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Effects of the Phenolic Contents of Mauritian Endemic Plant Extracts on Promoter Activities of Antioxidant Enzymes

SHINYA TOYOKUNI[a,*], TOMOYUKI TANAKA[a], WAKA KAWAGUCHI[a], NEIL RYAN LAI FANG[b], MUNETAKA OZEKI[a], SHINYA AKATSUKA[a], HIROSHI HIAI[a], OKEZIE I. ARUOMA[c] and THEESHAN BAHORUN[b,*]

[a]*Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan;* [b]*Department of Biological Sciences, Faculty of Science, University of Mauritius, Mauritius, Republic of Mauritius;* [c]*Department of Diabetes and Metabolic Medicine, Queen Mary University of London, Medical Unit, Royal London Hospital, Whitechapel, London E1 1BB, UK*

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Oxidative stress has been associated with a variety of pathologic conditions in humans. Increasing the transcriptional activities of antioxidant enzymes might be a strategy to prevent oxidative stress-associated diseases such as atherosclerosis and cancer. In the present paper, we studied the effects of extracts from 12 Mauritian endemic plants on the promoter activities of antioxidant enzymes; Cu, Zn-superoxide dismutase (Cu,Zn-SOD), Mn-superoxide dismutase (Mn-SOD), catalase, and glutathione dismutase (GPx). The levels of total phenolic compounds, total flavonoids, and proanthocyanidins were measured. Four luciferase expression vectors (pGL3-Basic) with promoter region of each enzyme were constructed, transfected to COS7 cells followed by an exposure to each extract (25 μg/ml, 24 h, non-toxic dose). Thereafter, luciferase activities were evaluated in comparison with a control luciferase vector with a herpes simplex virus thymidine kinase promoter. Mauritian endemic plants contained high amounts of total phenols, flavonoids and proanthocyanidins. Total phenols and flavonoids were proportionally associated with Cu,Zn-SOD promoter activity, whereas they were inversely correlated with catalase promoter activity. These results suggest that the chemopreventive potentials of the extracts might reside in their abilities to modulate the expression of the antioxidant enzyme genes.

Keywords: Promoter; Superoxide dismutase; Catalase; Glutathione peroxidase; Mauritian endemic plant extract; Total phenols

Abbreviations: ROS, reactive oxygen species; SOD, superoxide dismutase; GPx, glutathione peroxidase; DMSO, dimethylsulfoxide; NAC, N-acetylcysteine; PMA, phorbol 12-myristate 13-acetate; TLC, thin layer chromatography; TNF-α, tumor necrosis factor-α; PAC, proanthocyanidins

INTRODUCTION

Oxygen is a double-edged sword that can potentially cause cellular damage through excessive production of reactive oxygen species (ROS). Oxidative stress is an important contributor to the pathophysiology of a variety of human pathological conditions including cardiovascular dysfunctions, atherosclerosis, inflammation, carcinogenesis, drug toxicity, reperfusion-injury and neurodegenerative diseases.[1,2]

The human body has multiple mechanisms to protect cellular molecules, especially the genome, against ROS-induced damage.[3] These include repair enzymes (DNA glycosylases, AP Endonucleases, etc.), antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase), and intra- as well as extra-cellular antioxidants (glutathione, uric acid, ergothioneine, vitamin E, vitamin C and circulating diet-derived phenolic compounds). However, the innate defences may not be enough for severe and/or continued oxidative stress. The implication of free radical mechanisms in the pathogenesis of human diseases has led to a suggestion that antioxidants might have health benefits as prophylactic agents.[4]

Based on this idea, there has been a strong demand for the development of therapeutic and chemopreventive antioxidant agents with limited cytotoxicity to enhance the antioxidant capacity of the body and help attenuate the damage induced by ROS.

*Corresponding authors. Shinya Toyokuni: Tel.: +81-75-753-4423. Fax: +81-75-753-4432. E-mail: toyokuni@path1.med.kyoto-u.ac.jp; Theeshan Bahorun: Fax: + 230-454-6942. E-mail: tbahorun@uom.ac.mu

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There are mainly two strategies proposed for this. One would be to use antioxidants with direct radical scavenging activity,[5] and the other approach would be to identify antioxidants that would increase the expression of antioxidant enzymes such as phase 2 enzymes.[6] However, there is a paucity of data on natural phytochemicals that modulate the expression of enzymes metabolising ROS. In the present paper, we focus on the induction of the antioxidant enzymes; superoxide dismutases (SODs), catalase and glutathione peroxidase (GPx).

SOD catalyses the dismutation of superoxide to hydrogen peroxide whereas both catalase and GPx catalyse the conversion of H_2O_2 to water, preventing the generation of hydroxyl radicals through the Fenton reaction. GPx is in general thought to be more important as a H_2O_2-removing system in humans than catalase since no clinical effects are apparent in acatalasemic patients.[7] Catalase is located in peroxisomes, whereas GPx is localized in the mitochondria and cytosol, a similar distribution to those of Mn-superoxide dismutase (Mn-SOD) and Cu, Zn-superoxide dismutase (Cu,Zn-SOD).[2] The antioxidant properties of phenolic compounds from dietary or herb plants have aroused much attention.[8,9] Plants rich in these compounds are ideal sources of natural antioxidants and literature data abound in examples where fruits,[10,11] vegetables,[12] teas,[13] wines,[14] medicinal plants[15] and *in vitro* plant cell cultures[16,17] have been studied for their antioxidant capacities.

