JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Antioxidant and Antimutagenic Properties of *Hibiscus* Tiliaceus L. Methanolic Extract

RENATO MOREIRA ROSA,[†] MARIA INÉS S. MELECCHI,[‡]
RAFAEL DA COSTA HALMENSCHLAGER,[†] FERNANDA C. ABAD,[‡]
CRISTINA ROSAT SIMONI,[†] ELINA B. CARAMÃO,[‡]
JOÃO ANTONIO PÊGAS HENRIQUES,[†,§] JENIFER SAFFI,[*,†,§] AND
ANA LÍGIA LIA DE PAULA RAMOS[*,†]

Departamento de Biofísica and Instituto de Química, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil, and Laboratório de Genética Toxicológica, Universidade Luterana do Brasil, ULBRA, Canoas, RS, Brazil

The genus *Hibiscus* thrives in a variety of climates and produces a diversity of natural compounds with bioactive properties. We have studied the chemical composition and the in vivo antioxidant properties of *Hibiscus tiliaceus* L. methanolic flower extract, as well as its mutagenic/antimutagenic effects. Vitamin E and some stigmasterol derivatives that might confer an antioxidant effect to the extract were present. Treatment with this extract protected several *Saccharomyces cerevisiae* strains defective in antioxidant defenses against H_2O_2 and *t*-BOOH cytotoxicities, showing a clear antioxidant activity. The effect is the same for all strains used, independent of the antioxidant defense disrupted, suggesting that protection may be due to molecules that act as versatile and wide spectrum nonenzymatic antioxidants, such as vitamins or phytosterols. The extract was not mutagenic in either *Salmonella typhimurium* or *S. cerevisiae* and showed a significant antimutagenic action against oxidative mutagens in *S. cerevisiae*.

KEYWORDS: Phytotherapics; antioxidant; *Hibiscus tiliaceus* L.; *Saccharomyces cerevisiae*; antimutagenesis; Ames

INTRODUCTION

In recent times, focus on plant research has increased all over the world and a large body of evidence has been collected to show the immense potential of medicinal plants used in traditional systems. Various medicinal plants have been studied using modern scientific approaches, and the results have revealed the potential of medicinal plants in the area of pharmacology (*1*).

There is a considerable amount of epidemiological evidence revealing an association between diets rich in fruits and vegetables and a decreased risk of cardiovascular disease and certain forms of cancer (*2*−*4*). It is generally assumed that components of higher plants are active dietary constituents that contribute to these protective effects. For instance, α-tocopherol, β-carotene, and polyphenolic compounds may act as antioxidants or in other ways protect against cancers and cardiovascular diseases (*5*).

* To whom correspondence should be addressed. (J.S.) Tel: +55 51 3477 4000 ext. 2774. Fax: +55 51 3477 9239. E-mail: jenifer.saffi@ulbranet.com.br. (A.L.L.P.R.) Tel: +55 51 3316 7605. Fax: +55 51 3316 7003. E-mail: ana.ramos@ufrgs.br.
[†] Departamento de Biofísica, Universidade Federal do Rio Grande do Sul.
[‡] Instituto de Química, Universidade Federal do Rio Grande do Sul.
[§] Laboratório de Genética Toxicológica, Universidade Luterana do Brasil.

Species of the genus *Hibiscus* have been used in several applications, for example, as an antidote to poisoning with chemicals and venomous mushrooms in traditional medicine and as a source of fiber in the pulp and paper industries. Members of the genus *Hibiscus* thrive in a variety of climates and produce a diversity of natural compounds with bioactive properties, such as lignanamides, naphthalenes, polyphenol compounds, carotenoids, tochopherols, flavonoids, anthocyanins, phytosterols, and long-chain fatty esters (*6*). *Hibiscus tiliaceus* L. is a typical plant of tropical climates found in the regions of mangroves in significant quantities (*7*). It is also known as "Algodoeiro da Praia" (beach cotton) and occurs in mangroves in Santa Catarina state, in the south of Brazil. Their flowers are widely used in birth control in Asian and African countries (*8*−*10*). An infusion of the dried wood was used in folk medicine to expel the placenta and to combat postparturition disorders (*11*). An aqueous extract of wood and fresh flowers is registered for skin diseases (*12*−*15*). However, its chemical composition and biological and pharmacological effects are still poorly defined.

The aim of the present work was to evaluate the antioxidant and antimutagenic properties of *H. tiliaceus* L. methanolic flower extracts (HME) in vivo and to correlate these properties with the chemical composition of the extract. For the investiga-

10.1021/jf061407b CCC: $33.50 © 2006 American Chemical Society
Published on Web 08/29/2006

Table 1. *S. cerevisiae* Strains Used in This Study

strain	genotype	enzymatic defense lacking	source
BY4741	*Matα his3Δ1 leu2Δ0 lys2Δ0 ura 3Δ0*	none	EUROSCARF
cat1Δ	Like BY4741 except *cat1*::KanMx	cytosolic catalase	EUROSCARF
gpx3Δ	Like BY4741 except *gpx3*::KanMx	glutathione peroxidase 3	EUROSCARF
glr1Δ	Like BY4741 except *glr1*::KanMx	glutathione reductase	EUROSCARF
yap1Δ	Like BY4741 except *yap1*::KanMx	yAP-1 transcription factor	EUROSCARF
gsh1Δ	Like BY4741 except *gsh1*::KanMx	gluthatione sintetase 1	EUROSCARF
SOD+	*Matα his3Δ1 leu2Δ0 trp1–289 ura3–52*	none	E. B. Gralla, Los Angeles
sod1Δ	Like SOD+ except *sod1*::URA3	Cu–Zn superoxide dismutase	E. B. Gralla, Los Angeles
sod2Δ	Like SOD+ except *sod2*::TRP1	Mn superoxide dismutase	E. B. Gralla, Los Angeles
sod1Δsod2Δ	Like SOD+ except *sod1*::URA3 sod2::TRP1	all superoxide dismutases	E. B. Gralla, Los Angeles
N123	Mata his*1–7*	none but exhibits low glutathione content	J. A. P. Henriques, Porto Alegre
XV185-14c	*Matα his1–7 ade2–2 arg4–17 trp5–48 lys1–1 hom3–10*	none	R. C. Von Borstel

tion of the effect in living systems, we have used hydrogen peroxide (H_2O_2) and *t*-butyl-hydroperoxide (*t*-BOOH) to induce oxidative damage in *Saccharomyces cerevisiae* strains defective in several antioxidant defenses. We have also evaluated the effects of the extract on genomic stability in both *Salmonella typhimurium* and *S. cerevisiae* models.

