Antioxidant fractions of *Khaya grandifoliola* C.DC. and *Entada africana* Guill. et Perr. induce nuclear translocation of Nrf2 in HC-04 cells

Frédéric Nico Njyou^{1,3} · Atsama Marie Amougou¹ ·
Romeo Fouemene Tsayem¹ · Jacqueline Njikam Manjia¹ · Swetha Rudraiah³ ·
Bolling Bradley² · José Enrique Manautou³ · Paul Fewou Moundipa¹

Received: 20 April 2015 / Revised: 21 July 2015 / Accepted: 24 July 2015 / Published online: 14 August 2015
© Cell Stress Society International 2015

Abstract The in vitro antioxidant properties, cytoprotective activity, and ability to induce nuclear translocation of nuclear factor E2-related factor-2 (Nrf-2) of five solvent fractions of the methylene chloride/methanol (1:1 v/v) extract of *Khaya grandifoliola* (Meliaceae) and *Entada africana* (Fabaceae) were evaluated. Five antioxidant endpoints were used in the antioxidant activity investigation. The total phenolic content of the fractions was assessed as to the Folin–Ciocalteu method and the profile of interesting fractions analyzed by high-performance liquid chromatography (HPLC). The cytoprotective activity of fractions was determined by H₂O₂-induced oxidative stress in HC-04 cells by measuring lactate dehydrogenase (LDH) leakage into culture medium. HC-04 cells were used to investigate the ability to induce nuclear translocation of Nrf2. For both plants, the methylene chloride/methanol (90/10; v/v) fraction (F10), methylene chloride/methanol (75/25; v/v) (F25), and the methanolic fraction (F100) were found to have the highest total polyphenol content and exhibited high antioxidant activity strongly correlated with total polyphenol content. The cytoprotective activity of fraction F25 from both plants was comparable to that of quercetin (3.40±0.05 µg/mL), inhibiting LDH leakage with a low half inhibition concentration (IC₅₀) of 4.05±0.03 and 3.8±0.02 µg/mL for *K. grandifoliola*

and *E. africana*, respectively. Lastly, fraction F25 of *K. grandifoliola* significantly ($P<0.05$) induced nuclear Nrf2 translocation by sixfold, whereas that from *E. africana* and quercetin was only twofold. The results indicate for the first time that fraction F25 of the studied plants is more antioxidant and cytoprotective and induces nuclear translocation of Nrf2 in a human hepatocyte cell line.

Keywords *K. grandifoliola* · *E. africana* · Fraction F25 · Antioxidant · Nrf2

Abbreviations

CE	Plant crude extracts
F0	Methylene chloride fraction
F5	Methylene chloride/methanol (95/5; v/v) fraction
F10	Methylene chloride/methanol (90/10; v/v) fraction
F25	Methylene chloride/methanol (75/25; v/v) fraction
F100	Methanolic fraction
RC	Reference compound
PE	Plant extracts
AA	Ascorbic acid
DPPH 2	2-Diphenyl-picryl-hydrazyl (DPPH) free-radical scavenging assay
LP	Inhibition of rat liver lipid peroxidation assay
HR	Hydroxyl radical scavenging assay
LDH	Lactate dehydrogenase
HPLC-UV	High-performance liquid chromatography-ultraviolet
ROS	Reactive oxygen species
Nrf2	Nuclear factor E2-related factor-2
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide

✉ Paul Fewou Moundipa
pmoundipa@hotmail.com

¹ Laboratory of Pharmacology and Toxicology, Department of Biochemistry, Faculty of Science, University of Yaoundé 1, P.O. Box 812, Yaounde, Cameroon

² Department of Food Science, University of Wisconsin-Madison, Madison, WI 53706, USA

³ Department of Pharmaceutical Sciences, University of Connecticut, Storrs, Connecticut 06269, USA

MTS	3-(4, 5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt
CAE	Chlorogenic acid equivalents
AAE	Ascorbic acid equivalent
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
EDTA	Ethylenediaminetetraacetic acid
DTT	Dithiotreitol
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ANOVA	Analysis of variance

Introduction

Oxidative stress is linked to excessive production of reactive oxygen species (ROS). This plays a key role in promoting progression of liver diseases (Zhu et al. 2012). The liver possesses a range of antioxidant systems to neutralize ROS (Casarett et al. 2008). The expression of a series of antioxidant genes is regulated by the nuclear factor E2-related factor-2 (Nrf2) to protect cells against oxidative stress (Aleksunes et al. 2006; Tosi et al. 2011; Tang et al. 2014). Nrf2 activation is also essential for liver regeneration via alleviation of oxidative stress and regulation of hepatocyte proliferation (Shin et al. 2013). Many substances of plant origin are known to be Nrf2 activators (Zhao et al. 2010). *Khaya grandifoliola* and *Entada africana* are traditionally used in Cameroon and elsewhere in Africa to treat liver-related diseases and, pharmacological and phytochemical investigations conducted so far on these plants are reported (Njyou et al. 2013a). The anti-hepatitis C (Tietcheu et al. 2014), anti-angiogenic (Germanò et al. 2014), immunomodulatory, and anti-inflammatory (Owona et al. 2013a, 2013b) in vitro activities of *E. africana* are also documented. Our group has demonstrated that the methylenechloride/methanol (1:1 v/v) extract of the stem bark possesses strong hepatoprotective and antioxidant activities in vitro and in vivo (Njyou et al. 2013a, 2013b, 2013c). Since oxidative stress is involved in the development of most liver diseases, the objective of the present study was to evaluate in vitro antioxidant properties and cytoprotective activity of five solvent fractions of the methylene chloride/methanol (1:1 v/v) extract of both plants as well as their ability to induce nuclear translocation of Nrf2.