Mauritius is a tropical island in the Indian Ocean belonging to the Mascarene archipelago, which is characterised by a unique flora. It has been reported that 45% of the flora are strict endemics and account for 67% of the Mascarene endemic plants. A number of these endemics are reported in the traditional pharmacopoeia as potential prophylactic agents. A great number of these plants have been used traditionally to treat a wide range of physical ailments such as bronchitis, diabetes, asthma, dysentery and inflammatory diseases.[18] However, data concerning their indications and the nature of phytochemicals in the active extracts still need to be substantiated. In the present study, the levels of total phenols, flavonoids and proanthocyanidins (total, oligomeric and polymeric) and the presence of specific catechins and flavonol molecules were determined in twelve Mauritian endemic plant extracts. A novel system that enables the evaluation of the effect of the plant extracts on the gene promoter activity of antioxidant enzymes was established by the use of COS7 cells. The total phenolic and flavonoid contents in these plant extracts were proportionally associated with Cu,Zn-SOD promoter activity whereas they were inversely correlated with catalase promoter activity. The results suggest that the ability of phenolic compounds to regulate antioxidant enzyme expression might have chemopreventive properties in ROS-associated diseases.

MATERIALS AND METHODS

Chemicals

Acetone, acetonitrile, aluminum chloride, 2-aminoethyldiphenyl borate, dichloromethane, dimethylsulfoxide (DMSO), ethanol, ethyl acetate, formic acid, methanol, and toluene were obtained from Merck and of analytical quality. N-acetylcysteine (NAC) and dithiothreitol were form Wako (Osaka, Japan) and phorbol 12-myristate 13-acetate (PMA) was from Sigma (St Louis, MO, USA).

Plant Materials

Leaves of *Syzygium glomeratum*, *S. petrinense*, *S. venosum*, *S. commersonii*, *S. coriaceum* were obtained from Le Pétrin Conservation management area. *S. mauritianum* leaves were collected from Brise Fer Forest. Leaves of *Eugenia pollicina*, *E. elliptica*, *E. fasciculata*, *Monimiastrum globusum* were harvested at Le Mondrain Natural reserve while those of *E. orbiculata* and *M. acutisepalum* were obtained from Le Petrin Broadwalk. Leaves of *S. guehoii* were from the Arboretum of the Mauritius Herbarium, Réduit. All leaf specimens were harvested in 1999 and frozen at −30°C until use. Voucher specimens (depending on the availability of flowers) have been deposited at the Mauritius Herbarium, Mauritius Sugar Industry Research Institute, Réduit and at the Department of Biological Sciences, Faculty of Science, University of Mauritius.

Preparation of Plant Extracts

Total Extract

A 25 g of frozen mature leaves were crushed in a Waring blender, and macerated at 4°C, first in 500 ml methanol/water (70/30, v/v), then in acetone/water (70/30, v/v), and finally in 1 l absolute methanol for 24 h, respectively. The combined filtrates were reduced to the aqueous phase *in vacuo* at 37°C, followed by washing with dichloromethane (3 × 150 ml) to remove fat-soluble substances and chlorophylls. The aqueous extract was concentrated and divided into two equal aliquots. Part 1, corresponding to 10 g fresh weight total extract, was carefully freeze-dried and the residue was dissolved in absolute methanol (1/5, plant fresh weight/volume ratio) for storage at −30°C for subsequent analyses, or in DMSO for reporter assay. Part 2, corresponding to 10 g fresh weight,

was used to obtain the ethyl acetate extracts as described below.

Ethyl Acetate Extract

The total aqueous extract was partitioned with ethyl acetate (6 × 200 ml). Ethyl acetate layers, dehydrated with Na_2SO_4 were evaporated under low-pressure to dryness and dissolved in absolute methanol to yield a 1/5 (plant fresh weight/volume) ratio.

Aqueous Extract

Aqueous phase which remained following the ethyl acetate extraction was freeze-dried and dissolved in absolute methanol to obtain a 1/5 (plant fresh weight/volume) ratio. The three extract types were used in the assays described.

Thin Layer Chromatography (TLC)

The plant extracts were examined using a one-dimensional TLC on silica gel plates (Merck) for flavonoids and proanthocyanidins. Proanthocyanidins were analysed after migration in toluene/acetone/formic acid (3/3/1, v/v/v)[19] and visualised by vanillin/HCl spray reagent. Flavonoids were separated in ethyl acetate/formic acid/water (8/1/1, v/v/v)[20] and revealed by 1% 2-aminoethyldiphenyl borate solution in methanol followed by 5% polyethylene glycol 4000 in absolute ethanol at 365 nm.

Determination of Total Phenol Content

Total phenol contents in the extracts were determined by a colorimetric method adapted from Singleton and Rossi[21] at 680 nm using the Folin–Ciocalteu reagent. Results were expressed in mg of gallic acid equivalent/100 g of dry plant material.

Determination of Total Flavonoid Content

The flavonoid contents were estimated by the aluminium chloride method according to Lamaison *et al.*[20] Aliquots of 1 ml of methanolic extract were added to 1 ml of 2% methanolic $AlCl_3 \cdot 6\ H_2O$. After 10 min, the absorption was read at 360 nm. The flavonoid contents of leaves were expressed in mg of quercetin equivalent/100 g dry weight.

Determination of Proanthocyanidin Content

The standard method used was adapted from Porter *et al.*[22] To each tube was added 0.5 ml of plant methanolic extract, 6 ml of *n*-butanol/HCl (95/5, v/v) and 0.2 ml of 2% (w/v) solution of $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$ in 2 M HCl. The tubes were capped, thoroughly mixed and heated 40 min in a constant level water bath run at 95 ± 0.2°C. Solutions were cooled and extinctions recorded at 550 nm. The amount of proanthocyanidin was expressed in mg of cyanidin chloride equivalent/100 g of dry weight. Total, oligomeric and polymeric proanthocyanidins were determined from total, ethyl acetate and residual aqueous plant extracts, respectively.

