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Content of Polyphenolic Compounds in the Nigerian Stimulants *Cola nitida* ssp. *alba*, *Cola nitida* ssp. *rubra* A. Chev, and *Cola acuminata* Schott & Endl and Their Antioxidant Capacity

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Varieties of kola nuts (*Cola nitida alba*, *Cola nitida rubra* A. Chev, and *Cola acuminata* Schott & Endl), a group of popular Nigerian and West African stimulants, were analyzed for their content of secondary plant metabolites. The three varieties of the kola nuts contained appreciable levels of (+)-catechin (27–37 g/kg), caffeine (18–24 g/kg), (−)-epicatechin (20–21 g/kg), procyanidin B_1 [epicatechin-(4β→8)-catechin] (15–19 g/kg), and procyanidin B_2 [epicatechin-(4β→8)-epicatechin] (7–10 g/kg). Antioxidant capacity of the extracts and purified metabolites was assessed by two HPLC-based and two colorimetric in vitro assays. Extracts of all varieties exhibited antioxidant capacity with IC_{50} values in the range 1.70–2.83 and 2.74–4.08 mg/mL in the hypoxanthine/xanthine oxidase and 2-deoxyguanosine HPLC-based assays, respectively. Utilization of HPLC-based assays designed to reflect in situ generation of free radicals (e.g., $HO^{\bullet}$), as opposed to general assays (DPPH, FRAP) in common use which do not, indicate that, of the major secondary plant metabolites present in kola nut extracts, caffeine is potentially the more effective cancer chemopreventive metabolite in terms of its antioxidant capacity.

KEYWORDS: Caffeine; catechins; HPLC; kola nut; HPLC-ESI-MS; procyanidins; reactive oxygen species

INTRODUCTION

The stimulants *Cola nitida* A. Chev and *Cola acuminata* Schott & Endl (family: *Sterculiaceae*), commonly referred to as “kola nut” or “gworo” in Nigeria, are native to West Africa. *C. acuminata* is multicotyledonous while *C. nitida* is dicotyledonous. *C. nitida* has two subspecies, *alba* and *rubra*, which are white and reddish, respectively (*1*). In addition to their stimulant properties, kola nuts have become essential to the cultural and social well-being of the Nigerian people; no social or traditional ceremony is considered complete without the breaking of the kola nut (*2*). For thousands of years, West Africans have traditionally chewed kola nuts for their stimulant effects. Kola nuts are also gaining popularity in the Arabic world, where they were first introduced during the trans-Saharan trade. In Nigeria, *C. acuminata* consumption is more popular in the southern and middle belt of the country, while *C. nitida* is more popular in the north.

Similar to the use of Echinacea root among people of the first world, kola nuts are chewed on long distance journeys by travelers, truck drivers, and soldiers as appetite and thirst suppressants and in relieving physical fatigue and melancholy. In Nigeria, kola nut chewing is very popular among students and sedentary office workers to remain active, and extracts are currently used in many parts of the world as a component of carbonated drinks. Kola nuts are believed to possess aphrodisiac and medicinal properties (*3*). Experimental evidence suggests that kola nut extracts induce biphasic changes in the locomotor activity of mice depending on the dose and the treatment duration (*4*) and possess significant ability to induce gastric acid secretion (*1*).

Considering the widespread consumption of kola nuts in Nigeria and elsewhere (*2–4*), we investigated the profile of secondary plant metabolites in *C. nitida* and *C. acuminata* and assessed the capacity of methanolic extracts and purified metabolites to elicit antioxidant effects, which are now widely accepted as a potential protective mechanism in the chemoprevention of cardiovascular disease (*5*), diabetes (*6*), and cancer (*7, 8*).