Materials and methods

Chemicals

All reagents used in this study were of analytical grade and purchased from Sigma Chemicals Company

(Hamburg, Germany; St. Louis, MO, USA) and Prolabo (Paris, France).

Preparation of chemical plant fractions

Stem barks of the plants were collected in June 2012 in Fouban (West Cameroon). The botanical identification of the plants was done at the Cameroon National Herbarium, where voucher specimens are kept under the reference numbers 23434 YA for *K. grandifoliola* and 52661 YA for *E. africana*. The name of the plants has been checked with <http://www.theplantlist.org>. The plant crude extracts (CEs) and five fractions, namely methylene chloride (F0), methylene chloride/methanol (95/5; v/v) (F5), methylene chloride/methanol (90/10; v/v) (F10), methylene chloride/methanol (75/25; v/v) (F25), and methanolic fraction (F100) were prepared as described elsewhere (Tietcheu et al. 2014).

Phytochemical screening, determination of total phenolic content, and chemical profile of fractions

Phytochemical screening of the different fractions was performed according to a procedure described by Harbone (1976), Odebeyi and Sofowora (1978). Total phenolic content determination was performed according to the Folin–Ciocalteu method as described by Blažeković et al. (2010). Briefly, 50 µL of the test sample mixed with 2.4 mL distilled water and 200 µL of Folin–Ciocalteu's reagent (1/10) were added to 200 µL of Na₂CO₃ 20 %. The reaction mixture was incubated at 25 °C for 40 min and the absorbance read at 725 nm. The results were compared to a chlorogenic acid calibration curve, and the total phenolic content was expressed as milligrams of chlorogenic acid equivalents (CAEs) per gram of extract. Fractions with high polyphenol content were further characterized using high-performance liquid chromatography (HPLC). A Gilson semi-preparative HPLC with a GX-271 automated liquid handler and a 155 dual wavelength UV/visible detector were used to inject 250 µL of the fractions dissolved in DMSO-H₂O (1:1; v/v) mixture onto an Agela Venusil ASB C18 column (10×250 mm, 5 µm particle size). The solvent system used was (A) 10 % acetic acid in water and (B) acetonitrile with 100 % A at 0 min, linearly increasing to 100 % B from 0 to 30 min, then holding 100 % B to 40 min. Eluate was monitored at 280 and 340 nm.

Chemical antioxidant studies

For the following assays, plant samples and ascorbic acid (positive control) were tested in triplicate at concentrations of 0.1, 1, 10, and 100 µg/mL.

2, 2-Diphenyl-picryl-hydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging activity of plant samples was assessed using the method described by Moyo et al. (2010). A volume of 3.1 mL of a DPPH solution (40 µg/mL) in pure methanol was mixed with 50 µL of the test sample to achieve the desired concentration. Then, the mixture was incubated at room temperature for 30 min, and the absorbance recorded at 517 nm against a blank. Control samples were prepared containing the same volume without any plant extract (or ascorbic acid as positive control). The percentage of DPPH scavenging activity was calculated according to the following equation, which calculates the half scavenging concentration.

$$\text{Scavenging activity (\%)} = 100 \times (\text{Ac} - \text{As}) / (\text{Ac})$$

Ac: absorbance of control; *As*: absorbance of sample

Hydroxyl (OH[•]) radical scavenging assay

Hydroxyl radical scavenging assay was performed according to the method described by Su et al. (2009). Hydroxyl radicals were generated by the Fenton reaction of Fe²⁺ and H₂O₂. The reaction mixture consisted of 8 mM FeSO₄, 6 mM H₂O₂, distilled water, 50 µL of the test sample, and 20 mM sodium salicylate. The mixture was then incubated at 37 °C for 1 h, and the absorbance recorded at 562 nm. The scavenging activity was calculated using the same equation described above for the DPPH radical scavenging activity assay.

Reducing ability assay

The reducing ability of plant samples was assessed as described by Varshneya et al. (2012). Various concentrations of plant extracts, their fractions, or ascorbic acid were added to 1.1 mL of phosphate buffer (200 mM, pH 6.6) and 1 mL of potassium ferrocyanate [K₃Fe(CN)₆] (1 %). After incubation at 50 °C for 20 min, 1 mL of trichloroacetic acid (10 %) was added to the mixture before centrifugation (1000 rpm, 10 min). The supernatant was mixed with 1 mL of distilled water and 1 mL of ferrichloride (1 %). The absorbance was measured at 700 nm.

Total antioxidant capacity assay

Determination of total antioxidant capacity was done by the phosphomolybdenum method according to Blažeković et al. 2010. The tubes containing the plant extracts and reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) were incubated at 95 °C for

90 min. After the mixture had cooled to room temperature, the absorbance of each solution was measured at 695 nm against a blank. The antioxidant capacity was expressed as ascorbic acid equivalent (AAE).

Inhibition of rat liver lipid peroxidation assay

The inhibition of lipid peroxidation by plant extracts and ascorbic acid was determined according to the thiobarbituric acid method. FeCl₂–H₂O₂ was used to induce lipid peroxidation in liver homogenates (Su et al. 2009). Each plant sample was mixed with 1.0 mL of a 10 % liver homogenate, and then, an appropriate volume of FeCl₂ (0.5 mM) and H₂O₂ (0.5 mM) was added. The mixture was incubated at 37 °C for 60 min, and then, 1.0 mL each of trichloroacetic acid (15 %) and thiobarbituric acid (0.67 %) were added, and the mixture was heated to 100 °C for 15 min. After centrifugation (3500 rpm, 5 min), the absorbance of the supernatant was recorded at 532 nm. The percentage inhibition was calculated according to same equation described above.