High Performance Liquid Chromatography Analysis

A Hewlett-Packard 1100-series liquid chromatography system including vacuum degasser, quaternary pump, auto-sampler, thermostat-regulated column compartment and diode array detector was used. Analyses were carried out at 25°C after filtration of methanolic stock extracts on Millipore filter (0.22 μm) and injection (30 μl) on a Lichrosorb RP 18 column (0.5 μm; 4.6 mm id × 150 mm) by an acidified acetonitrile–water gradient. Elution (flow rate: 0.7 ml/min) was performed in the following order: 0–30 min, 0–15% B in A; 30–50 min, 15% B in A; 50–60 min, 15–25% B in A; 60–90 min, 25–100% B in A; 90–100 min, 100–0% B in A [Solvent A: acetonitrile/water, 1/9, v/v, pH 2.6; Solvent B: acetonitrile/water, 1/1, v/v, pH 2.6]. Absorption wavelengths were selected at 280 nm for flavan-3-ol derivatives and at 360 nm for flavonoids. (−)-Epicatechin, (+)-catechin, proanthocyanidin B2 dimer, quercetin-3-*O*-galactoside (hyperoside) and quercetin-3-*O*-glucoside (isoquercetin) were identified by comparison with authentic standards (Extrasynthèse, Genay, France). The UV spectra obtained for each peak, after subtraction of the corresponding UV-based spectrum, were computer normalised and the plots were superimposed. Peaks were considered to be chromatographically pure when there was exact coincidence to their corresponding UV spectra.

Cell Lines and Animals

COS7 and HeLa cell lines were obtained through the American Type Culture Collection (Rockville, MD). These cells were maintained in Dulbecco's modified Eagle's medium (GIBCO BRL, Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum (FCS; Hyclone Laboratories, Inc., Logan, Utah) under 5% CO_2 in air at 37°C. Specific pathogen-free 5-week-old male Wistar rats were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan).

Genomic DNA

Rat or human genomic DNA was isolated from rat liver or HeLa cells (human) with a standard

phenol–chloroform extraction method as described previously.[23]

Reporter Constructs

Promoter regions of Cu,Zn-SOD (human), Mn-SOD (rat), catalase (rat) and GPx (human) were amplified with polymerase chain reaction by using genomic DNA as a template (10 ng in a system of 50 μl; 30 cycles of denaturing at 95°C for 30 s, and annealing/extention at 68°C for 3 min; LA-PCR kit, TakaraBio, Shiga, Japan). The used primers were as follows. Each primer was designed to include a site for restriction enzyme to facilitate cloning into pGL3-Basic Vector (Promega, Tokyo); human Cu,Zn-SOD promoter (−1499 to +27, GenBank AP001711), forward-5′-CCGAC*TCGAG*CCCTTGGCAAGTTTACAATC-3′ (XhoI), reverse-5′-CCGA*AAGCTT*GAGACTACGACGCAAACCAG-3′ (HindIII); rat Mn-SOD promoter (−2425 to +44, GenBank X56600), forward-5′-CGG*GGTACC*AGGTAATGTGGTCCATCTCCAAGGTGG-3′ (KpnI), reverse-5′-CCGA*AAGCTT*TCTCCTCAGAACACGGCCGTTCGCTAGC-3′ (HindIII); rat catalase promoter (−3307 to +2, GenBank AH004967), forward-5′-CGG*GGTACC*AAGTTTGCCTCTGGTGTCTGTGTTTCCCC-3′ (KpnI), reverse-5′-CCGA*CTCGAG*ATGGTGTAGGATTGCGGAGCTGCAGAGC-3′ (XhoI); human GPx promoter (−1389 to +565, GenBank M83094), forward-5′-CGG*GGTACC*GTTCATGCAGCTGTGGCAGAGTTAC-3′ (KpnI), reverse-5′-CCGA*AAGCTT*TCGTTCTTGGCGTTCTCCTACAGGA-3′ (HindIII). Each cloned construct was confirmed by sequencing with an ABI PRISM 377 DNA sequencer.

Reporter Assay

One or 5×10^5 COS7 cells were plated on 12-well plastic plate, and incubated for 24 h. Each reporter construct together with phRL-TK (thymidine kinase) vector (Promega) was transfected with Effectine according to the manufacturer's instructions (Qiagen, Tokyo), and incubated for 24 h. Then, each plant extract (final concentration 25 μg/ml) was added to each well. Solvent of the extract (DMSO) or other chemicals as well as medium was used as controls. Twenty-four hours later, cells were lysed, collected and subjected to dual-luciferase reporter assay system (Promega). Renilla luciferase from phRL-TK served as a control. Luciferase activity was measured with Gene Light 55 (Microtec Nichion, Funabashi, Japan). Triplicate measurements were performed for each sample.

Detection of Peroxides by Confocal Microscopy

In order to corroborate the results of reporter assay the technique of confocal microscopy was used to assess the levels of peroxides. The procedure was performed as previously described[24] with a modification to microscopic use. COS7 cells (5×10^4) were plated on each chamber of collagen I-coated 4-chamber slides (Iwaki, Tokyo). Medium change was performed after 24 h, and plant extracts (*E. pollicina* and *E. elliptica*) were added for an incubation of 24 h. Mouse recombinant tumor necrosis factor-α (TNF-α, final concentration 10 ng/ml; Sigma) was added and incubated for 1 h,[25] and this served as the positive control. Then, after washing twice with phosphate-buffered saline, 2′, 7′-dichloroflurescein, laser-grade (final concentration, 5 μM; Across Organics, NJ) was added and incubated for 5 min at 37°C, followed by an examination with a confocal laser scanning microscopy (Fluoview, Olympus, Osaka).