MATERIALS AND METHODS

Chemicals. All of the solvents were bidistilled and stored in dark flasks. Anhydrous sodium sulfate was analytical grade and heated at 300 °C before used. H_2O_2, *t*-BOOH, 4-nitroquinoline-oxide (4-NQO), D-biotin, aflatoxin B1, aminoacids (L-histidine, L-threonine, l-metionine, L-tryptophan, L-leucine, and L-lysine), nitrogen bases (adenine and uracil), l-canavanine, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). The S9 fraction, prepared with the polychlorinated biphenyl mixture Aroclor 1254, was purchased from Moltox (Annapolis, MD), and glucose-6-phosphate and NADP were obtained from Sigma. Oxoid nutrient broth no. 2, used in the Ames test, was obtained from Oxoid USA Inc. (Maryland).Yeast extract, bacto-peptone, and bacto-agar were obtained from Difco Laboratories (Detroit, MI).

Plant Material. *H. tiliaceus* L. flowers were collected in the mangroves from Santa Catarina, Brazil, in December 1999 and January 2000. The plant was identified by Dr. B. Irgand (Instituto de Biociências, UFRGS, RS, Brazil). A dried specimen (ICN: 113936) was deposited in the herbarium of UFRGS. Flowers were dried at 40 °C, ground, and stored in dark bags to protect them from humidity and light.

Preparation of Methanol Extracts. Fifteen grams of dried and ground flowers was continuously extracted for 48 h with methanol in a Soxhlet apparatus. The extract was filtered and concentrated in a rotary evaporator at 30−40 °C to obtain a semisolid material. The viscous residue thus obtained was kept in a vacuum desiccator over phosphorus pentoxide for 24 h to obtain a completely dry solid mass. This mass was denominated HME.

Chemical Characterization of the Extract. Because of its complex nature, the extract was submitted to the following PLC (preparative liquid chromatographic) fractionation on silica: Two grams of silica was slurry packed in a glass column (20 cm × 1 cm) with *n*-hexane. Twenty milliliters of hexane, hexane/benzene (1:1), dichloromethane, ethyl acetate, and methanol was used to elute 500 mg of the extract from the column. The first three fractions were obtained only to clean up the extract, and they are composed mainly of hydrocarbons (*16*). The last two fractions were concentrated under nitrogen to 1 mL and analyzed. Analyses were performed using an QP5050A gas chromatograph coupled to a GCT mass spectrometer (Shimadzu Co., Japan). Adequate separation of the analytes was achieved using an OV-5 (polymethylsiloxane with 5% phenyl groups) fused open tubular column (30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas with a linear velocity of 1.0 mL/min. Sample aliquots of 2 μL were injected. The oven temperature program was as follows: initial temperature, 50 °C for 2 min; 50−280 °C at 3 °C/min, and 30 min at 280 °C. The split/splitless injector, in the split mode (1:20), was kept at 280 °C, and the interface was kept at 280 °C. The conditions used in EI+ mode were as follows: ion source temperature, 280 °C; electron energy, 70 eV; filament current, 500 μA; and resolution, 7000 fwhm.

Mutagenicity Testing by the *Salmonella*/Microsome Assay. *S. typhimurium* TA98 and TA102 strains were kindly provided by B. M. Ames (University of California, Berkeley, CA) and M. Blanco (Instituto de Investigaciones Citológicas, Valencia, Spain), respectively. Mutagenicity was assayed by the preincubation procedure described (*17*, *18*). HME was dissolved in distilled water. One hundred microliters of test bacterial cultures ($1-2 \times 10^9$ cells/mL) was incubated at 37 °C with different amounts of HME in the presence or absence of S9 mix for 20 min, without shaking. Subsequently, 2 mL of soft agar (0.6% agar, 0.5% NaCl, 50 μM histidine, and 50 μM biotin, pH 7.4, 45 °C) was added to the test tube and poured immediately onto a plate of minimal agar (1.5% agar, Vogel−Bonner E medium, containing 2% glucose). Aflatoxin B_1 (1.0 μg/plate) was used as a positive control for all strains in the metabolic assay with S9 mix. In the absence of S9 mix, the positive control was 4-NQO (0.5 μg/plate) for TA98 and TA102. The plates were incubated in the dark at 37 °C for 48 h before the revertant colonies were counted. Mutagenicity data were analyzed with Salmonel software (*19*). A compound was considered positive for mutagenicity only when (a) the number of revertants was at least double the spontaneous yield ($MI \geq 2$; MI = mutagenic index: no. of induced colonies in the sample/no. of spontaneous in the negative control); (b) a significant response for analysis of variance ($p \leq 0.05$) was found; and (c) a reproducible positive dose−response ($p \leq 0.01$) was present, evaluated by the Salmonel software (*19*). A cytotoxic effect was considered when $MI \leq 0.6$.