Calculation of half efficient/inhibitory concentration (EC₅₀/IC₅₀) of plant fractions and study of correlations between total polyphenol content and antioxidant activity

Different EC₅₀/IC₅₀ values were automatically calculated using STATGRAPHICS plus version 5.0 software. Correlation between total polyphenol content of fractions and their antioxidant activity were determined by linear regression. For each plant fraction, four different solutions were prepared at the tested concentrations (0.1, 1, 10, and 100 µg/mL) and total polyphenol content of each solution was assayed. Then, for each antioxidant model, the activity of the fraction at each tested concentration was plotted against the polyphenol content using the Microsoft Excel 2007, and the coefficient *r*² value was deduced from the graph.

Cellular viability and antioxidant activity study

Cell culture and treatment

The spontaneously immortalized human hepatocyte HC-04 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Fisher Scientific; PA, USA) supplemented with 10 % fetal bovine serum, 10 mM glucose, and 1 % antibiotic-antimycotic (100 units/mL penicillin G sodium, 100 µg/mL streptomycin sulfate, and 0.25 µg/mL amphotericin B) in a 5 % CO₂ and humidified environment (95 % relative humidity) at 37 °C. To study the cell viability, cells were seeded at 25,000 cells per well in complete DMEM in a 96-well plate and incubated for 24 h. Afterwards, cells were treated with plant fractions or quercetin (positive control) at concentration of 0, 4, 20, 100, and 500 µg/mL or vehicle

controls (0.1 % DMSO) and incubated for 24 h. In cellular antioxidant experiments, cells were plated at a density of 2×10^5 per well in a 24-well plate (plating volume is 1 mL per well) for 24 h. Following 24 h, cells were treated with plant fractions or quercetin (1, 4, 20, 50, and 100 $\mu\text{g/mL}$ concentration) or vehicle and incubated for another 24 h. Cells were further treated with 1 mM H_2O_2 for an additional 24 h. To analyze the effect of selected plant fractions on the induction of Nrf2 nuclear translocation, cells were treated with vehicle or 40 μM of quercetin or the highest active determined concentrations of selected fractions, 20 and 50 $\mu\text{g/mL}$ for *E. africana* and *K. grandifoliola*, respectively. Cells were harvested 12 h after treatment.

Cell viability assay

Cell viability was quantified using the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium salt (MTS) assay kit (CellTiter 96 Aqueous Cell Proliferation Assay; Promega, Madison, WI) according to the manufacturer's instructions using microplate reader.

Cellular antioxidant study

The fractions from each with higher polyphenol content were chosen for cellular antioxidant study. Cell integrity was determined by monitoring lactate dehydrogenase (LDH) leakage into medium using a commercial LDH kit assay (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions using microplate reader.

Subcellular fractionation of Nrf2 protein and Western blot analysis for Nrf2

Cells were washed twice with ice-cold PBS and suspended in hypotonic buffer (10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 1.5 mM MgCl_2 , 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail) for 30 min, vortexed for 10 s, and centrifuged at $10,000 \times g$ for 15 min at 4°C . The supernatants comprised the cytoplasmic fraction. The nuclear pellets were further resuspended in hypertonic buffer [20 mM HEPES-KOH (pH 7.9), 400 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 1 mM DTT, 5 % glycerol, and protease inhibitor] and incubated for 30 min on ice. The lysates were vortexed for 10 s and centrifuged at $18,000 \times g$ for 15 min at 4°C . The resulting supernatants comprising the nuclear fraction were stored at -80°C until assayed. Protein content was determined by Lowry reagent using bovine serum albumin as a standard. Equal amounts of nuclear proteins ($\sim 30 \mu\text{g}$) boiled in Laemmli sample buffer containing 10 % β -mercaptoethanol for 10 min at 90°C were separated by electrophoresis in a 10 % SDS-polyacrylamide gel and were transferred onto PVDF

membranes. The membranes were blocked with 5 % non-fat dry milk and further incubated with rabbit anti-human Nrf2 primary antibody (1/5000 in 5 % BSA) overnight at 4°C . This was followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1/2000 in 2 % milk) for 2 h. Protein-antibody complexes were detected using a chemiluminescent kit (Thermo Scientific, IL).

Statistical analysis

Statistical analyses were performed by one-way ANOVA followed by Dunnett's post hoc test using the Graphpad InStat 3 software for Windows. The results were considered statistically significant at $P < 0.05$.

Results

Phytochemical compounds, total phenolic content, and fingerprint of the fractions

As presented in Table 1, all fractions of *K. grandifoliola* and *E. africana* revealed the presence of polyphenols, among other classes of compounds detected. For both plant fractions, the total phenolic content ranged from 6.031 to 41.372 mg CAE/g extract. Fraction F25 of *K. grandifoliola* and *E. africana* was found to contain 38.401 ± 0.033 and 41.372 ± 0.201 CAE/g extract, respectively. Representative HPLC chromatograms for fraction F25 of *K. grandifoliola* and *E. africana* are presented in Fig. 1a, b, respectively. Several peaks with UV absorption at 280 nm were detected, indicating the presence of aromatic compounds.