Statistical Analyses

Statistical analyses were performed with an unpaired *t*-test, which was modified for unequal variances when necessary. $p < 0.05$ was considered as statistically significant. Correlation coefficients and regression lines were also calculated.

RESULTS

Constructs for Reporter Assays

In the first experiments, H_2O_2, N-acetyl cysteine and dithiothreitol were added to the system in order to check the responses of the reporter constructs (Table I). PMA (50 or 150 nM, 16 h) increased the transcriptional activity of Cu,Zn-SOD, which was consistent with the results of Minc *et al.*[26], whereas dithiothreitol (DTT, 1 mM, 4 h) increased that of Mn-SOD in the COS7 cells.[27] Hydrogen peroxide (1 or 10 μM, 24 h) increased the transcriptional activities of both catalase and GPx. Transcriptional activity of thymidine kinase was regarded as a control, and its decrease indicated loss or damage to the cells. Transcriptional activities of catalase and GPx were increased upon mild oxidative stress loading on the cells. On the contrary, severe oxidative damage either reduced the transcriptional activity or lead to erratic results as seen in Table I. This enabled a careful selection of the ideal condition for each of the extracts where they either induced very little or no damage to the cells in the following experiments.

Analysis of Phenolic Compounds

Total phenolic contents of Mauritian endemic plants ranged from 30.9 to 93.9 mg/g dry weight (Table II). Flavonoids were present in the range of 6.2 to 56.0 mg/g dry weight. Total proanthocyanidins

TABLE I Basal and induced promoter activity of antioxidant enzymes in COS7 cells

	Cu,Zn-SOD	Cu,Zn-SOD	Cu,Zn-SOD	Mn-SOD	Mn-SOD
Basal level (H_2O; vs. TK promoter)	0.80	0.44	1.45	1.46	1.09
NAC 5 mM (vs. basal level)	0.97 ± 0.01 (102%)	ND	ND	1.06 ± 0.04 (94%)	ND
NAC 10 mM (vs. basal level)	0.95 ± 0.02 (102%)	ND	ND	1.03 ± 0.01 (95%)	ND
DTT 0.5 mM (vs. basal level)	ND	1.02 ± 0.02 (97%)	ND	ND	1.03 ± 0.06 (107%)
DTT 1 mM (vs. basal level)	ND	0.93 ± 0.05 (78%)	ND	ND	1.11 ± 0.04* (79%)
PMA 50 nM (vs. basal level)	ND	ND	1.53 ± 0.21** (78%)	ND	ND
PMA 150 nM (vs. basal level)	ND	ND	1.78 ± 0.24** (72%)	ND	ND
Medium (vs. basal level)	0.93 ± 0.06 (101%)	1.02 ± 0.06 (96%)	1.03 ± 0.10 (95%)	0.99 ± 0.04 (98%)	0.99 ± 0.03 (110%)
	Catalase	Catalase	GSH-Px	GSH-Px	
Basal level (H_2O; vs. TK promoter)	1.08	0.39	0.68	0.24	
H_2O_2 1 μM (vs. basal level)	0.89 ± 0.03 (107%)	1.21 ± 0.17* (86%)	1.02 ± 0.04 (100%)	1.14 ± 0.43 (58%)	
H_2O_2 10 μM (vs. basal level)	0.92 ± 0.09 (109%)	1.61 ± 1.18 (41%)	1.04 ± 0.07 (99%)	1.50 ± 0.51 (21%)	
H_2O_2 100 μM (vs. basal level)	0.97 ± 0.06 (85%)	0.91 ± 0.33 (2.7%)	1.18 ± 0.03* (99%)	1.89 ± 0.88 (9.6%)	
Medium (vs. basal level)	0.95 ± 0.05 (107%)	0.97 ± 0.04 (102%)	1.08 ± 0.03 (100%)	1.05 ± 0.04 (102%)	

Cells were transfected doubly with pRL-TK vector and a different pGL3-X construct. Cells were treated with either hydrogen peroxide (24 h), phorbol 12-myristate 13-acetate (16 h), N-acetylcysteine or dithiothreitol (4 h) prior to havesting. Parenthesis (%) shows TK activity vs. basal level TK activity, which reflects survived cell population. Refer "Materials and methods" section for details. ND, not done; SOD, superoxide dismutase; GSH, glutathione; Px, peroxidase; NAC, N-acetylcysteine; DTT, dithiothreitol; PMA, phorbol 12-myristate 13-acetate; TK, thymidine kinase (*$p < 0.05$; **$p < 0.01$ vs. Medium; means ± SD, $n = 3$). Refer "Materials and methods" section for detail.

varied between 1.4 and 47.2 mg/g dry weight while oligomeric or polymeric proanthocyanidins varied between 0 and 20.5, or between 1.2 and 26.7 mg/g dry weight, respectively.

Leaves of *E. pollicina* were the richest in phenolic content not only within the *Eugenia* genus but also among the other Myrtaceae endemics studied. The polyphenolic contents of *E. elliptica*, *E. fasciculata* and *E. orbiculata* were within the range of 26.2–28.1 mg/g dry weight. Leaves of *S. venosum* and *S. glomeratum* showed the highest levels of phenols within the *Syzygium* genus of 86.3 and 67 mg/g dry weight, respectively, while *S. mauritianum* leaves contained the lowest amount (30.9 mg/g dry weight). Leaves of *S. guehoii*, *S. petrinense*, *S. coriaceum* showed intermediate amounts of 53.8, 51.5 and 51.2 mg/g dry weight, respectively. Leaves of *M. globusum* were found to be richer in phenol (40.9 mg/g dry weight) than those of *M. acutisepalum* (35.4 mg/g dry weight).

