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Evaluation of antioxidant activity of Brazilian plants

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Abstract

In this work, 22 alcoholic extracts, obtained from 14 species of plants belonging to four families, used for different food and medicinal purposes in Brazil, were evaluated for their capacity to inhibit the reduction of the free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and to protect *Saccharomyces cerevisiae* cells, an eukaryotic cell model, against the lethal oxidative stress caused by *tert*-butylhydroperoxide (TBH). Five extracts, two from Lamiaceae family (ethanol and butanol extracts from aerial parts of *Hyptis fasciculata*) and three from Palmae family (*Copernicia cerifera* leaves and mesocarp of fruits and the endocarp/mesocarp of fruits from *Orbignya speciosa)* were able to increase the tolerance of *S. cerevisiae* to TBH and showed to be active as DPPH radical scavengers, thus indicating that these plant extracts could be considered as potential sources of antioxidants. With the exception of ethanol extract of *H. fasciculata*, the remainder four extracts exhibited a DPPH radical scavenging activity higher than that obtained from *Ginkgo biloba,* a reference plant with well documented antioxidant activity. Interestingly, the ethanol extract of *G. biloba* were not effective for yeast cell protection, reinforcing the antioxidant potential of these extracts.
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1. Introduction

Chemistry of natural products is a research field with endless potential, and is especially important in countries possessing great biodiversity, as Brazil. In the last years, interest in the antioxidant activity of plant extracts, or isolated substances from plants, has grown, due to the fact that free radicals have been related to some diseases, as well as to the aging process [1]. Reactive oxygen species (ROS) are generated by normal metabolic processes in all oxygen utilizing organisms. It is estimated that about 1% of the total oxygen consumed by mitochondria is transformed into superoxide anion [2]. Damage induced by ROS includes DNA mutation, protein oxidation and lipid peroxidation, contributing to the development of cancer, diabetes, atherosclerosis, inflammation, and premature aging [3]. About 95% of the pathologies observed in people above 35 years of age are associated with production and accumulation of free radicals [4].

Antioxidant properties elicited by plant species have a full range of perspective applications in human healthcare. In recent years, the prevention of cancer and cardiovascular diseases has been associated with the ingestion of fresh fruits, vegetables or teas rich in natural antioxidants [5] what suggests that a higher intake of such compounds should lower the risk of mortality from these diseases [1]. In search for sources of novel antioxidants, in the last few years some medicinal plants have been extensively studied for their

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radical scavenging activity [6]. However, in vivo assays are also necessary to have a more precise evaluation of the potenciality as an antioxidant agent of the plant extracts. *Saccharomyces cerevisiae,* the preferred model for studies of response to stress in eukaryotic cells, is a useful organism for the identification of antioxidant agents. The use of lower organisms, like yeast cells, as model systems is particularly attractive because of the facility in genetic manipulation, the availability of the complete *S. cerevisiae* genomic sequence and the apparent conservation of molecular mechanisms between yeast and human cells. About 30% of the human disease-associated genes significantly match yeast genes and, in contrast to humans, yeast genes can be easily manipulated through molecular biology techniques [7].

The aim of this work was to analyze the antioxidant activity of Brazilian medicinal plant extracts by two complementary approaches: evaluation of the capacity of these extracts to eliminate free radicals in vitro, and analysis of their potential in protecting *S. cerevisiae* cells against oxidation. The present paper deals with a preliminary screening of some Brazilian plants from four families, Palmae, Leguminosae, Polygalaceae and Lamiaceae, chosen due to their uses as human food or as phytomedicines in the treatment of different pathologies.

Among the plants of the Tropics it is difficult to find a family of plants more useful to people than the Palmae family. In fact, this family has been called the most versatile of all due to its many uses. Brazilian palms are a source of a useful leaf fiber and a brittle yellowish wax [8]. Generally, carotenoids are in greater concentrations in palm fruits, as buriti (*Mauritia vinifera*), which could help to explain their use as an anti-tumoral agent [9]. *Bauhinia* species (Leguminosae family) have been used as forage, human food or for the treatment of diabetes [10]. Plants from the Polygalaceae family have been used for their analgesic ability, while plants from Lamiaceae have been found to possess anti-inflammatory activity. It has been well established that all of these diseases have one or more components that point at their etiology/onset as being caused by oxidative damage [3].

Brazil is one of the greatest centers in the world when biodiversity is considered. Furthermore, it should be taken into account that this biodiversity could become lost in a short period of time due to the lack of knowledge of its chemistry and pharmacological potentials. Having theses aspects in mind, a study of Brazilian folk medicinal plants has been undertaken.

2. Materials and methods

2.1. General

Commercial rutin was obtained from Merck® and *Ginkgo biloba* extract (Egb 761) from Tanakan, as a 40 mg (concentration) oral solution. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was purchased from Sigma. Recordings were made in a UV–vis Spectrometer Shimadzu UV-2200. All reagents used, including solvents, were of analytical grade and obtained from Tedia.