Chemical antioxidant activities

Table 2 shows the concentration of each fraction required to scavenge radical/inhibit LP by 50 % ($\text{EC}_{50}/\text{IC}_{50}$). Fractions F25 and F100 of *K. grandifoliola* are most active given their respective low IC_{50} values in radical-scavenging and LP-inhibiting activities. Similarly, fraction F100 of *E. africana* efficiently scavenged the DPPH free radical and also inhibited LP. The total antioxidant capacity of the plant extracts, expressed as equivalents of ascorbic acid milligram per gram of extract (AAE mg/g extract) is shown in Table 3. All the extracts showed an increase in antioxidant capacity with increasing concentrations. At 100 $\mu\text{g/mL}$, total antioxidant capacity of fractions F25 was found to be 283.700 ± 0.004 and 259.158 ± 0.004 AAE mg/g extract for *K. grandifoliola* and *E. africana*, respectively. Figure 2 depicts the reducing capacity of the plant fractions and ascorbic acid. The reducing ability of all fractions increased with increasing concentrations.

Table 1 Fractionation yield, phytochemical compositions, and polyphenol content of different fractions of the plants

Fractions	<i>Khaya grandifoliola</i>			<i>Entada africana</i>		
	Yields (%)	Phytochemical classes of compounds tested present	Polyphenol content (mg CAE/g of extract)	Yields (%)	Phytochemical classes of compounds tested present	Polyphenol content (mg CAE/g of extract)
F0	5.163	Sugar, terpenes, polyphenols	10.324±0.202	14.881	Sterols, terpenes, polyphenols,	6.031±0.062
F5	12.204	Sugar, polyphenols	13.101±0.104	5.453	Sterols, terpenes, polyphenols, flavonoids	27.433±0.571
F10	7.987	Sugar, polyphenols, tanins	32.205±2.062	8.563	Sugar, polyphenols, saponins, flavonoids	29.013±0.714
F25	59.692	Sugar, polyphenols, tanins, flavonoids, leucoanthocyanes, terpenes	38.401±0.033	3.822	Sugar, polyphenols, saponins, flavonoids	41.372±0.201
F100	77.363	Polyphenols, tanins, flavonoids, leucoanthocyanes, terpenes	32.722±0.604	8.863	Polyphenols, flavonoids, saponins	39.292±0.681

Values are expressed as mean ± SD of two experiments in triplicate

F0 methylene chloride fraction, F5 methylene chloride/methanol (95/5; v/v) fraction, F10 methylene chloride/methanol (90/10; v/v) fraction, F25 methylene chloride/methanol (75/25; v/v) fraction, F100 methanol (100 %) fraction, CAE chlorogenic acid equivalent

Correlations between total polyphenol content and antioxidant activity

The all r^2 values are presented in Table 4. The correlation coefficient for the selected fractions was found to be ranged from 0.804 to 0.996 in at least three out of five oxidation models used. This is indicative of a very strong and positive correlation between the total polyphenol content and antioxidant endpoints studied.

Fraction F25 protects cells against H₂O₂-mediated cell damage

Except for the fraction F0, cell viability remained unchanged by treatment for 24 h with all other plant fractions at concentrations of 4, 20, and 100 µg/mL (data not shown). In cytoprotective experiments, and as expected, lactate dehydrogenase (LDH) activity in culture medium of HC-04 cells was significantly ($p<0.05$) increased when cells were treated with H₂O₂ in

Fig. 1 HPLC chromatogram of the F25 [methylene chloride/methanol (75/25; v/v)] fraction. **a** Chromatogram of *K. grandifoliola* F25 and **b** chromatogram of *E. africana* F25. Volume injected 250 µL. Agela Venusil ASB C18 column (10×250 mm, 5 µm particle size). The

solvent system consisted of [A] 10 % acetic acid in water and [B] acetonitrile with 100 % A at 0 min, linearly increasing to 100 % B from 0 to 30 min, then holding at 100 % B to 40 min. Flow rate 0.5 mL/min

Table 2 Scavenging/inhibiting effect of the plant extracts in different oxidation models as determined by half efficient (EC₅₀)/inhibition (IC₅₀) concentration

RC and PE	Oxidation models and EC ₅₀ /IC ₅₀ (μg/mL) values					
	Effects of <i>Khaya grandifoliola</i> extracts			Effects of <i>Entada africana</i> extracts		
	DPPH	HR	LP	DPPH	HR	LP
CE	4.543±0.281	18.101±6.933	13.202±1.704	44.881±1.926	>100	95.521±1.701
AA	3.553±0.004	4.252±0.072	14.646±1.488	3.554±0.002	4.253±0.074	14.653±1.491
F0	>100	>100	49.802±0.002	>100	>100	>100
F5	>100	>100	>100	85.873±0.471	>100	>100
F10	19.232±2.087	>100	7.002±0.003	64.004±2.273	>100	60.432±2.122
F25	5.623±0.002	22.202±2.832	8.901±0.003	47.154±1.932	>100	53.564±2.401
F100	9.471±0.002	28.353±5.304	8.703±0.002	41.062±0.657	>100	79.952±1.303

Values are expressed as mean ± SD of two experiments in triplicate

F0 methylene chloride fraction, F5 methylene chloride/methanol (95/5; v/v) fraction, F10 methylene chloride/methanol (90/10; v/v) fraction, F25 methylene chloride/methanol (75/25; v/v) fraction, F100 methanol (100 %) fraction, RC reference compound, PE plant extracts, CE crude extract, AA ascorbic acid, DPPH DPPH free-radical scavenging assay, LP inhibition of rat liver lipid peroxidation assay, HR hydroxyl radical scavenging assay, EC₅₀/IC₅₀ concentration of the plant extract required to scavenge/inhibit 50 %

comparison to untreated controls. LDH activity in medium was significantly ($p<0.05$) lower in a concentration-dependent manner following pretreatment with quercetin and fraction of *E. africana* at 4, 10, and 20 μg/mL and at all tested concentrations with regard to *K. grandifoliola* as compared to the H₂O₂-only treated group (Fig. 3). The half inhibition concentration values (IC₅₀), which is defined as the concentration of sample required to inhibit LDH leakage by 50 % from the H₂O₂-only treated cells, were found to be 4.051±0.032, 3.821±0.023, and 3.401±0.054 μg/mL for fraction F25 of *K. grandifoliola*, *E. africana*, and quercetin, respectively.

