_{50} similar to that of the standardized extract of *Ginkgo biloba* [5] and the highest polyphenol content (81.5 mg/ml), even higher than the content of *G. biloba*. On the basis of this initial screening, the ethyl acetate extract of sheaths was subjected to isolation techniques, which might justify the aforementioned activity.

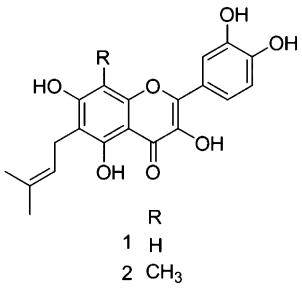

Fig. 1 Structures of flavonoids **1** and **2**

Table 2 ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) and ^{1}H–^{13}C HMBC for flavonoid **2** (DMSO-d_6)

Position	δ_C	δ_H (*J*, Hz)	HMBC
2	147.1, C		
3	136.2, C		
4	176.7, C		
5	155.5, C		
6	111.3, C		
7	159.7, C		
8	102.1, C		
9	152.2, C		
10	103.6, C		
1′	123.0, C		
2′	115.5, CH	7.7 d (2.6)	2, 3′, 6′
3′	145.7, C		
4′	148.2, C		
5′	116.3, CH	6.9 d (8.6)	1′, 3′, 4′
6′	120.6, CH	7.6 dd (2.6, 8.6)	2, 2′
1″	21.9, CH_2	3.3 d (6.0)	5, 6, 7, 2″, 3″
2″	123.1, CH	5.2 br t (6.0)	4″, 5″
3″	131.2, C		
4″	18.3, CH_3	1.7 s	2″, 3″, 5″
5″	26.1, CH_3	1.6 s	2″, 3″, 4″
Me-(C_8)	8.9, CH_3	2.3 s	7, 8, 9
OH (C_5)		12.6 s	5, 6, 10

Chemical shifts are given in ppm. Assignments were confirmed by HSQC and HMBC (see Supporting Information)

Flavonoids **1** and **2** were isolated from ethyl acetate extracts of sheaths and were the major compounds from the original fraction (Fig. 1).

Compound **2** showed $[M–H]^-$ ions at *m/z* 384.1137 in its HR-ESI–MS, consistent with the molecular formula $C_{21}H_{20}O_7$ (*m/z* Calc.: 384.1136). The spectral data of compound **2** were similar to those of compound **1**. The ^{1}H NMR spectrum of **2** (Table 2) showed a deshielded singlet, characteristic of a chelated OH group on ring A (δ 12.6, s). The compatibility of the ^{1}H NMR spectrum of **2** with a 1′,3′,4′-trisubstituted ring was based on the chemical shifts and coupling constants for H-2′ (δ 7.7, d, $J = 2.6$ Hz), H-5′

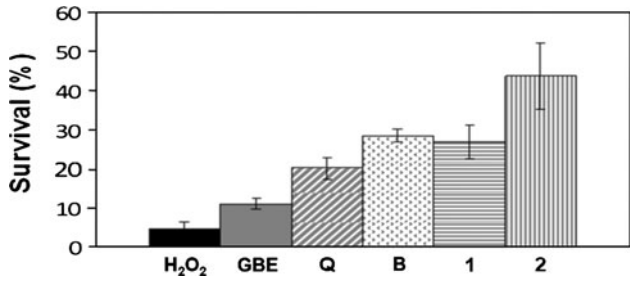

Fig. 2 Effect of *V. kolbekii* and flavonoids on cell viability after stress with 2 mM hydrogen peroxide. *GBE 761*® standard extract of *Ginkgo biloba*, *B* ethyl acetate extract of sheaths of *V. kolbekii*, and *Q* quercetin. The experiments were performed as described in "Materials and methods"

(δ 6.9, d, $J = 8.6$ Hz), and H-6′ (δ 7.6, dd, $J = 2.6$ and 8.6 Hz). The ^{1}H NMR showed signals related to a vinyl proton, CH (δ 5.2 br, t, $J = 6.0$ Hz), allylic protons, CH_2 (δ 3.3, d, $J = 6.0$ Hz), and vinyl methyls (δ 1.7, s and δ 1.6, s), characteristic of an isoprenyl unit. The assignment of the ^{1}H NMR spectrum of **2** was supported by the observation of carbon signals in its ^{13}C NMR spectra (Table 2) characteristic of flavonols. The location of the isoprenyl unit at C-6 can be confirmed by the HMBC spectrum, which indicates the existence of correlations between methylene protons at H-1″ (δ 3.3) and carbons at C-5 (δ 155.5), C-6 (δ 111.3), and C-7 (δ 159.7) (Table 2).