2.2. Plant material

Table 1 lists the plants used in this study. All of these plants were formerly mentioned in the treatment of some diseases. They were collected at different periods of time. For each plant a herbarium sample has been deposited in the herbarium of the Botanical Garden of Rio de Janeiro, Brazil.

2.3. Sample preparation

Crude extracts were obtained from different parts of plants after maceration with 96% ethanol, at room temperature for at least 24 h, and repeated until exhaustion of the material. Thereafter, the ethanol crude extract (ECE) was dried under reduced pressure. *Hyptis fasciculata* ECE was also submitted to a liquid–liquid *n*-butanol extraction procedure in order to obtain another extract of different polarity. Therefore, our analysis were conducted with a total of 21 ECE and one butanol extract. The dry extracts were dissolved in 96% ethanol to a final concentration of 1.0 mg ml^{-1}. The standard samples (*G. biloba* and rutin) were prepared using the same dilution procedure.

2.4. DPPH photometric assay

The extracts were measured in terms of hydrogen donation or radical scavenging ability using the stable radical DPPH, which is purple at room temperature [11]. Ethanol solution of the extract (2.5 ml), at different concentrations, were mixed with 1.0 ml of a (0.3 mM) DPPH ethanol solution. The absorbance was measured at 518 nm (ABS_{SAMPLE}) after 30 min of reaction at 25 °C. Ethanol (1.0 ml) plus plant extract solution (2.5 ml) were used as blank. DPPH solution (1.0 ml) plus ethanol (2.5 ml) were used as a negative control. Positive controls were those using the standard solutions of *G. biloba* and rutin. The percentage of DPPH discoloration of the sample was calculated according to the equation % Discoloration $= [1 - (ABS_{SAMPLE}/ABS_{CONTROL})] \times 100$. Discoloration was plotted against sample extract concentration, and a linear regression curve was established in order to calculate the IC_{50} (inhibitory concentration 50 μg ml^{-1}), which means the amount of sample necessary to decrease the absorbance of DPPH in 50%.

2.5. Yeast growth conditions and in vivo antioxidant analysis

The *S. cerevisiae* strain BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) used in the in vivo assays was acquired from Euroscarf, Frankfurt, Germany. Stocks of this strain

Table 1
Plants used in this study

Family	Plant	Part used	Extract	Abbrev.
Palmae	*Copernicia cerifera*	Leaves	Ethanol	CCL
		Mesocarp of fruits	Ethanol	CCM
		Epicarp of fruits	Ethanol	CCEP
	Mauritia vinifera	Mesocarp of fruits	Ethanol	MVM
		Epicarp of fruits	Ethanol	MVEP
	Syagrus oleracea	Epicarp of fruits	Ethanol	SOEP
		Epicarp/mesocarp of fruits	Ethanol	OSM
	Orbigynia speciosa	Endocarp of fruits	Ethanol	OSEN
		Flowers	Ethanol	OSF
		Leaves	Ethanol	OSL
Leguminosae	*Bauhinia variegata*	Aerial parts	Ethanol	BV
	Bauhinia purpurea	Aerial parts	Ethanol	BP
	Bauhinia candida	Aerial parts	Ethanol	BC
	Bauhinia monandra	Aerial parts	Ethanol	BM
	Bauhinia angulosa	Aerial parts	Ethanol	BA
Polygalaceae	*Polygala paniculata*	Aerial parts	Ethanol	PPA
		Roots	Ethanol	PPR
Lamiaceae	*Hyptis fasciculata*	Aerial parts	Ethanol	HF
		Aerial parts	Ethanol	HFB
	Hyptis heterodon	Aerial parts	Ethanol	HH
Ginkgoaceae	*Ginkgo biloba*	Leaves	Ethanol	GB

were maintained on solid YPD medium (1% yeast extract, 2% glucose, 2% peptone and 2% agar) in appropriate conditions to avoid selection of petites or suppressors. Cells were grown up to the middle of exponential phase (1.0 mg dry weight ml^{-1}) in liquid YPD medium, using an orbital shaker at 28 °C and 160 rpm, with the ratio of flask volume/medium of 5/1 [12]. Thereafter, cells were reinoculated in fresh medium (control), and in fresh medium containing, either 3 mM *tert*-butylhydroperoxide (TBH) or 5 mg ml^{-1} plant extract, as well as fresh medium containing 3 mM TBH plus 5 mg ml^{-1} plant extract and incubated, for 3 h, at 28 °C/160 rpm, in the dark (to avoid peroxide degradation). The initial cell concentration was 0.1 mg ml^{-1}.