Fraction F25 induces nuclear translocation of Nrf2

Fraction F25 from both plants induced nuclear translocation of Nrf2 (Fig. 4) at 12 h after treatment. Compared to the

control, fraction F25 from *K. grandifoliola* significantly ($p<0.05$) induced nuclear Nrf2 translocation by sixfold, whereas both quercetin and fraction F25 from *E. africana* produced a twofold induction.

Discussion

In the present study, the antioxidant properties of five solvent fractions of *K. grandifoliola* and *E. africana* were evaluated to determine their ability to protect against oxidative stress, a causative factor in liver diseases (Zhu et al. 2012). Both plant fractions were preliminary screened phytochemically and tested for total polyphenol content, free radical scavenging activity, inhibition of lipid peroxidation, total antioxidant capacity, and reducing ability.

Table 3 Total antioxidant capacity (AAE mg/g extract) of the plant extracts

PE	Concentrations (μg/mL)							
	Effects of <i>Entada africana</i> extracts				Effects of <i>Khaya grandifoliola</i> extracts			
	0.1	1	10	100	0.1	1	10	100
CE	1.343±0.125	5.067±0.172	39.133±0.604	224.786±1.121	2.442±0.345	21.91±0.086	128.327±1.386	255.617±1.466
F0	0.000±0.000	0.855±0.345	5.189±1.121	35.472±0.086	0.000±0.00	0.488±0.172	10.256±1.552	66.972±4.007
F5	0.183±0.654	3.053±0.845	19.78±1.325	135.857±5.235	0.193±0.021	1.099±0.325	5.189±0.776	68.926±4.915
F10	2.076±0.086	14.469±0.862	144.567±0.175	294.567±1.762	0.733±0.123	4.823±0.086	73.871±0.692	286.022±0.604
F25	0.122±0.253	14.347±0.431	115.507±3.104	259.158±0.004	1.038±0.086	6.166±0.259	75.092±2.415	283.700±0.004
F100	2.076±0.054	14.469±1.293	147.009±4.657	294.567±0.0857	0.855±0.172	1.771±0.162	34.005±0.431	202.321±0.104

Values are expressed as mean equivalents ascorbic acid per gram of extract ± SD of two experiments in triplicate

F0 methylene chloride fraction, F5 methylene chloride/methanol (95/5; v/v) fraction, F10 methylene chloride/methanol (90/10; v/v) fraction, F25 methylene chloride/methanol (75/25; v/v) fraction, F100 methanol (100 %) fraction, PE plant extracts, CE crude extract, AAE ascorbic acid equivalents

Fig. 2 Reducing power of the plant extracts. **a** Experiment with *K. grandifoliola* extracts. **b** Experiment with *E. africana* extracts. Values are expressed as mean $\pm$ SD of two experiments in triplicate, *CE* crude extract, *F0* methylene chloride fraction, *F5* methylene chloride/methanol (95/5; v/v) fraction, *F10* methylene chloride/methanol (90/10; v/v) fraction, *F25* methylene chloride/methanol (75/25; v/v) fraction, *F100* methanol fraction, *AA* ascorbic acid

The results of our preliminary phytochemical screening revealed the presence of multiple polar and non-polar chemical constituents (Table 1). Steroids, triterpens, flavonoids, polyphenols tannins, sugars among others were tested positive in the different fractions from these plants. Polyphenols present in polar plant fractions were quantitatively prominent (Table 1). Based on polyphenolic content, plant fractions can be ranked as follows: F25 > F100 > F10 > F5 > F0. The HPLC-UV fingerprint of fraction F25 from both plants was determined, indicating the presence of a group of aromatic compounds. Since polyphenols are aromatic, the results presented in this manuscript confirm the presence of aromatic compounds in these plant fractions. The fractions are mixtures containing at least one predominant compound(s) that can be eluted at approximately 7 and 23 min for *K. grandifoliola* (Fig. 1a) and at 5, 8, and 23 min for *E. africana* (Fig. 1b). The existing literature supports the notion that the antioxidant activity of these plant extracts can be attributed to the presence of phenolic compounds, which function as free radical

scavengers, hydrogen-donating sources, singlet oxygen quenchers, or metal ion chelators (Quideau *et al.* 2011).

When examining chemical antioxidants, more than one assay should be performed to determine antioxidant activity and mechanism (Wong *et al.* 2006). This is important for natural products since their antioxidant action can be the combined result of a mixture of compounds working through different mechanisms. Accordingly, DPPH radical, hydroxyl radical, lipid peroxidation, total antioxidant capacity, and reducing power ability assays were used in the current studies (Chanda and Dave 2009). The scavenging of radicals, inhibition of LP, and reduction of potassium ferrocyanate and phosphomolybdate by the different samples from *K. grandifoliola* and *E. africana* indicate that they possess strong antioxidant properties.