Assays with *S. cerevisiae*. The relevant genotypes of *S. cerevisiae* strains used in this work are given in **Table 1**. Media, solutions, and buffers were prepared as previously described (*21*). Complete medium YPD containing 0.5% yeast extract, 2% bacto-peptone, and 2% glucose was used for routine growth of yeast cells. For plates, the medium was solidified with 2% bacto-agar. The minimal medium (MM) contained 0.67% yeast nitrogen base without amino acids and 2% glucose, and 2% bacto-agar was supplemented with the appropriate amino acids. The synthetic complete medium (SC) was MM supplemented with 2 mg of adenine, 2 mg of arginine, 5 mg of lysine, 1 mg of histidine, 2 mg of leucine, 2 mg of methionine, 2 mg of uracil, 2 mg of tryptophan, and 24 mg of threonine per 100 mL MM. For mutagenesis in the strain XV-185-14c, the omission media lacking lysine (SC-lys), histidine (SC-his), or homoserine (SC-hom) were used. For determination of forward mutation frequency induced by H_2O_2 or *t*-BOOH, in strain N123, cells treated with this chemical were plated onto SC plates lacking arginine and supplemented with 60 μg/mL canavanine.

Stationary phase cultures were obtained by the inoculation of an isolated colony in liquid YPD. After 48 h at 30 °C with aeration by shaking, the cultures contained $2-3 \times 10^8$ cells/mL. Exponential phase cultures were obtained by inoculation of 5×10^5 cells/mL of the same YPD culture in stationary phase into 5 mL of fresh YPD medium. After 18 h of incubation, at 30 °C, with aeration by shaking, the cultures contained $1-2 \times 10^7$ cells/mL with 20−30% budding cells (*20*).

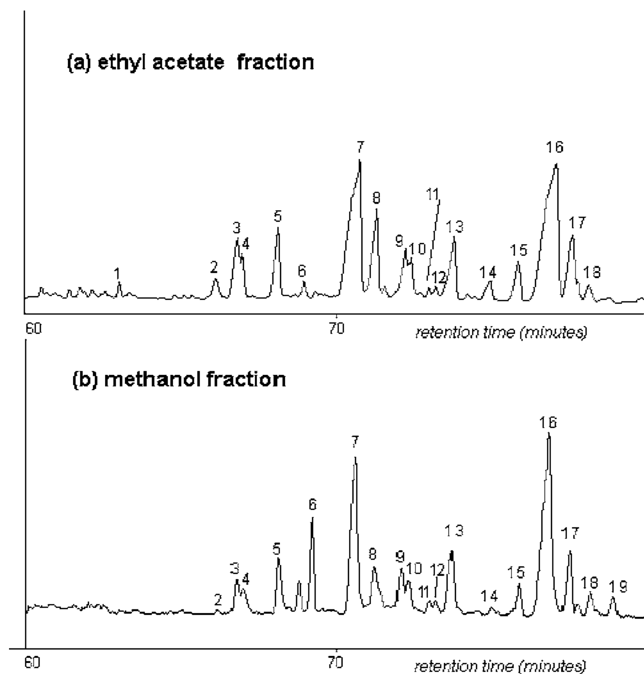

Figure 1. TIC of the fraction of the methanol extract of *H. tiliaceus* L. flowers: (**a**) ethyl acetate fraction and (**b**) methanol fraction.

Antioxidant Assays in *S. cerevisiae*. Yeast cells were grown overnight in YPD medium at 28 °C in an orbital shaker to the stationary growth phase. A suspension containing 1 × 10^8 cells/mL was shaken for 3 h at 30 °C with several concentrations of HME in phosphate buffer (PB, 0.067 M, pH 7.0). Cells were then harvested by centrifugation and washed twice with PB. The cell density was determined using a Neubauer counting chamber, and the sensitivity to H_2O_2 or *t*-BOOH was estimated by the spot test. In this case, after treatment, a 10 μL drop of each decimal dilution (10^8−10^3 cells/mL) was spotted onto SC plates containing the oxidant agents. After incubation for 2 days at 30 °C, each resistant drop received one point in the survival score, according to Lewinska et al. (*22*). Data represent the average of at least three independent experiments.

Detection of HME-Induced Reverse and Frame Shift Mutation in *S. cerevisiae*. Mutagenesis was measured in *S. cerevisiae* strain XV185-14c. A suspension of 2 × 10^8 cells/mL in either exponential or stationary phase was incubated for 18 h at 30 °C with various concentrations of extract. Cell survival was determined on SC (3−5 days, 30 °C) and mutation induction (LYS, HIS, or HOM revertants) on the appropriate omission media (7−10 days, 30 °C). The allele *his1−7* is a nonsuppressible missense allele, and reversions result from mutation at the locus itself (*23*), whereas *lys1−1* is a suppressible ochre nonsense mutant allele (*24*), which can be reverted either by locus-specific or by a forward mutation in a suppressor gene (*25*, *26*). True reversions and forward (suppressor) mutations at the *lys1−1* locus were differentiated according to ref *27*, indicating that the reduced adenine content of the medium SC-lys shows locus reversions as red and suppressor mutations as white colonies. It is believed that *hom3−10* contains a frameshift mutation due to its response to a range of diagnostic mutagens (*26*). Assays were repeated at least four times, and plating was in triplicate for each dose. Data from mutagenesis, recombinogenesis, and survival assays in *S. cerevi*siae were expressed as means and standard deviations and were statistically analyzed using Student's *t*-test. Differences were considered significant when $P < 0.05$ (*28*).

Detection of Potential Antimutagenic Activity of HME in Oxidative Mutagenesis in *S.cerevisiae*. Strain N123 was used for this analysis because it is very responsive to H_2O_2-induced mutagenesis since it shows a low glutathione content (*29*). Yeast cells were cultured overnight in YPD medium at 30 °C in an orbital shaker until the cell suspension reached a density of 1−2 × 10^8 cells/mL. Cells were harvested and washed twice by centrifugation with PB and submitted to H_2O_2 or *t*-BOOH and HME at 28 °C for 2 h in the dark with shaking.