2.6. Viability determinations

Cell viability was analyzed by plating, in triplicate, on solid YPD medium, after proper dilution. Plates were incubated at 28 °C for 72 h and the colonies counted [12]. Survival rates were calculated in relation to the viability of cells harvested immediately after reinoculation.

2.7. Statistical analysis

Experiments were done in triplicate. The results are given as a mean ± standard deviation (S.D.). Student's *t*-test was used for comparison between two means and a one-way analysis of variance (ANOVA) was used for comparison of more than two means. A difference was considered statistically significant when $p \leq 0.05$.

3. Results

DPPH is a free radical, stable at room temperature, which produces a violet solution in ethanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncolored ethanol solutions. The use of DPPH provides an easy and rapid way to evaluate antioxidant activity. Results of DPPH reduction by extracts are shown in Table 2. The IC_{50} values were obtained by linear regression and showed a very good coefficient of determination ($r^2 \geq 0.95$). Statistical analysis (ANOVA) of data from the three separate tests showed that all experiments made with each sample, were statistically equivalent ($p \leq 0.05$).

Nine extracts (CCL, CCM, CCEP, OSM, BV, BC, BF, HF and HFB) exhibited IC_{50} values below 60 μg ml^{-1}, indicating a good potential as free radical scavengers (Table 2). Among them, six extracts showed a higher capacity of scavenging the DPPH free radical (three extracts from the Palmae family, two extracts from the Leguminosae family and one extract from the Lamiaceae family) than *G. biloba* ethanol extract used as a standard (positive control). Two extracts of the Palmae family exhibited an outstanding DPPH scavenging potential: CCM, whose IC_{50} was almost the same as purified rutin, (rutin IC_{50} was 11.9 ± 0.4 μg ml^{-1}); and CCL that showed a twofold higher, in vitro, antioxidant activity than the *G. biloba* standard extract. The unusual high IC_{50} found for some extracts (Table 2) were achieved by mathematical equation due to the fact that those ones had a very low activity in DPPH assay.

To analyze the capacity of extracts in protecting *S. cerevisiae* cells against oxidation, viability was determined after

Table 2
Yeast survival rates after incubation with TBH, in the presence or not of plant extracts, and DPPH radical scavenging activities (expressed as IC_{50})

Extract	In vivo assay survival (%)			In vitro assay
	Control (no drugs)	TBH	TBH + extract	EC_{50} ($\mu g\,ml^{-1}$)
CCL	100	0	42.0 ± 2.7	23.5 ± 0.1
CCM	100	0	36.1 ± 3.6	15.3 ± 0.4
CCEP	100	0	0	41.9 ± 0.8
MVM	100	0	15.5 ± 2.2	538.3 ± 2.0
MVEP	100	0	0	71.0 ± 1.0
SOEP	100	0	16.8 ± 1.8	425.5 ± 1.9
OSM	100	0	15.0 ± 2.3	27.0 ± 0.2
OSEN	100	0	0	4104.3 ± 6.7
OSF	100	0	0	427.4 ± 1.8
OSL	100	0	0	895.9 ± 2.3
BV	100	0	0	37.0 ± 0.4
BP	100	0	0	137.9 ± 1.1
BC	100	0	0	45.4 ± 0.9
BM	100	0	0	199.8 ± 1.6
BA	100	0	0	106.4 ± 1.1
BB	100	0	0	68.4 ± 1.0
BF	100	0	0	35.1 ± 0.3
PPA	100	0	0	135.4 ± 1.1
PPR	100	0	0	325.6 ± 1.6
HF	100	0	7.3 ± 0.5	57.9 ± 0.9
HFB	100	0	4.0 ± 0.7	35.0 ± 0.3
HH	100	0	0	233.4 ± 1.3
G. biloba EGb 761®	100	0	0	41.5 ± 0.1

oxidative stress with or without plant extracts. To verify their possible toxic effects, cells were also grown in YPD medium supplemented with 5 mg ml^{-1} extracts. None of them caused growth inhibition (results not shown). The oxidative stress generator used in this study was TBH, which leads to lipid peroxidation, a parameter associated with cell death [12]. As expected, cells showed extreme sensibility to 3 mM TBH, and were unable to survive when exposed to this condition (Table 2). However, seven extracts increased cell tolerance to TBH: CCL, CCM, MVM, SOEP, OSM, HF and HFB. Furthermore, the ECE of *Copernicia cerifera* leaves showed the best protective effect, and even after 17 h of exposure to TBH, 11% of cells survived (results not shown).

With the exception of the extracts obtained from MVM and SOEP, which exhibited IC_{50} higher than 400 $\mu g\,ml^{-1}$, all others, that increased cell tolerance to TBH, showed low IC_{50} values (Table 2). On the other hand, four extracts that were found to be more active as DPPH scavengers (CCEP, BV, BC and BF) did not exert any protective effect upon *S. cerevisiae*. Likewise, the ethanol extract of *G. biloba*, used as standard during in vitro analysis, was inactive for cell protection against TBH.