Besides the characteristic signals of an isoprenyl group at high field in the ^{1}H NMR spectrum of compound **2**, the signal of a third methyl group (δ 2.3, s) was verified [6] (Table 2). The position of this methyl group was found to be on C-8 through chemical shift of this carbon atom, δ 102.1(Table 2), which was similar to that found in 3′,4′,5,7-tetrahydroxy-3,6-dimethoxy-8-methylflavone isolated from *V. candida* (δ 101.98) [6]. The results of the HMBC spectrum confirmed the position of the methyl group, which exhibits a heteronuclear long-range coupling between carbon atoms C-7, C-8 and C-9 and hydrogen atoms of the methyl group (Table 2). After careful examination using spectroscopic methods and literature data [6, 7], the structure was determined as 8-methyl-6-prenylquercetin (**2**). The difference of 14 Da between the flavonoids **1** and **2** confirmed the presence of a methyl group bonded to C-8 of compound **2**, which is typical of flavonoids isolated from Velloziaceae [6, 8].

According to Fig. 2, the ethyl acetate extract of sheaths and the flavonoids **1** and **2** isolated from it increased cellular tolerance to H_2O_2 stress. Although quercetin, a known antioxidant [9], and flavonoids **1** and **2** had produced an increase in tolerance that was not statistically different, flavonoid **2** seems to be a better protector against peroxide stress than flavonoid **1** and quercetin, indicating that the methyl group in carbon-8 might have a role in the antioxidant action.

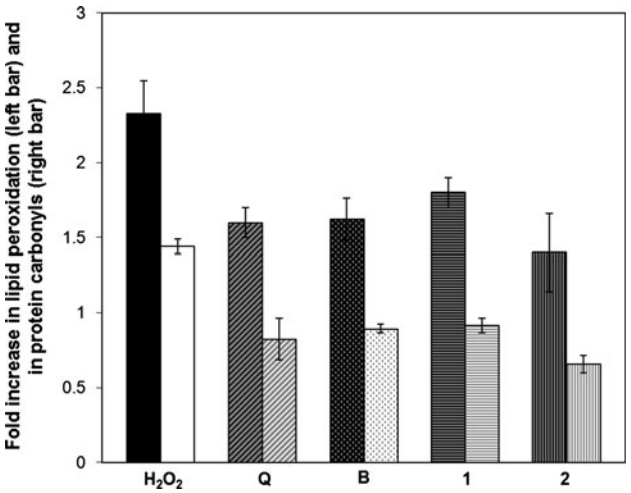

Fig. 3 Effect of *V. kolbekii* and flavonoids on lipid peroxidation (*left bars*) and on carbonylation of proteins (*right bars*), after a stress with 2 mM hydrogen peroxide. *B* ethyl acetate extract of sheaths of *V. kolbekii* and *Q* quercetin. The experiments were performed as described in "Materials and methods"

According to Fig. 3, the extract **B** and the flavonoids isolated from this extract were able to protect cell membranes and proteins from yeast against H_2O_2-induced oxidative stress. Again, no difference between the flavonoids **1** and **2** was found with respect to the protection of the membranes (Fig. 3). These results suggest that the presence of isoprenyl and methyl groups in different flavonoids does not influence the protection of the biomembranes against peroxide stress. Although all the antioxidants tested have reduced the levels of protein oxidation, flavonoid **2** promoted an even greater reduction, as attested by the highest survival rate shown in Fig. 2. The greater antioxidant potential of flavonoid **2** might be explained by the fact that the methyl group confers increased lipophilicity to the molecule, thereby increasing its absorption by cells and its availability to protect biomolecules against oxidative stress. Protection against protein carbonylation requires absorption of antioxidants, contrary to protection against lipid peroxidation. The level of carbonyl groups is measured in the soluble fraction of yeast extracts, i.e., in intracellular proteins [10]. On the other hand, antioxidants which are not (or poorly) absorbed can decrease lipid peroxidation by protecting the outer layer of the cytoplasmic membrane [11].