Table 2. Identification of Compounds in TIC and Respective Retention Time (T_r)

compounds	T_r
1. vitamin E	63.033
2. ergostatrienol	66.158
3. fucosterol	66.585
4. ergostenol	67.009
5. stigmastadienol (isomer 1)	68.149
6. androstanone-acetyloxyl	69.008
7. stigmastenol	70.799
8. stigmastadienol (isomer 2)	71.349
9. fucostenone	72.250
10. stigmastenone (isomer 1)	72.458
11. stigmastadienone (isomer 1)	73.067
12. stigmastadienol acetate	73.358
13. stigmastadienone (isomer 2)	73.826
14. hydroxy ergostenone	74.958
15. ergostenedione	75.875
16. stigmastenone (isomer 2)	77.069
17. ergostadienone	77.597
18. sigmastadienone (isomer 3)	78.175
19. stigmasterol	79.95

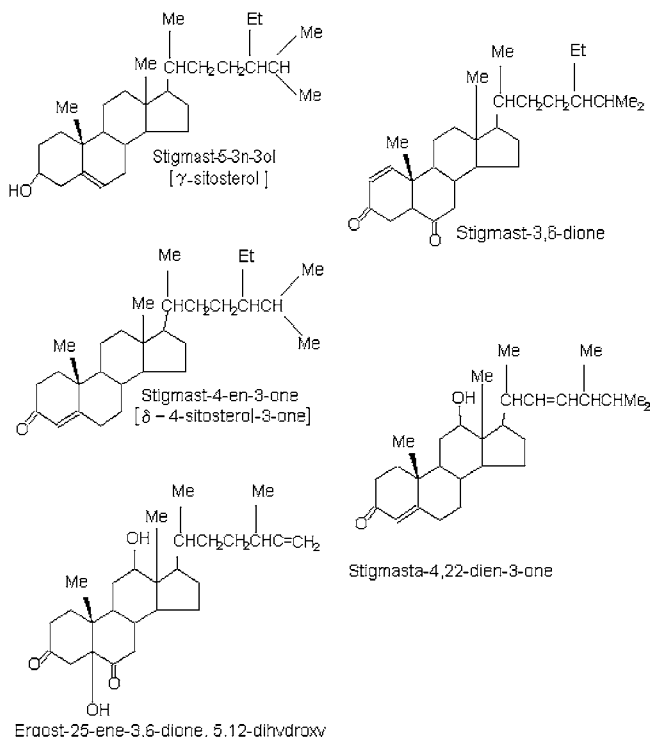

Figure 2. Some structures of identified compounds in the *H. tiliaceus* L. extract.

The cell concentration and percentage of budding cells in each culture were determined by microscope counts using a Neubauer chamber. After treatment, appropriate dilutions of cells were plated onto SC plates to determine cell survival and 100 μL aliquots of cell suspension (2 × 10^8 cells/mL) were plated onto SC media supplemented with 60 μg/mL canavanine and incubated for 4−5 days at 30 °C. Data represent the average of at least three experiments.

RESULTS

Chemical Analysis of HME. Figure 1 shows the total ion chromatogram (TIC) for the ethyl acetate and methanol fractions of the HME, and **Table 2** shows the identification of numbered peaks. **Figure 2** shows the structure of some of the identified compounds. The main compounds identified were stigmasterol derivatives with very similar structures.

Table 3. Protective Effect of HME in Yeast Strains Against H_2O_2 Toxicity[a]

H_2O_2 (mM)	treatment			
	PBS	0.1% HME	0.25% HME	0.5% HME
		BY4741		
1	+++	+++	+++	+++
2	++	++	+++	+++
		*cat1*Δ		
0.25	++	++	++	++
0.5	+	++	++	++
		*gpx3*Δ		
1.5	+	+++	+++	+++
2	+	+++	+++	+++
		*glr1*Δ		
1.5	+	+++	+++	+++
2	+	+++	+++	+++
		*gsh1*Δ		
1	++	+++	+++	+++
1.5	–	++	+++	+++
		*yap1*Δ		
0.05	++	++	++	++
0.1	+	+	+	+
0.2	–	–	–	–
0.3	–	–	–	–
		SOD+		
0.75	+++	+++	+++	+++
1.0	+++	+++	+++	+++
1.5	++	++	++	+++
2.0	+	++	++	+++
		*sod1*Δ		
0.75	+	+++	+++	+++
1.0	–	++	+++	+++
1.5	–	++	+++	+++
2.0	–	++	++	+++
		*sod2*Δ		
0.75	++	++	++	++
1.0	–	–	+	+
1.5	–	+	++	++
2.0	–	+	+	++
		*sod1*Δ *sod2*Δ		
0.75	++	++	++	++
1.0	+	+++	+++	+++
1.5	–	+	++	+++
2.0	–	+	++	+++

[a] Key: +++, growth not inhibited; ++, partial inhibition; +, strong inhibition; and –, complete inhibition of growth.

Table 4. Protective Effect of HME in Yeast Strains against *t*-BOOH Toxicity[a]

t-BOOH (mM)	treatment			
	PBS	0.1% HME	0.25% HME	0.5% HME
		BY4741		
0.5	+++	+++	+++	+++
1	++	+++	+++	+++
		*cat1*Δ		
0.15	+	+	++	++
0.30	+	++	+++	+++
		*gpx3*Δ		
0.4	+	+++	+++	+++
0.8	–	++	+++	+++
		*glr1*Δ		
0.5	+	++	+++	+++
1.0	–	+	++	+++
		*gsh1*Δ		
0.5	–	–	++	+++
1.0	–	+	+++	+++
		*yap1*Δ		
0.025	+	+	+	+
0.05	+	+	+	+
0.075	–	–	–	–
0.1	–	–	–	–
		SOD+		
0.25	+++	+++	+++	+++
0.5	++	+++	+++	+++
0.75	++	++	++	+++
1.0	+	+	++	++
		*sod1*Δ		
0.25	+++	+++	+++	+++
0.5	++	+++	+++	+++
0.75	+	+++	+++	+++
1.0	–	+	++	+++
		*sod2*Δ		
0.25	++	++	++	++
0.50	+	+	+	+
0.75	–	+	++	++
1.0	–	+	+	++
		*sod1*Δ *sod2*Δ		
0.25	++	+++	+++	+++
0.50	+	++	+++	+++
0.75	–	++	++	+++
1.0	–	++	++	+++

[a] Key: +++, growth not inhibited; ++, partial inhibition; +, strong inhibition; and –, complete inhibition of growth.