4. Discussion

We have studied several plants belonging to different families, trying to validate some of their uses by Brazilian people. A review pointed out that, today, approximately 60% of the anti-tumoral and anti-infective agents, either commercially available or in late stages of clinical trials are of natural origin [13].

Based on the mechanism of reduction of the DPPH molecule, extensively described in the literature, and on previous knowledge of the chemistry of some plants, it is possible to infer that the strong antioxidant activity of some polar extracts is due, at least in part, to the presence of substances with an available hydroxyl group, flavonoids, in this case [14,15].

We have also attempted to evaluate the antioxidant activity of plant extracts, using the yeast, *S. cerevisiae*, as an experimental model. The usefulness of lower eukaryotic organisms, such as yeast, in drug activity screening has been largely demonstrated [16]. In analyzing results obtained, the capacity of some extracts of protecting yeast cells against TBH, in most cases, correlated with their anti-radicalar activity. However, results of the screening tests indicated that the antioxidant potential estimated by the in vitro DPPH assay, though present in 40% of the extracts tested, did not confer protection to yeast cells, per se, against oxidative stress. Out of nine extracts that positively reduced the DPPH radical ($IC_{50} \leq 60\ \mu g\,ml^{-1}$), only five were able of increasing yeast cell tolerance to TBH (Table 2).

This occurs because the mechanism involved in the reduction of DPPH free radicals is based on a scavenging activity. In this system, the structure (both planar and spatial) of the antioxidant compound, present in the extract, is important for its capacity of donating hydrogen ions. Compounds able to donate hydrogen are derived from the shikimate pathway, as for example, flavonoids [17]. These molecules are not produced by plants whose extracts display a very high IC_{50} in the DPPH test. Such plants are, in fact, very rich in compounds of the acetate pathway, like terpenoids and fatty acids, which are unable of scavenging the DPPH free radical, but are able to avoid oxidative damage of cell membranes [18]. This is the case of extracts from the mesocarp of fruits from *M. vinifera* and from the epicarp of fruits from *Syagrus oleracea* (Table 2). Antioxidants involved in the protection against TBH, which leads to lipid peroxidation [12], are those available at the site of radical attack. They break the chain of oxidation by being preferentially oxidized by the attacking radical, thereby preventing oxidation of the adjacent fatty acid of cell membrane [1]. The plant extracts that scavenge the DPPH radical and also protect *S. cerevisiae* against oxidative damage are probably rich in both compounds, encompassing both pathways. This is the case of CCL, CCM, OSM, HF and HFB extracts.

Another important result of our work is that these two methodologies were able to infer about the possible oxidative etiology of the diseases for which the mentioned plants are used: generally, the extracts tested exhibited antioxidant potential as a radical scavenger or as a cell protector, or both. Few extracts (HH, BM, PPR, OSL, OSF and OSEN) did not show either in vitro or in vivo antioxidant activities. This

null effect could be explained by the fact that the species from which the extracts were obtained are not exactly the species used popularly for the purpose. Therefore, although some *Hyptis* species are used to treat inflammatory diseases, *Hyptis heterodon* (HH) seems to be an ineffective species. Some *Bauhinia* species are employed to lower blood glucose levels, but *Bauhinia monandra* (BM) showed a very weak effect. *Polygala paniculata* leaves have anti-nociceptive activity, but not the roots (PPR). The mesocarp of the fruit from *O. speciosa* is used in prevention and treatment of leukemia. However, its leaves (OSL), flowers (OSF) as well as the endocarp of fruits (OSEN) have no effect.

It is noteworthy to mention that apparent unexpected result with the EGb 761 (*G. biloba* standard extract) has a coherent explanation. The molecules present in this extract (oxidized diterpenes and glucosylated flavonoids) are not able to protect the damage induced by TBH on *S. cerevisiae* membranes, making them inactive in this methodology.

Finally, according to our results, the yeast *S. cerevisiae* was confirmed as being useful in evaluating the antioxidant activity of plant extracts. There may, of course, be limitations in extrapolating results obtained with yeast cells, due to differences in the molecular environment and the more complex genetic interactions in mammals. However, *S. cerevisiae* could be proposed as a cell model to provide an initial and rapid screening of the antioxidant potential of plants. Furthermore, this in vivo methodology could be complemented by in vitro assays, producing a more complete analysis of the antioxidant capacity of plant extracts.

There are several other in vitro methodologies that measure different kinds of free radical species such as lipid peroxidation, deoxyribose damage assay, superoxide scavenger capacity, among others [11,19]. Nevertheless, it is important to use an in vivo methodology to assess the real capacity of an antioxidant to protect cells. This way, the present work should be complemented with further studies in order to understand the mechanistic features of each used methodology.

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