An extract exhibits its free radical scavenging activity through neutralization of free radical by either a transfer of hydrogen or an electron (Amarowicz *et al.* 2004). Hydroxyl radical is one of the reactive oxygen species found in

Table 4 The r^2 (correlation coefficient) values between antioxidant activities and total polyphenolic content

PE	Correlation coefficient r^2									
	Effects of <i>Khaya grandifoliola</i> extracts					Effects of <i>Entada africana</i> extracts				
	DPPH	HR	LP	TAC	RPA	DPPH	HR	LP	TAC	RPA
CE	0.879	0.857	0.857	0.883	0.995	0.965	NC	0.862	0.899	0.943
F0	NC	NC	0.307	0.993	0.971	NC	NC	NC	0.947	0.980
F5	NC	NC	NC	0.985	0.994	0.758	NC	NC	0.974	0.955
F10	0.985	NC	0.953	0.915	0.981	0.856	NC	0.656	0.934	0.869
F25	0.978	0.698	0.804	0.810	0.976	0.965	NC	0.956	0.924	0.972
F100	0.977	0.804	0.857	0.896	0.996	0.945	NC	0.632	0.915	0.977

F0 methylene chloride fraction, *F5* methylene chloride/methanol (95/5; v/v) fraction, *F10* methylene chloride/methanol (90/10; v/v) fraction, *F25* methylene chloride/methanol (75/25; v/v) fraction, *F100* methanol (100 %) fraction, *PE* plant extracts, *CE* crude extract, *DPPH* DPPH free-radical scavenging assay, *LP* inhibition of rat liver lipid peroxidation assay, *HR* hydroxyl radical scavenging assay, *TAC* total antioxidant capacity, *RPA* reducing power ability, *NC* not computed because of the weak activity of the plant fraction in the oxidation model

Fig. 3 Protective effect of plant-selected fractions and quercetin on H_2O_2 -induced LDH leakage in HC-04 cells. Values are mean $\pm$ SD of samples in triplicate. Significantly different ($p < 0.05$) compared to untreated control (a) and 1 mM H_2O_2 treated (b). E25/K25 Entada/khaya F25 fraction

biological systems. It oxidizes and damages polyunsaturated fatty acids of cell membranes leading to lipid peroxidation (Battu et al. 2011). Free radical scavenging is one of the mechanisms by which antioxidants inhibit the lipid peroxidation (Amarowicz et al. 2004).

Fig. 4 Effect of plant fractions on nuclear Nrf2 accumulation in HC-04 cells. **a** After treatment with DMSO (control), fraction F25 of *Entada africana* at 20 μ g/mL, fraction F25 of *Khaya grandifoliola* at 50 μ g/mL, or quercetin at 40 μ M for 12 h. Nuclear proteins were extracted, and equal amounts were separated by SDS-PAGE and immunoblotted with antibodies specific to Nrf2 or to transcription factor II B (TFIIB) as loading control. Blots represent experiments performed in triplicate ($n=3$). **b** Histogram showing the densitometric analysis of Nrf2 expression normalized to TFIIB, using Image J software. *Significantly different ($p < 0.05$) compared to untreated control

In radical-scavenging and inhibitory tests, the low EC_{50}/IC_{50} values obtained for the plant samples we tested are indicative of their potent antioxidant activity. Regarding the reducing power and total antioxidant ability experiments, a higher absorbance and quantity of ascorbic acid equivalent/g extract are indicative of increased reducing power and total antioxidant ability, respectively.

In this study, a plant fraction was selected, if it exhibited good activity in at least four of the antioxidant assays used. Therefore, based on EC_{50}/IC_{50} values (Table 2), quantity of ascorbic acid equivalent/g extract values (Table 3), and absorbance (Fig. 2), fractions F10, F25, and F100 from both plants were considered to be most active. Many previous studies support the concept that phenolic compounds are in great part responsible for the antioxidant activity of plant extracts (Wong et al. 2006; Moyo et al. 2010). Similarly, other studies have documented the correlation between total phenolic content in plant tissue and antioxidant activity (Kumar and Hemalatha 2011). In the current investigation, a positive correlation was noted between the antioxidant activity of selected fractions and their total polyphenol content (Table 4). Therefore, we are very confident that the strong antioxidant activity of fractions F10, F25, and F100 could be attributed to the higher polyphenolic content of these fractions.

A major contributor to oxidative damage is H_2O_2 , which is formed from superoxide leaking from mitochondria (Xu and Zhou 2013). Oxidative stress and cytotoxicity is routinely induced in vitro by treating cultured cells with H_2O_2 (Subramaniam and Ellis 2011). To further study the antioxidant activity of our plant fractions, we evaluated their capacity to protect the human hepatocyte HC-04 cells from H_2O_2 -induced cytotoxicity. The effect of these fractions on cell viability was determined by the reduction test of MTS. Apart from fraction F0 of *K. grandifoliola*, all other plant

fractions tested did not affect cell viability at concentrations below 100 µg/mL. Because of their high polyphenol content and potent antioxidant activity, fractions F25 and F100 of both plants were selected for the cytoprotective study.

As expected, intoxication of HC-04 cells with H₂O₂ significantly ($p < 0.05$) increased LDH leakage into media by 56 % compared to controls. Treatment of cells with fraction F25 of *K. grandifoliola* and *E. africana* or quercetin significantly reduced H₂O₂-induced LDH leakage (Fig. 3). Since these fractions are active in inhibiting lipid peroxidation, we propose that a potential mechanism of cytoprotection is by preventing oxidation of membrane lipids. In fact, H₂O₂ is known to produce cellular and molecular liabilities leading to lipid peroxidation, which subsequently gives rise to the production of a variety of ROS that can cause further damage to DNA, proteins, and lipids (Esterbauer 1993). In contrast to this protective effect of F25 fraction of *K. grandifoliola*, exposure of HC-04 cells to crude extracts at the concentrations of 20, 50, and 100 µg/mL, F25 fraction of *E. africana*, and quercetin at 50 and 100 µg/mL, all seemed to induce LDH leakage. This suggests that at these concentrations, the polyphenols in these extracts stimulate H₂O₂ production, resulting in cytotoxicity. In fact, production of H₂O₂ by polyphenols in culture media has been previously demonstrated (Long et al. 2000; Erlank et al. 2011). In the same line, high-dose green tea polyphenols was reported to be nephrotoxic in mice (Inoue et al. 2011).