TLC and HPLC data indicated that the main polyphenolic classes identified in the leaf extracts from *Eugenia, Syzygium* and *Monimiastrum* species were flavan-3-ols (catechins, oligomeric and polymeric proanthocyanidins), flavonol glycosides and phenolic acids. The identification of flavanol derivatives in all the species studied is shown in Table II. The major catechin found in all Eugenia species, in *M. acutisepalum* and all *Syzygium* species except *S. petrinense* and *S. glomeratum* was (+)-catechin, whereas (−)-epicatechin was observed only in *S. glomeratum* and *E. pollicina*. (−)-epicatechin gallate was detected in a few species (*S. glomeratum, S. coriaceum, E. pollicina*, and *M. globusum*). Dimeric (B1 and/or B2 derivatives), oligomeric and polymeric proanthocyanidins were detected in all *Syzygium* and *Eugenia* species. *M. globusum* and *M. acutisepalum* were characterised by the absence of B1 and B2 dimers. However, oligomeric and polymeric proanthocyanidins were present in *M. acutisepalum* leaf extract while only polymeric proanthocyanidins were detected in *M. globusum*. These observations were confirmed by proanthocyanidin quantitative analysis data (Table II). Proanthocyanidin contents of the *Eugenia* species leaves followed a similar trend as total phenolic contents. Maximum proanthocyanidins was recorded in *E. pollicina* (47.2 mg/g dry weight) and intermediate levels were observed in *E. elliptica*, *E. fasciculata* and *E.orbiculata* (17.3–20.3 mg/g dry weight). Leaves of the newly found *S. guehoii* species were the richest in proanthocyanidin content (22.1 mg/g dry weight) within the *Syzygium* species. It was followed by *S. venosum*, *S. glomeratum* and *S. mauritianum*, which contained comparable amounts (12.8, 13.5 and 14.8 mg/g dry weight). The lowest proanthocyanidin yield was observed in *S. coriaceum* (3.72 mg/g dry weight).

TABLE II Contents of Mauritian plant extracts

Plant species	Contents (mg/g dry weight)					
	Total Phenols	Flavonoids	Total PAC	Oligomeric PAC	Polymeric PAC	Major phenolic compounds*
Eugenia species						
E. elliptica	46.2	17.9	20.3	1.2	18.6	1,3,4,6,9
E. fasciculata	54.0	24.8	17.3	7.3	10.8	1,3,4,6,9,0
E. orbiculata	58.7	16.7	18.9	5.8	15.9	1,2,3,4,6,9
E. pollicina	93.9	56.0	47.2	20.5	26.7	1,3,4,6,7,8,9
Monimiastrum species						
M. acutisepalum	35.4	28.4	8.8	1.3	7	1,3,4,6
M. globusum	40.9	19.7	10.2	0.0	9.9	1,3,4,8
Syzygium species						
S. coriaceum	51.2	6.2	3.7	0.7	1.9	1,2,3,4,6,8,9
S. glomeratum	67.0	17.3	13.5	1.9	10.4	3,4,5,7,8,9
S. guehoii	53.9	16.4	22.1	3.2	20.7	1,3,4,5,6,0
S. mauritianum	30.9	9.6	14.8	0.9	10.7	2,3,4,5,6
S. petrinense	51.6	24.2	1.4	0.6	1.2	1,2,3,4,5,9
S. venosum	86.3	38.1	12.8	1.2	8.7	1,2,3,4,6,0

PAC, proanthocyanidins. *Major phenolic compounds: 1, quercetin-3-*O*-galactoside (hyperoside); 2, quercetin-3-*O*-rutinoside (rutin); 3, kaempferol-3-glucoside (astragalin); 4, quercetin-3-*O*-glucoside (isoquercitrin); 5, chlorogenic acid; 6, (+)-catechin; 7, (−)-epicatechin; 8, (−)-epicatechin gallate; 9, procyanidin B1 dimer; 0, procyanidin B2 dimer. Measurements were done in triplicate, and mean values are shown.

Quantitative flavonoid data presented in Table II shows that *E. pollicina* leaf extracts contained the highest amount (56 mg/g dry weight). High contents were also noted in *S. venosum* (38 mg/g dry weight), *M. acutisepalum* (28.4 mg/g dry weight), *E. fasciculata* (24.8 mg/g dry weight) and *S. petrinense* (24.2 mg/g dry weight). *E. elliptica*, *E. orbiculata*, *S. glomeratum*, *S. guehoii* and *M. globusum* contained comparable amounts within the range of 16.7–19.7 mg/g dry weight while the low values were observed for *S. mauritianum* and *S. coriaceum* with 9.6 and 6.2 mg/g dry weight, respectively.

The main flavonoid derivatives identified by TLC and HPLC in the leaf extracts are also shown in Table II. Kaempferol-3-glucoside (astragalin) and quercetin-3-glucoside (isoquercitrin) were present in all the species studied. Quercetin-3-*O*-glucoside (hyperoside) was observed in most of the *Eugenia*, *Monimiastrum* and *Syzygium* species. The presence of quercetin-3-*O*-rutinoside (rutin) was noted in *S. venosum*, *S. petrinense* and *S. mauritianum* within the *Syzygium* genus, in *E. orbiculata* in the *Eugenia* genus, but it was not detected in the *Monimiastrum* species studied. The presence of chlorogenic acid, a main phenolic acid, was also examined and shown in Table II. This caffeoylquinic derivative was observed in all *Syzygium* species except *S. venosum* and *S. coriaceum*, and was absent in *Eugenia* and *Monimiastrum* species.