Oxidative DNA damage can be induced by reactive oxygen species and therefore it can cause mutagenicity and, consequently, carcinogenicity [12]. Carcinogenesis is a multistep process that requires activation of oncogenes and inactivation of tumor suppressor genes. Concerning the latter, *TP53* represents one of the most studied tumor suppressor genes in biology, which is mutated and non-functional in more than 50% of cases of human cancer

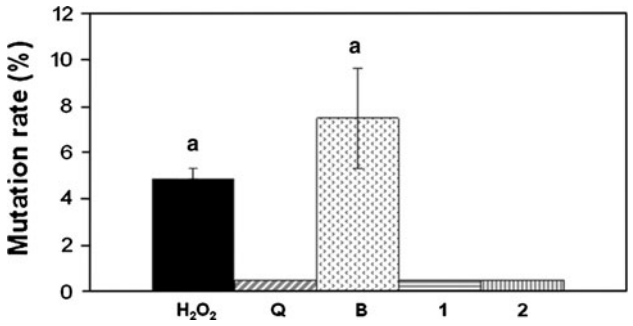

Fig. 4 Effect of *V. kolbekii* and flavonoids on mutation in yeast cells expressing the p53 tumor suppressor factor, after a stress with 2 mM hydrogen peroxide. *B* ethyl acetate extract of sheaths of *V. kolbekii* and *Q* quercetin. The experiments were performed as described in "Materials and methods". Values with same letters are considered statistically equal

[12]. We used an adaptation of the functional analysis of separated allele in yeast (FASAY) procedure to verifying an inhibitory effect of mutations mediated by H_2O_2 in p53 [13]. This assay allows the study of transcriptional *TP53* activity, which is based on expression of the p53 human protein in yeast [13]. The yeast strain yIG397, expressing human p53, contains an integrated plasmid with the *ADE2* open reading frame under the control of a p53-responsive promoter. Thus, when the wild-type form of human p53 is produced, yeast cells express *ADE2* and grow normally even in the absence of adenine. Cells expressing a non-functional p53 fail to express *ADE2* [13]. Consequently, colonies with mutated p53 only grow in medium supplemented with adenine, because they cannot produce it. As can be seen in Fig. 4, both flavonoids **1** and **2** reduced to zero the mutation rate of 4.4% caused by H_2O_2. The standard quercetin also provided a similar result (Fig. 4). An ethyl acetate extract of sheaths was not found to be antimutagenic.

In conclusion, these results indicate that the use of *Saccharomyces cerevisiae* as a model is an interesting strategy for the investigation of in vivo natural antioxidant sources. On the basis of the antimutagenic and antioxidant activities found, flavonoids **1** and **2** of Velloziaceae seem to have a great potential as prophylactic agents for cancer prevention, suggesting that other biological activities can be found.

Materials and methods

General experimental procedures

UV and IR spectra were recorded on a Varian Cary 1E UV–visible spectrophotometer and a Nicolet Magna IR 760 spectrometer, respectively. The NMR spectra were recorded in DMSO-d_6, using a Bruker DRX-500 instrument operating at 500 (^{1}H) and 125 (^{13}C) MHz. ESI–MS spectra were recorded by direct inlet using an ultrOTOF-ESI–TOF Mass Spectrometer (Bruker Daltonics), negative mode, and electrospray as the ion source. LiChroprep RP-18 (25–40 μm, Merck) and Sephadex LH-20 (Pharmacia Biotech AB) were used for column chromatography. Semipreparative HPLC was performed on an Agilent 1100 liquid chromatograph with a Zorbax Eclipse XDB C_{18}, 9.4 mm × 25 cm, 5 μm, column with a flow rate of 3 ml min^{-1} and UV–vis detection. Fractions were monitored by TLC on Si gel 60 F254 Al sheets (Merck), and spots were visualized by spraying with NP-PEG (diphenyl boric acid–polyethylene glycol). Reagents and standard samples were purchased from Sigma-Aldrich.

Plant material, extraction, and isolation

Leaves and sheaths of *V. kolbekii* Alves were collected in the São José mountain range, Minas Gerais, Brazil, in September 2008. The plant material was identified as to species by the author, and a voucher specimen (R.J.V. Alves 7909) was deposited at the herbarium of the Museum Nacional, University Federal of Rio de Janeiro.

The air-dried powdered leaves (162 g) and sheaths (565 g) of *V. kolbekii* were first extracted with hexane, then EtOAc, and afterwards EtOH, by maceration for 48 h, affording, respectively 4.4, 4.6, 3.0 g (leaves) and 52.0, 34.8, 13.0 g (sheaths) of extracts. The ethyl acetate extract of sheaths of *V. kolbekii* (**B**,10 g) was fractionated by VLC (vacuum liquid chromatography) containing Si C_{18}, eluting with gradient mixtures of H_2O/MeOH to give six fractions. Fractions 4 and 5 showed orange spots by TLC revealed with NP-PEG (indicative of flavonoids) affording, respectively, 0.5 and 2.3 g. Then, fraction 5 (0.84 g) was subjected to gel filtration on Sephadex LH-20, with MeOH to give a total of 47 fractions. Fractions 31–37 (114 mg) provided a mixture in which two flavonoids were the major compounds, requiring an additional chromatographic purification by semipreparative HPLC, eluted with MeOH/H_2O (75:25) to afford **1** (27 mg) and **2** (22 mg). Physical and spectral data of **1** were in agreement with those reported in the literature for 6-prenylquercetin, which was previously isolated from *Glycyrrhiza uralensis* by Fukai et al. [7]. Flavonoid **2** had not previously been described in the scientific literature.