In Vivo Antioxidant Assays. Tables 3 and **4** clearly show that incubation of the strains with HME results in an increase of the survival after H_2O_2 and *t*-BOOH treatment, respectively, for strains of *S. cerevisiae* defective in antioxidant defense, except for the strain defective in Yap1 transcription factor. This increase of survival score does not occur in a dose-related manner and is identical for all strains used, independent of the type of enzymatic defense lacking.

Mutagenicity/Antimutagenicity of HME. As can be seen in **Table 5**, mutagenicity was not found in TA98 (detects frameshift mutation in the DNA target -C-G-C-G-C-G-C-G-), for HME either in the absence or in the presence of metabolic activation. Negative results for mutagenicity were also observed in TA102, which detects oxidative agents, alkylating mutagens, and reactive oxygen species. HME did not induce mutagenicity in *S. cerevisiae* (**Table 6**). The frequency of point (HIS1+, LYS1+) and frameshift (HOM3+) mutations during treatment is not significant. In **Table 7**, it can be seen that HME treatment increases the survival of strain N123 during H_2O_2 or *t*-BOOH treatment and simultaneously reduces, in a dose-dependent manner, the forward mutagenesis induced in yeast, as measured by the number of canavanine resistant colonies.

DISCUSSION

Pharmacological investigations of the genus *Hibiscus* have demonstrated interesting biological activities. *Hibiscus sabdarifa*, a traditional chinese rose tea, is used against hypertension, inflammation, liver disorders, and mutagenicity (*30*−*33*). Studies have shown that *Hibiscus rosa sinensis* possesses anticomplementary, antidiarrheic, antiphlogistic, antispermatogenic, androgenic, antitumor, antidiabetic, and anticonvulsant activities (*34*−*39*). Furthermore, the dried flower of *Hibiscus syriacus*, widely distributed over East Asia, is used as an antipyretic and anthelmintic (*40*).

In this work, a methanolic extract of *H. tiliaceus* flowers was studied to determine its in vivo antioxidant activity. The possible antioxidant effect of HME may be explained by the presence of vitamin E and several derivatives of stigmasterol, which were identified in the samples. Vitamin E is a well-studied molecule

Table 5. Induction of His+ Revertants in *S. typhimurium* TA98 and TA102 Strains Treated with Methanolic Extract of *H. tiliaceus* L. Flowers with (+S9) and without (–S9) Metabolic Activation

	strains of *S. typhimurium*							
	TA98 (–S9)		TA98 (+S9)		TA102 (–S9)		TA102 (+S9)	
dose (μg/plate)	rev/plate[b]	MI[a]	rev/plate	MI	rev/plate	MI	rev/plate	MI
NC[c]	41.33 ± 7.77	1.00	43.33 ± 5.86	1.00	214.00 ± 11.14	1.00	216.00 ± 10.58	1.00
100	36.33 ± 6.35	0.88	52.00 ± 5.29	1.20	210.67 ± 12.22	0.98	218.67 ± 40.46	1.01
200	43.00 ± 6.08	1.04	41.00 ± 7.00	0.95	197.33 ± 32.08	0.92	224.67 ± 48.43	1.04
500	36.00 ± 15.72	0.87	50.00 ± 6.00	1.15	180.67 ± 16.04	0.84	217.33 ± 44.06	1.01
1000	45.67 ± 8.62	1.11	51.50 ± 9.19	1.18	202.67 ± 42.39	0.95	197.33 ± 8.33	0.91
2000	41.67 ± 12.10	1.01	46.33 ± 5.69	1.06	229.33 ± 28.10	1.07	204.00 ± 24.33	0.94

[a] Mutagenic index = no. of His+-induced in the sample/no. of spontaneous His+ in the negative control (zero). [b] Number of His+/plate, mean values at least of two experiments ± SD in triplicate. [c] Negative control: sterile distilled water. Positive control: TA98 (–S9), 4-NQO (0.5 μg/plate), 517 ± 105.53; (+S9) aflatoxin B1 (10 μg/plate), 1031 ± 139.00; TA102 (–S9), 4-NQO (0.5 μg/plate), 346 ± 45.2; (+S9) aflatoxin B1 (10 μg/plate), 972 ± 133.7.

Table 6. Induction of Point Mutation for (*his*1–7), Ochre Allele (*lys*1–1), and Frameshift Mutations (*hom*3–10) in the Haploid *XV185-14c* Strain of *S. cerevisiae* after Treatment with the Methanolic Extract of *H. tiliaceus* L. Flowers in the Stationary Phase of Growth

dose	survival (%)	His1/10^7 survivors[a]	Lys1/10^7 survivors[b]	Hom3/10^7 survivors[a]
0	100	7.45 ± 2.19	1.95 ± 0.63	6.50 ± 2,40
0.1%	106.5	3.55 ± 2.19	1.75 ± 0.63	8.45 ± 0.63
0.5%	97.10	6.85 ± 0.49	2.30 ± 1.13	7.55 ± 2.47
0.5 μg/mL 4-NQO[c]	62.06	93.0 ± 7.62*	28.9 ± 7.35*	13.0 ± 1.60*

[a] Locus-specific revertants. [b] Locus nonspecific revertants (forward mutation). [c] Positive control. Mean and standard deviation per three experiments independents in triplicate; *data significant in relation to negative control group (solvent) at $P < 0.001$/two-tailed Students' *t*-test.