Reporter Assay of Plant Extracts

Each plant extract was assessed for its ability to modulate the transcriptional activities of the four antioxidant enzymes after 24 h-incubation. The assay conditions were carefully chosen after preliminary experiments, so indicated no apparent cellular toxicity at 24 h. The data is summarized in Table III. The alteration of the transcriptional activity ranged from 0.57 to 1.56. None of the extracts showed a one-sided increased or decreased transcriptional activities of all the four antioxidant enzymes. Phenolic content was proportionally associated with the transcriptional activity of Cu,Zn-SOD but the polymeric proanthocyanidins were inversely associated with the transcriptional activity of GPx (Table IV). Statistical analysis of our data groups, namely, SODs; peroxidases, Pxs; total summation of SODs and Pxs; SODs minus Pxs; SODs/Pxs, indicated that the levels of the phenolic compounds were associated with the cellular environments, in particular, the ability to accumulate H_2O_2 in the cells ($r = 0.72$ or 0.75).

Detection of Peroxides

Incubation of COS7 cells with TNF-α induced significant cytoplasmic fluorescence, whereas faint signal was observed in the untreated COS7 cells (Fig. 1A and B). Cytoplasmic accumulation of H_2O_2 was expected by an exposure of the extracts from *E. pollicina* to COS7 cells based on the data in Table III. Extracts from *E. pollicina* indeed induced significantly high cytoplasmic fluorescence. In contrast, extracts from *E. elliptica*, in which lower accumulation of H_2O_2 was expected, revealed significantly lower fluorescence than those from *E. elliptica* (Fig. 1C and D).

DISCUSSION

The tropical island of Mauritius harbours a unique flora, which has resulted from colonization and

TABLE III Effects of Mauritian plant extracts on the promoter activity of antioxidant enzymes

	Reporter assay*				
Plant species	Cu,Zn-SOD	Mn-SOD	Catalase	GSH-Px	Survival (%)
Basal (DMSO; vs. TK promoter)	3.34	2.81	3.77	0.30	100
Eugenia species					
E. elliptica	0.87 ± 0.18	0.77 ± 0.12*	1.2 ± 0.18	1.22 ± 0.18	96.7
E. fasciculata	0.97 ± 0.27	0.93 ± 0.03*	0.84 ± 0.07*	1.56 ± 0.29	128.4
E. orbiculata	1.16 ± 0.27	0.99 ± 0.29	1.13 ± 0.14	1.17 ± 0.23	101.35
E. pollicina	1.38 ± 0.11**	0.79 ± 0.04**	0.96 ± 0.05	1.13 ± 0.21	96.65
Monimiastrum species					
M. acutisepalum	1.07 ± 0.27	0.67 ± 0.09**	1.54 ± 0.43	1.40 ± 0.21	147.15
M. globusum	1.09 ± 0.30	0.90 ± 0.11	1.26 ± 0.13	1.29 ± 0.13	133.45
Syzygium species					
S. coriaceum	1.10 ± 0.16	0.85 ± 0.16	1.05 ± 0.06	1.27 ± 0.03*	130.1
S. glomeratum	1.24 ± 0.20	0.74 ± 0.06**	1.07 ± 0.20	1.62 ± 0.22*	136.3
S. guehoii	0.91 ± 0.19	0.61 ± 0.07**	0.96 ± 0.15	1.00 ± 0.36	125.7
S. mauritianum	1.14 ± 0.11*	0.75 ± 0.09**	1.08 ± 0.13	1.20 ± 0.11	125.75
S. petrinense	1.06 ± 0.07	0.57 ± 0.08**	0.96 ± 0.15	1.48 ± 0.18*	146.55
S. venosum	1.27 ± 0.09**	1.24 ± 0.43	0.92 ± 0.12	1.36 ± 0.10*	125.75
Medium	0.95 ± 0.02	1.10 ± 0.10	1.10 ± 0.11	1.09 ± 0.09	114.3

Reporter assay: Cells were transfected doubly with pRL-TK vector and a different pGL3-x construct. Cells were treated with 25 mg/ml of Mauritian plant extracts for 24 h prior to harvesting. Data is shown as a fraction vs. basal level of each reporter. Refer to "Materials and mehods" section for details ($p < 0.05$; **$p < 0.01$ vs. Medium; means ± SD, $n = 3$).

adaptive radiation of plant species from different sources. Around 70% of the phanerogam genera are derived from Madagascar and the mainland Africa, 8% from Asia, 12% of pan Indo-Pacific origin and 8% are endemic.[28] Tropical plants are tolerant of high levels of environmental stress induced by ultraviolet radiation and pollutants including smoke that contains high levels of free radicals. This may explain the high levels of phenolic compounds in the endemic plant species studied.

A simple system to assess the transcriptional activity of antioxidant enzymes was established, and the effects of plant extracts on the promoter activity of antioxidant enzymes were evaluated. Substantive amounts of phenolic compounds were detected in *E. pollicina*, *S. venosum* and *S. glomeratum*. These amounts are comparable or even higher to the amounts cited in the literature for phenol-rich plants. Detailed analysis of these phenolics revealed the presence of catechins, oligomeric procyanidins including B dimers, polymeric proanthocyanidins, conjugated flavonoids and chlorogenic acid as the major phenolic compounds (Table II).