3,5,7,3′,4′-Pentahydroxy-8-methyl-6-prenylflavonol (8-methyl-6-prenylquercetin) (**2**): yellow amorphous powder. IR ν_{max} (KBr) cm^{-1}: 3398, 2926, 1648, 1605, 1552, 1348. UV λ_{max} (MeOH) nm (log ε): 274 (4.05), 377 (4.01). ^{1}H, ^{13}C NMR, and HMBC data, see Table 2. High resolution negative-ion HR-ESI–MS *m/z* 383.1137 [M–H]$^-$ (Calcd for $C_{21}H_{19}O_7$: 383.1131).

DPPH photometric assay and phenolic compound content

All extracts and isolated flavonoids **1** and **2** were assessed for the ability to donate hydrogen atoms to the DPPH radical, according to Blois [14]. The results were expressed as $IC_{50} \pm$ standard deviation. Tests for the flavonoids **1** and **2** were made on a reduced scale with the same method, and the absorbance was measured in an ELISA reader [15]. The total phenolic content of the extracts was determined by the Folin–Ciocalteu method [16].

Yeast strains, media, and growth conditions

Wild-type strain BY4741 (*MATa*, *his3*, *leu2*, *met15*, *ura3*) was acquired from Euroscarf, Germany. Stocks of this strain were maintained on rich and solid YPD (yeast peptone dextrose) medium. Cells were grown up to the middle of the first exponential growth phase (1×10^6 cells/ml) in rich and liquid YPD medium. The haploid *S. cerevisiae* strain yIG397 (*MATa ade2-1 leu2-3,112 trp1-1 his3-11,15 can1-100 ura3-1 URA3* 3xRGC::p*CYC1*::*ADE2*), which contains the *ADE2* reporter gene under control of three p53 binding sites, was a kind gift from Dr. Richard Iggo (Swiss Institute for Experimental Cancer Research, Basel, Switzerland). This strain was routinely maintained on solid and liquid SD (minimal) medium (dextrose and yeast nitrogen base) supplemented with leucine, tryptophan, histidine, and 200 µg/l adenine. The yeast strain yIG397 was co-transformed according to the procedures described by Ito et al. [17] and Flaman et al. [13].

In vivo antioxidant analysis

Cells were directly stressed with 2 mM hydrogen peroxide for 1 h at 28°C/160 rpm, or previously treated with 50 µg/ml of **B** (ethyl acetate extract of sheaths of *V. kolbekii*) or 5 mg/ml GBE 761® (standard extract of *Ginkgo biloba*) or 10 µg/ml of flavonoids (**1**, **2**, or quercetin) for 1 h at 28°C/160 rpm before being stressed. The extracts and flavonoids were solubilized in DMSO in all experiments using yeast. Cell viability was analyzed by plating, in triplicate, after proper dilution. Plates were incubated at 28°C for 72 h and the colonies counted. Survival was expressed as a ratio between stressed cells, treated or not, and nonstressed cells [18].

Biomarkers of oxidative stress

For lipid peroxidation and protein carbonylation assays, 50 mg of cells was used (dry weight) and were determined according to the protocols of Steels et al. [19] and Dalle-Donne et al. [10], respectively. The amount of protein was determined according to Stickland [20]. More details are found in the Supporting Information.

Analysis of mutation rate

The antimutagenic effect of the extract and flavonoids **1** and **2** on *TP53* was tested by plating cells on SD medium supplemented with histidine and tryptophan with (ADE+) and without (ADE−) adenine (100 µg/ml). Plates were done in triplicate and colonies were counted after incubation. Percentages of mutation were calculated by the following equation: mutation rate (%) = [(number of cells/ml plated on ADE+) − (number of cells/ml plated on ADE−)]/(number of cells/ml plated on ADE+) [13].

Statistical analyses

Results were expressed as mean ± standard deviation of at least three independent experiments. Statistical differences were tested using Student's *t* test considering parametric data. The latter denotes the homogeneity between experimental groups at $p < 0.05$.

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