Table 7. Effect of Methanolic Extract of *H. tiliaceus* L. Flowers on H_2O_2– and *t*-BOOH Cytotoxicity and -Induced Forward Mutation in N123 Yeast Strain[a]

	survival (%)	*CAN1* mutants/ 10^7 survivors
negative control	100	10.13 ± 4.79
4 mM H_2O_2	45.60 ± 0.26	377.45 ± 41.68
4 mM H_2O_2 plus 0.1% HME	40.20 ± 0.07**	371.49 ± 44.53
4 mM H_2O_2 plus 0.25% HME	61.67 ± 7.70**	256.6 ± 38.20**
4 mM H_2O_2 plus 0.5% HME	68.12 ± 4.91**	45.5 ± 27.60***
1.5 mM *t*-BOOH	36.49 ± 12.8	154.12 ± 33.30
1.5 mM *t*-BOOH plus 0.1% HME	61.25 ± 0.15	126.11 ± 22.04
1.5 mM *t*-BOOH plus 0.25% HME	68.44 ± 2.22*	101.0 ± 2.10**
1.5 mM *t*-BOOH plus 0.5% HME	72.2 ± 5.12**	84.13 ± 17.10***

[a] Mean and standard deviation per three experiment independents in triplicate. Data significant in relation to positive control (H_2O_2 or *t*-BOOH) and the mutagenic agent plus HME treatment at *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$ two-tailed Students' *t*-test.

that acts as an effective lipophilic antioxidant and potent radical scavenger, stabilizing cell membranes and reducing free radical-induced DNA damage (*41*). Phytosterols such as stigmasterol, stigmastadienol, and stigmastadienone, present in this extract, are recognized as antioxidants (*42*) and have a potential role in the chemoprevention of DNA damage in human cells induced by oxidative radicals (*41*).

In order to evaluate the protective effect in vivo, we used several *S. cerevisiae* strains defective in antioxidant defenses, such as superoxide dismutases; glutathione peroxidase; catalase; glutathione redutase; transcription factor Yap1p, which activates antioxidant defense gene expression during oxidative stress; and a strain blocked in glutathione biosynthesis (*43*). The H_2O_2 or *t*-BOOH concentration used in these assays was appropriate for the differential sensitivity of each strain, which is dependent on their genetic background, and also exerts an influence on the response to HME treatment and on the disrupted gene (*44*).

Treatment with HME protected *S. cerevisiae* strains against oxidative cytotoxicity, showing a clear antioxidant action. This protective property, shown by the increase in survival, is the same for all strains, independent of the antioxidant defense disrupted. Therefore, we suggest that this protection is due to molecules present in HME that act as versatile and wide spectrum nonenzymatic antioxidants, such as vitamins or phytosterols. However, HME treatment does not increase the survival of strain *yap1*Δ after H_2O_2 and *t*-BOOH exposure. The transcription factor Yap1p is very important for activation of antioxidant enzyme gene expression during oxidative stress; hence, only potent antioxidants will be able to protect this strain in this model (*43*). Therefore, we cannot exclude the possibility that the antioxidant protective effects observed in the other strains are not due to an adaptative response of these cells.

Some *Hibiscus* spp. have been shown to elicit antioxidant properties in both in vitro and in vivo systems. *Hibiscus sabdariffa* extracts inhibit low-density lipoprotein oxidation in vitro, decreases serum lipids in cholesterol- and high fructose-fed rats, and efficiently protects against *t*-BOOH-induced hepatic toxicity and in rats and in cultures of rat hepatocytes, indicating antioxidant activity (*31*, *45*−*47*). A coumarino-lignan and cleomiscosin C, isolated from the root bark of *Hibiscus syriacus*, showed lipid peroxidation inhibitory activity comparable to vitamin E in an in vitro model using rat microsome liver fraction (*40*). In our in vivo model, we were able to show that HME also has antioxidant activity.

HME was not mutagenic in either *S. typhimurium* or *S. cerevisiae*, differing from *H. sabdariffa* Linn ethanolic extract (*32*). HME strongly inhibited the mutagenic action of H_2O_2 or *t*-BOOH in *S. cerevisiae*, mainly by an increase in cell survival, in spite of its weak antioxidant effect. Similarly, *H. rosa sinensis* extract inhibited *t*-BOOH-induced genotoxicity in rat primary hepatocytes culture but not rat hepatic cytotoxicty nor lipid peroxidation, suggesting that these might represent different biological mechanisms (*48*).

Recently, protocatechoic acid, a polyphenolic compound from *H. sabdariffa*, was shown to be an efficacious agent in inhibiting the carcinogenic action of various chemicals in different tissues (*31*, *32*). The metabolite 9,90-O-feruloyl-secoisolaricinresinol, isolated from *Hibiscus taiwanensis*, showed strong cytotoxic activity against human lung carcinoma (A549) and breast carcinoma (MCF-7) cell lines (*49*). Here, we show that HME possesses antimutagenic activity that can be independent of its

antioxidant effect, and future work will be necessary to identify the main substances responsible for these effects.

ACKNOWLEDGMENT

We thank Dr. Eloy Garcia and Dr. Christine Gaylard for a critical English revision.

LITERATURE CITED

(1) Dahanukar, S. A.; Kulkarmi, R. A.; Rege, N. N. Pharmacology of medicinal plants and natural products. *Indian J. Pharmacol.* **2000**, *32*, S81−S118.

(2) Ames, B. N. Dietary carcinogens and anticarcinogens: Oxygen radicals and degenerative diseases. *Science* **1983**, *221*, 1256−1264.

(3) Block, G. The data support a role for antioxidants in reducing cancer risk. *Nutr. Rev.* **1992**, *50*, 207−213.

(4) Beckman, K. B.; Ames, B. N. The free radical theory of aging matures. *Physiol. Rev.* **1998**, *78*, 547−581.

(5) Newmark, H. L. Plant phenolic compounds as inhibitors of mutagenesis and carcinogenesis. In *Phenolic Compounds in Food and Their Effects on Health II: Antioxidants and Cancer Prevention*; Huang, M. T., Ho, C. T., Lee, C. Y., Eds.; ACS Symposium Series 507; American Chemical Society: Washington, DC, 2000; pp 48−52.