Fraction F25 from both plants is known to contain polyphenols that can activate Nrf2 (Zhao et al. 2010). This transcription factor has been demonstrated to play an important role in protecting against liver injury (Copple et al. 2008) by regulating a battery of antioxidant and cytoprotective genes in response to cellular stress (Mohammadzadeh et al. 2012; Bryan et al. 2013). The oxidative stress sensing and transcriptional response by Nrf2 has a relatively rapid onset (Zhao et al. 2010; Erlank et al. 2011). Therefore, we investigated the effect of fraction F25 from both plants on Nrf2 activation status at 12 h before intoxication with H₂O₂. Consistent with the effect produced by quercetin (Fig. 4), a known Nrf2 inducer (Zhao et al. 2010), fraction F25 also significantly ($p < 0.05$) induced the nuclear translocation of Nrf2. These results are also in agreement with previous studies documenting Nrf2 activation by the ethanolic extract of *Alismatis rhizoma* (Han et al. 2013), thus suggesting that the cytoprotective effect of these fractions is mediated in part by Nrf2.

These results not only demonstrate the potent antioxidant activity, but for the first time, the ability of F25 fraction of both *K. grandifoliola* and *E. africana* to promote the nuclear translocation of Nrf2 in a human hepatocyte cell line. Future studies are aimed at analyzing the phytochemical composition of these fractions, to isolate the active molecule(s) in these fractions and to conduct more in depth and comprehensive investigation of their mechanism of action as cytoprotectants.

Acknowledgments Grants from the International Foundation for Sciences and the Organisation for the Prohibition of Chemical Weapons (F/4223-2) and National Institute of Health (DK069557) awarded to Drs. Njayou and Manautou, respectively, supported this work. Dr. Njayou thanks the Fulbright Program for the award which allowed him to carry out experiments on HC-04 cells in the laboratory of Dr. Manautou at the School of Pharmacy, Connecticut, USA.

References

- Aleksunes LM, Slitt AL, Maher JM, Dieter MZ, Knight TR, Goedken M, Cherrington NJ, Chan JY, Klaassen CD, Manautou JE (2006) Nuclear factor-E2-related factor 2 expression in liver is critical for induction of NAD(P)H:quinone oxidoreductase 1 during cholestasis. *Cell Stress Chaperones* 11(4):356–363
- Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA (2004) Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem* 84:551–562
- Battu CR, Ethadi SR, Vede PG, Swathi PK, Chandrika K, Rao VA (2011) Evaluation of antioxidant and anti-inflammatory activity of *Euphorbia heyneana* Spreng. *Asian Pac J Trop Biomed* 1:191–194
- Blažeković B, Vladimir-Knežević S, Brantner A, Štefan MB (2010) Evaluation of antioxidant potential of *Lavandula x intermedia* Emeric ex Loisel ‘Budrovka’: a comparative study with *L. angustifolia* Mill. *Molecules* 15:5971–5987
- Bryan HK, Olayanju A, Goldring CE, Park BK (2013) The Nrf2 cell defence pathway: Keap1-dependent and -independent mechanisms of regulation. *Biochem Pharmacol* 85:705–717
- Casarett LJ, Doull J, Klaassen CD (2008) Casarett and Doull’s toxicology: the basic science of poisons, 7th edn. McGraw-Hill, New York
- Chanda S, Dave R (2009) *In vitro* models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: an overview, review. *Afr J Microbiol Res* 3(13):981–996
- Copple IM, Goldring CE, Jenkins RE, Chia AJL, Randle LE, Hayes JD, Kitteringham NR, Park BK (2008) The hepatotoxic metabolite of acetaminophen directly activates the Keap1-Nrf2 cell defense system. *Hepatology* 48:1292–1301
- Erlank H, Elmann A, Kohen R, Kanner J (2011) Polyphenols activate Nrf2 in astrocytes via H₂O₂, semiquinones, and quinines. *Free Radical Bio Med* 51:2319–2327
- Esterbauer H (1993) Cytotoxicity and genotoxicity of lipid-oxidation products. *Am J Clin Nutr* 57:779–785, discussion 785S–786S
- Germanò MP, Certo G, D’Angelo V, Sanogo R, Malafronte N, De Tommasi N, Rapisarda A (2014) Anti-angiogenic activity of *Entada africana* root. *Nat Prod Res* 6:1–6. doi:10.1080/14786419.2014.987773
- Han WC, Kwun MJ, HaKim K, Choi JY, Oh SR, Ahn KS, Lee JH, Joo M (2013) Ethanol extract of *Alismatis rhizoma* reduces acute lung inflammation by suppressing NF-κB and activating Nrf2. *J Ethnopharmacol* <http://dx.doi.org/10.1016/j.jep.2013.01.010>
- Harbone J (1976) *Phytochemical methods. A guide to modern techniques of plants analysis*. Chapman and Hall, London, p 150
- Inoue H, Satoko A, Maeda-Yamamoto M, Nesumi A, Tanaka T, Murakami A (2011) High-dose green tea polyphenols induce nephrotoxicity in dextran sulfate sodium-induced colitis mice by down-regulation of antioxidant enzymes and heat-shock protein expressions. *Cell Stress Chaperones* 16:653–662. doi:10.1007/s12192-011-0280-8
- Kumar R, Hemalatha S (2011) *In-vitro* antioxidant activity of alcoholic leaf extract and subfractions of *Alangium lamarckii* Thwaites. *J Chem Pharm Res* 3(1):259–267
- Long LH, Clement MV, Halliwell B (2000) Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (–)-epigallocatechin,