Antioxidant activities of polyphenolic compounds are widely reported in the literature; for example, phenolic acids such as chlorogenic acid,[29] other phenylcarboxylic acids,[30–32] flavones, flavonol aglycones and their glycosides such as rutin and hyperoside,[33–35] catechins[36] and proanthocyanidins.[37–39] Use of plant phenolics in food preservation is increasingly suggested primarily because of their antioxidant effects.[40,41] It is important to note that many biological functions of these molecules such as antimutagenicity, anticarcinogenicity and antiaging, amongst others, at least partially stems from this antioxidant activity.[42,43]

To further understand the biological benefits of these phenolics, we have focused on the effects on the promoter activity of antioxidant enzymes. Kim *et al.*[44] have reported that ginsenoside Rb2 derived from Panax ginseng, activates transcription of Cu,Zn-SOD in human HepG2 hepatocellular carcinoma cells through AP2 site.[44] It is known that PMA, a strong promoter in skin carcinogenesis, induces the promoter activity of Cu,Zn-SOD in HeLa cells through Sp1, Egr-1 and WT1 binding sites.[26] However, use of neoplastic cells such as HepG2 and HeLa for the evaluation of antioxidants as chemopreventive agents often presents mixed results primarily as a consequence of the fact that metabolism and the signal pathways of neoplastic and non-neoplastic cells are different. For this reason, COS7 cells derived from non-neoplastic renal tubular cells of monkey were used in the studies discussed here.

The genes for the antioxidant enzymes are housekeeping genes in cultured cells, and as such, their expression were not greatly affected by the presence of usual oxidants and antioxidants (Table I), but oxidative cellular damage does. Moderate cellular damage induced the increased transcriptional activities of these antioxidant enzymes while severe cellular damage in contrast decreased the transcriptional activities (Table I). As the reader could reason, it would be useless if cellular damage is coexistent with the induction of antioxidant enzymes when thinking of using plant extracts as preventive or therapeutic agents. Thus,

TABLE IV Association of promoter activity of antioxidant enzymes with phenolic content of plant extracts

	Cu,Zn-SOD		Mn-SOD		Catalase		GSH-Px	
Contents	$Y = aX^{-3} + b$	r	$Y = aX^{-3} + b$	r	$Y = aX^{-3} + b$	r	$Y = aX^{-3} + b$	r
Total Phenols	5.22, 0.81	0.66	4.27, 0.58	0.44	−5.60, 1.39	0.56	−0.15, 1.32	0.00
Flavonoids	6.14, 0.96	0.55	2.91, 0.75	0.21	−2.4, 1.14	0.17	−0.075, 1.31	0.00
Total PAC	0.75, 0.81	0.045	4.35, 1.04	0.34	−3.97, 1.14	0.24	−7.47, 1.43	0.48
Oligomeric PAC	12.43, 1.06	0.48	1.49, 0.81	0.045	−10.8, 1.12	0.33	−8.27, 1.34	0.26
Polymeric PAC	1.75, 1.08	0.089	−0.32, 0.82	0.00	−3.53, 1.12	0.14	−14.80, 1.49	0.61
	SODs		Pxs		Total		H_2O_2-1	
	$Y = aX^{-3} + b$	r	$Y = aX^{-3} + b$	r	$Y = aX^{-3} + b$	r	$Y = aX^{-3} + b$	r
Total Phenols	9.49, 1.39	0.65	−5.75, 2.71	0.41	3.74, 4.10	0.20	15.25, −1.32	0.72
Flavonoids	9.05, 1.71	0.44	−2.52, 2.45	0.13	6.52, 4.16	0.24	11.57, −0.73	0.38
Total PAC	5.10, 1.84	0.21	−11.45, 2.57	0.51	−6.34, 4.41	0.21	16.55, −0.73	0.48
Oligomeric PAC	13.92, 1.87	0.29	−19.2, 2.46	0.42	−5.23, 4.33	0.084	33.07, −0.59	0.47
Polymeric PAC	1.42, 1.91	0.032	−18.33, 2.61	0.53	−16.91, 4.51	0.35	19.76, −0.70	0.37

	H_2O_2-2	
	$Y = aX^{-3} + b$	r
Total Phenols	6.05, 0.48	0.75
Flavonoids	5.34, 0.69	0.47
Total PAC	6.28, 0.71	0.48
Oligomeric PAC	13.14, 0.76	0.50
Polymeric PAC	6.89, 0.73	0.34

SODs, summation of Cu,Zn-SOD and Mn-SOD promoter activity; Pxs, summation of catalase and GSH-Px promoter activity; Total, summation of promoter activities of all the four antioxidant enzymes; H_2O_2-1, SODs minus Pxs; H_2O_2-2, SODs/Pxs; PAC, proanthocyanidins; r, correlation coefficient; bold and underlined, $r > 0.6$.

we carefully chose the experimental conditions that did not to give rise to damage to the cells (25 μg/ml concentration and 24 h-incubation).

Our results showed that promoter activity of Cu,Zn-SOD was proportionally associated with phenolic content whereas that of GSH-Px was inversely associated with the content of proanthocyanidins (Table IV). Rate constant of Cu,Zn-SOD for $O_2^{\cdot-}$ is $2.4 \times 10^9\,M^{-1}\,s^{-1}$, and that of catalase and glutathione peroxidase is 1.7 and $5 \times 10^7\,M^{-1}\,s^{-1}$, respectively.[45] Increased Cu,Zn-SOD activity and decreased catalase or glutathione peroxidase activities both lead to the accumulation of H_2O_2. As simple models, we have proposed two functions, namely [H_2O_2-1] = [Cu,Zn-SOD + Mn-SOD] − [catalase + GPx] and [H_2O_2-2] = [Cu,Zn-SOD + Mn-SOD]/[catalase + GPx] (each enzyme denotes the fraction of transcriptional activity in comparison to the basal level and obtained from Table III). Interestingly, these two functions revealed the best correlation of $r = 0.72$ and 0.75, respectively. Furthermore, we confirmed the increase in peroxides after addition of *E. pollicina* to COS7 cells. It is well established that low level of oxidative stress induces other cellular protective mechanisms as an adaptive response rather than causing cellular damage.[1,46] We suggest that a carefully chosen mix of endemic plant extracts could facilitate the regulation of oxidative stress levels in the cells.