(6) Holser, R.; Bost, G.; Van Boven, M. Phytosterol composition of hybrid *Hibiscus* seed oils. *J. Agric. Food Chem.* **2004**, *52*, 2546−2548.

(7) Liu, J.; Shigenaga, M. K.; Mori, A.; Ames, B. Free radicals and neurodegenerative diseases: Stress and oxidative damage. In *Free Radicals in Brain Physiology and Disorders*; Packer, L., Hiramatsu, M., Yoshikawa, T., Eds.; Academic Press Inc.: San Diego, 1996; pp 403−437.

(8) *Kirk-Othmer Encyclopedia of Chemical Technology*, 3rd ed.; John Wiley & Sons, Inc.: New York, 1981; Vol. 16, no. 23, pp 469−490.

(9) Brondegaard, V. J. Contraceptive plant drugs. *Planta Med.* **1973**, *23*, 167−172.

(10) Kholkute, S. D.; Mudgal, V.; Udupa, K. N. Studies on the ant fertility potentiality of *Hibiscus rosa sinensis. Planta Med.* **1977**, *31*, 35−39.

(11) Kobayashi, J. Early Hawaiian uses of medicinal plants in pregnancy and childbirth. *J. Trop. Pediatr. Environ. Child Health* **1976**, *22*, 260−262.

(12) Singh, Y. H.; Ikahihifo, T.; Panuve, M.; Slatter, C. Folk medicine in Tonga. A study on the use of herbal medicines for obstetric and gynecological conditions and disorders. *J. Ethnopharmacol.* **1984**, *12*, 305−329.

(13) Whistler, W. A. Traditional and herbal medicine in Cook Islands. *J. Ethnopharmacol.* **1985**, *13*, 239−280.

(14) Holdsworth, D.; Wamoi, B. Medicinal plants of the Admiralty Island, Papua New Guinea, Part I. *Int. J. Crude Drug Res.* **1982**, *20*, 169−181.

(15) Holdsworth, D. Traditional medicinal plants of Rarotonga, Cook Island. Part II. *J. Pharmacognosy* **1991**, *29*, 71−79.

(16) Melecchi, M. I. S.; Martinez, M. M.; Abad, F. C.; Zini, P. P.; Nascimento, Filho, I.; Caramão, E. B. Chemical composition of *Hibiscus tiliaceus* L. flowers: A study of extraction methods. *J. Sep. Sci.* **2002**, *25*, 86−90.

(17) Maron, D. M.; Ames, B. N. Revised methods for the S*almonella* mutagenicity test. *Mutat. Res.* **1983**, *113*, 173−215.

(18) Mortelmans, K.; Zeiger, E. The Ames *Salmonella*/microsome mutagenicity assay. *Mutat. Res.* **2000**, *455*, 29−60.

(19) Myers, L.; Adams, N.; Kier, L.; Rao, T. K.; Shaw, B.; Williams, L. Microcomputer software for data management and statistical analysis of the Ames/*Salmonella* test. In *Statistics in Toxicology*; Krewski, D., Franklin, C. A., Eds.; Gordon and Breach: New York, 1991; pp 265−279.

(20) Picada, J. N.; Maris, A. F.; Ckless, K.; Salvador, M.; Khromov-Borisov, N. N.; Henriques, J. A. P. Differential mutagenic, antimutagenic and cytotoxic responses induced by apomorphine and its oxidation product, 8-oxo-apomorphine-semiquinone, in bacterial and yeast. *Mutat. Res.* **2003**, *539*, 29−41.

(21) Burke, D.; Dawson, D.; Stearns, T. Methods in yeast genetics. *Cold Spring Harbor Laboratory Course Manual*; CSH Laboratory Press: Plainview, New York, 2000; pp 171−205.

(22) Lewinska, A.; Bilinski, T.; Bartosz, G. Limited effectiveness of antioxidants in the protection of yeast defective in antioxidant proteins. *Free Radical Res.* **2004**, *36*, 1159−1164.

(23) Snow, S. Absence of suppressible alleles at the *his* 1 locus of yeast. *Mol. Gen. Genet.* **1978**, *164*, 341−342.

(24) Hawthorne, D. C. Identification of nonsense codons in yeast. *J. Mol. Biol.* **1969**, *43*, 71−75.

(25) Hawthorne, D. C.; Mortimer, R. K. Suppressors in yeast. *Genetics* **1963**, *48*, 617−620.

(26) Von Borstel, R. C.; Cain, K. T.; Steinberg, C. M. Inheritance of spontaneous mutability in yeast. *Genetics* **1971**, *69*, 17−27.

(27) Schuller, R. C.; Von Borstel, R. C. Spontaneous mutability in yeast. I. Stability of lysine reversion rates to variation of adenine concentration. *Mutat. Res.* **1974**, *24*, 17−23.

(28) Camussi, A.; Moller, F.; Ottavianio, M.; Gorla, S. Distribuizone campionarie e stima dei parametri in una popolazione. *Metodi Statistici per la Sperimentazion Biologica*; Zanichelli: Bologna, 1986; Vol. 3.

(29) Brendel, M.; Grey, M.; Maris, A.; Hietkamp, J.; Fesus, Z.; Pich, C.; Dafré, L.; Schmidt, M.; Eckardt-Schupp, F.; Henriques, J. Low glutathione pools in the original pso3 mutant of *Saccharomyces cerevisiae* are responsible for its pleiotropic sensitivity phenotype. *Curr. Genet.* **1998**, *33*, 4−9.

(30) Dafallah, A. A.; al-Mustafa, Z. Investigation of the antiinflamatory activity of Acacia nilotica and *Hibiscus sabdariffa*. *Am. J. Chin. Med.* **1996**, *24*, 263−269.