- (-)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochem Bioph Res Co* 273:50–53
- Mohammadzadeh M, Halabian R, Gharehbaghian A, Amirzadeh N, Jahanian-Najafabadi A, Roushandeh AM (2012) Roudkenar MH (2012) Nrf-2 overexpression in mesenchymal stem cells reduces oxidative stress-induced apoptosis and cytotoxicity. *Cell Stress Chaperones* 17:553–565. doi:10.1007/s12192-012-0331-9
- Moyo M, Ndhala AR, Finnie JF, Staden JV (2010) Phenolic composition, antioxidant and acetylcholinesterase inhibitory activities of *Sclerocarya birrea* and *Harpephyllum caffrum* (Anacardiaceae) extracts. *Food Chem* 123:69–76
- Njaye FN, Aboudi ECE, Tandjang MK, Tchana AK, Ngadjui BT, Moundipa PF (2013a) Hepatoprotective and antioxidant activities of stem bark extract of *Khaya grandifoliola* (Welw) C.DC and *Entada africana* Guill. et Perr. *J Nat Prod* 6:73–80
- Njaye FN, Ngougoure FP, Tchana A, Moundipa PF (2013b) Protective effect of *Khaya grandifoliola* C.DC. stem bark extract on carbon tetrachloride-induced hepatotoxicity in rats. *Int J Ind Med Plants* 29(1):1161–1166
- Njaye FN, Tietcheu BRG, Ngadjui BT, Moundipa PF (2013c) Hepatoprotective activity of three Cameroonian medicinal plants against oxidative damages induced by paracetamol and CCl₄ in primary rat hepatocytes. *Int J Pharma Photon* 104:306–314
- Odebeyi O, Sofowora (1978) Phytochemical screening of Nigerian medicinal plants II *Llyordia* 41:234–246
- Owona BA, Njaye NF, Laufer SA, Schluesener HJ, Moundipa PF (2013a) *Entada africana* fraction CH₂Cl₂/MeOH 5% inhibits inducible nitric oxide synthetase and proinflammatory cytokines gene expression induced by lipopolysaccharide in microglia. *BMC Complement Altern Med* 13:254. <http://www.biomedcentral.com/1472-6882/13/254>
- Owona BA, Njaye NF, Laufer SA, Schluesener HJ, Moundipa PF (2013b) A fraction of stem bark extract of *Entada africana* suppresses lipopolysaccharide-induced inflammation in RAW264.7 cells. *J Ethnopharmacol* <http://dx.doi.org/10.1016/j.jep.2013.06.016>
- Quideau S, Deffieux D, Douat-Casassus C, Pouysegu L (2011) Plant polyphenols: chemical properties, biological activities, and synthesis. Review, natural products. *Angew Chem Int Edit* 50:586–621
- Shin SM, Yang JH, Ki SH (2013) Role of the Nrf2-ARE pathway in liver diseases. *Oxid Med Cell Longev* 1–9
- Su XY, Wang ZY, Liu W (2009) *In vitro* and *in vivo* antioxidant activity of *Pinus Koraiensis* seed extract containing phenolic compounds. *Food Chem* 117:681–686
- Subramaniam SR, Ellis EM (2011) Esculetin-induced protection of human hepatoma HepG2 cells against hydrogen peroxide is associated with the Nrf2-dependent induction of the NAD(P)H: quinone oxidoreductase 1 gene. *Toxicol Appl Pharm* 250:130–136
- Tang W, Jiang YF, Ponnusamy M, Diallo M (2014) Role of Nrf2 in chronic liver disease. *World J Gastroenterol* 20(36):13079–13087. doi:10.3748/wjg.v20.i36.13079
- Tietcheu BRG, Sass G, Njaye NF, Mkounga P, Tiegs G, Moundipa PF (2014) Anti-hepatitis C virus activity of crude extract and fractions of *Entada africana* in genotype 1b replicon systems. *T Am J Chin Med* 42(4):853–868
- Tosi MER, Bocanegra V, Manucha W, Lorenzo AG, Vallés PG (2011) The Nrf2–Keap1 cellular defense pathway and heat shock protein 70 (Hsp70) response. Role in protection against oxidative stress in early neonatal unilateral ureteral obstruction (UUO). *Cell Stress Chaperones* 16:57–68. doi:10.1007/s12192-010-0221-y
- Varshneya C, Kant V, Mehta D (2012) Total phenolic contents and free radical scavenging activities of different extracts of seabuckthorn (*Hippophae rhamnoides*) pomace without seeds. *Int J Food Sci Nutr* 63(2):153–9
- Wong SP, Leong LP, Koh JHW (2006) Antioxidant activities of aqueous extracts of selected plants. *Food Chem* 99:775–783
- Xu D, Zhou C (2013) Antioxidative effects of berberine pre-treatment on hydrogen peroxide-induced PC12 cell toxicity. *Neural Regen Res* 5(18):1391–1395
- Zhao CR, Gao ZH, Qu XJ (2010) Nrf2–ARE signaling pathway and natural products for cancer chemoprevention. *Cancer Epidemiol* 34:523–533
- Zhu R, Wang Y, Zhang L, Guo Q (2012) Oxidative stress and liver disease. *Hepatol Res* 42(8):741–749