Oxidative stress has been associated with a variety of human diseases including atherosclerosis and carcinogenesis, two major causes of death in the world.[47] There is an intense need for the prevention of these diseases via modulation of oxidative stress. Efforts are being directed at extending this study to cells from different origin including neuronal and endothelial cells. Endemic tropical plants could represent important sources of antioxidants which could be applied in the management of clinical conditions in which oxidative and nitrosative stress mechanisms are implicated.

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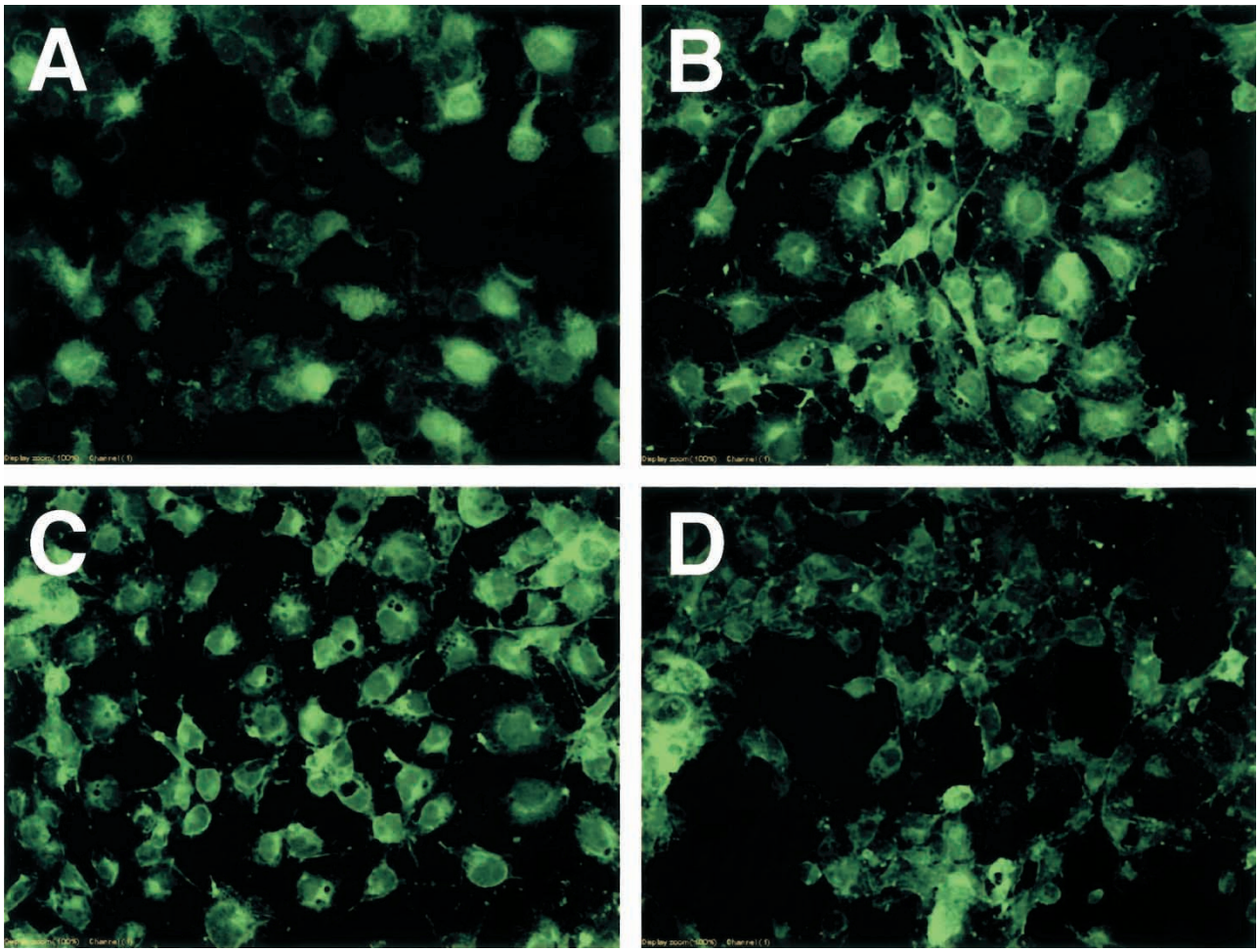

FIGURE 1 Detection of peroxides in COS7 cells after an exposure to Mauritian endemic plant extracts. COS7 cells were plated on a collagen I-coated 4-chamber slide. (A) untreated control, (B) as a positive control, mouse recombinant tumor necrosis factor-α (final concentration 10 ng/ml) was added and incubated for 1 h. Mauritian endemic plant extracts (C) *Eugenia pollicina* and (D) *Eugenia elliptica*; final concentration 25 μg/ml, were added for an incubation of 24 h. After washing, 2′, 7′-dichloroflurescein was added and incubated for 5 min at 37°C, followed by an examination with a confocal laser scanning microscopy. Extracts from *Eugenia pollicina* induced significant fluorescence, which was consistent with the results in Table III. Refer to "Materials and methods" section, and "Results" sections for more details.

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