(31) Tseng, T.-H.; Kao, E.-S.; Chu, C.-Y.; Chou, F.-P.; Lin, Wu H.-W.; Wang, C.-J. Protective effects of dried flower extract of *Hibiscus sabdariffa L.* against oxidative stress in rat primary hepatocytes. *Food Chem. Toxicol.* **1997**, *35*, 1159−1164.

(32) Chewonarin, T.; Kinouche, T.; Kataoka, K.; Arimochi, H.; Kuwahara, T.; Vinitketkummuen, U.; Ohnishi, Y. Effects of roselle (*Hibiscus sabdariffa* Linn.), a Thai medicinal plant, on the mutagenicity of various known mutagens in *Salmonella typhimurium* and on formation of aberrant crypt foci induced by the colon carcinogens azoxymethane and 2-amino-1-methyl-6-phenylimidazo(4,5-b) pyridine in F344 rats. *Food Chem. Toxicol.* **1999**, *37*, 591−560.

(33) Farayi, M. H.; Tarkhami, A. H. The effect of sour tea (*Hibiscus sabdariffa*) on essential hypertension. *J. Etnopharmacol.* **1999**, *65*, 231−236.

(34) Alam, M.; Siddiqui, M.; Hussain, W. Treatment of diabetes through herbal drugs in rural India. *Fitoterapia* **1990**, *61*, 240−242.

(35) Shimizu, N.; Tomoda, M.; Suzuki, T.; Takada, K. Plant mucilages XLIII. A representative mucilage with biological activities from the leaves of *Hibiscus rosa sinensis. Biol. Pharm. Bull.* **1993**, *16*, 735−739.

(36) Serrame, E.; Lin Sylianco, C. Y. Anti-tumor promoting activity of decoctions and expressed juices from Philippine medicinal plants. *Philipp. J. Sci.* **1995**, *124*, 275−281.

(37) Reddy, C. M.; Munthy, D. R. K.; Patil, S. B. Antispermatogenic and androgenic activities of various extracts of *Hibiscus rosa sinensis* in albino mice. *Indian J. Exp. Biol.* **1997**, *35*, 1170−1174.

(38) Kasture, U. S.; Chopde, C. T.; Deshmulch, V. K. Anticonvulsive activity of *Albizzia lebbeck*, *Hibiscus rosa sinensis* and *Butia monosperma* in experimental animals. *J. Etnopharmacol.* **2000**, *71*, 65−75.

(39) Sachdewa, A.; Khermani, L. D. Effect of *Hibiscus rosa sinensis* ethanol flower extract on blood glucose and lipid profile in streptozotocin induced diabetes in rats. *J. Etnopharmacol.* **2003**, *89*, 61−66.

(40) Yun, B.; Lee, I.; Ryoo, I.; Yoo, I. Coumarins with monoamine oxidase inhibitory activity and antioxidative coumarins-lignans from *Hibiscus syriacus. J. Nat. Prod.* **2001**, *64*, 1238−1240.

(41) Wang, H.-L.; Li, Q.-L.; Ying, J.; Luo, Z.-F.; Wang, Y.-Z. The protective effect and mechanism of soybean oil and its extracts in DNA damage in human ECV304 cells exposed to UV-C. *Biochim. Biophys. Acta* **2003**, *1626*, 19−24.

(42) De Jing, A.; Plat, J.; Mensink, R. Metabolic effects of plant sterols and stanols. *J. Nutr. Biochem.* **2003**, *14*, 362−369.

(43) Ikner, A.; Shiozaki, K. Yeast signaling pathways in the oxidative stress response. *Mutat. Res.* **2005**, *569*, 13−27.

(44) Higgins, V. J.; Alic, N.; Thorpe, G. W.; Breitenbach, M.; Larsson, V.; Dawes, I. W. Phenotypic analysis of gene deletant strains for sensitivity to oxidative stress. *Yeast* **2002**, *3*, 203−214.

(45) Chen, C. C.; Hsu, J. D.; Wang, S. F.; Chiang, H. C.; Yang, M. Y.; Kao, E. S.; Ho, Y. C.; Wang, C. J. *Hibiscus sabdariffa* L. extract inhibits the development of atherosclerosis cholesterol-fed rabbits. *J. Agric. Food Chem.* **2003**, *51*, 5472−5477.

(46) Hirunpanich, V.; Utaipat, A.; Morales, N.; Bunyapraphatsara, N.; Sato, H.; Herumale, A.; Suthisisang, C. Hypocholesterolemic and antioxidant effects of aqueous extracts from the dried calyx of *Hibiscus sabdariffa* L. in hypercholesterolemic rats. *J. Etnopharmacol.* **2006**, *103*, 252−260.

(47) Wang, C.-J.; Wang, J.-M.; Lin, W.-L.; Chu, C.-Y.; Chou, F.-P.; Tseng, T.-H. Protective effect of *Hibiscus* anthocyanins against tert-BOOH-induced hepatic toxicity in rats. *Food Chem. Toxicol.* **2000**, *38*, 411−416.

(48) Tseng, T.-H.; Kauo, T.-W.; Chu, C.-Y.; Chou, F.-P.; Lin, W.-L.; Wang, C.-J. Induction of apoptosis by *Hibiscus* protocatechuic acid in human leukemia cells via the reduction of retinoblastoma (RB) phosphorylation and Bcl-2 expression. *Biochem. Pharmacol.* **2000**, *60*, 307−315.

(49) Wu, P.-L.; Chuang, T.-H.; He, C.-X.; Wu, T.-S. Cytotoxicity of phenylpropanoid esters from the stems of *Hibiscus taiwanensis. Bioorg. Med. Chem.* **2004**, *12*, 2193−2197.

Received for review May 18, 2006. Revised manuscript received July 25, 2006. Accepted July 27, 2006. This work was supported by grants from the Brazilian Agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Rio Grande do Sul (FAPERGS) and GENOTOX—Genotoxicity Laboratory, Universidade Federal do Rio Grande do Sul.

JF061407B