MATERIALS AND METHODS

Reagents. Acetic acid, 2-deoxyguanosine, ethylenediaminetetraacetic acid, hypoxanthine, methanol, uric acid, xanthine, and xanthine oxidase were obtained from Merck (Darmstadt, Germany); Sephadex LH-20

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was from GE Healthcare Biosciences AB (Uppsala, Sweden); K_2HPO_4 and KH_2PO_4 were from Serva (Heidelberg, Germany); ascorbic acid, caffeine, DPPH (1,2-diphenyl-2-picrylhydrazyl), ethanol, $FeCl_3 \cdot 6H_2O$, formic acid, hexane, $FeSO_4 \cdot 7H_2O$, salicylic acid, 2,4,6-tripyridyl-*s*-triazine complex (TPTZ), tetrabutylammonium hydroxide, and Trolox were from Sigma-Aldrich Chemie (Seelze, Germany). Standard phenolic compounds were obtained from Extrasynthese (Lyon Nord, Genay, France). Doubly distilled water was used for all aqueous solutions.

Samples. Different kola nut varieties [*C. nitida alba* (HK-W:-Hausa kola, White), *C. nitida rubra* (HK-R:-Hausa kola, Red), and *C. acuminata* (IgK:-Igala kola)] were acquired from a retail outlet in the northern Nigerian city of Samaru-Zaria, the home base of one of the authors (S.E.A.). They were thoroughly washed with three portions of doubly distilled water, sliced into small pieces with a stainless steel knife, and freeze-dried, following which the samples were pulverized in a kitchen-type blender to a fine homogeneous powder.

Extraction. Freeze-dried samples (5 g) were defatted with hexane (100 mL) through a Soxhlet extraction for 3 h. The defatted material was dried under a stream of nitrogen, and the phenolic components were further extracted through a 3 h reflux with methanol (100 mL × 3). The methanol extracts were evaporated to constant weight *in vacuo* at 35 °C, and the residue was dissolved in 10 mL of methanol. The three extracts were pooled with rinsing, methanol was evaporated *in vacuo*, and the residue was dissolved in 10 mL of methanol (*9*). This solution was used with or without dilution for further analyses.

Column Chromatography (CC) on Sephadex LH-20. The dried residues, after suspension in absolute ethanol, were immobilized on Sephadex LH-20 and added to a glass column (38 × 4.5 cm) filled with Sephadex LH-20 in ethanol to a level ca. 5 cm from the top. The immobilized extracts were added to the free volume at the head of the column. After bedding down of the gel material, fractionation was conducted by successive applications of methanol (0%, 1%, 5%, 10%, 20%, 30%, 50%, and 100%) in ethanol (250 mL). Fractions (250 mL) were collected, and the solvent was removed by rotary evaporation *in vacuo* at 40 °C. Dried fractions were suspended in methanol (10 mL) and diluted, when necessary, prior to analytical HPLC. Individual phenolic compounds in relevant fractions were purified by semipreparative HPLC for structure elucidation by spectroscopic analyses.

Analytical High-Performance Liquid Chromatography (HPLC). Analytical HPLC was conducted on a Hewlett-Packard (HP) 1090 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) fitted with a 25 cm × 4 mm i.d., 5 μm C18 reversed-phase column (Latek, Eppelheim, Germany). Sample extracts of kola nuts were dissolved in methanol (5.0 mL) and, when necessary, further diluted prior to injection (20 μL) into the HPLC. The mobile phase consisted of 2% acetic acid in doubly distilled water (solvent A) and methanol (solvent B) with the following gradient: 95% A for 2 min; to 75% A in 8 min; to 60% A in 10 min; to 50% A in 10 min; and 0% A until completion of the run (*9*). The flow rate of the mobile phase was maintained at 1 mL/min, and phenolic compounds in the eluates were detected at 250, 278, and 340 nm with a HP 1040 M diode-array UV detector. The amount of phenolic compounds in the extracts was estimated (*9, 10*) from standard curves (0–4 mM) of authentic standards. Instrument control and data handling were by means of a HP Chemstation operating in the Microsoft Windows software environment.

High-Performance Liquid Chromatography–Electrospray Ionization–Mass Spectrometry (HPLC-ESI-MS). Reversed-phase HPLC-ESI-MS was conducted on an Agilent (Agilent Technologies, Waldbronn, Germany) 1100 HPLC coupled to an Agilent MSD (HP1101). Chromatographic separation was conducted utilizing the same column, mobile phase, and conditions as described for analytical HPLC. The analyses were conducted in both the negative and positive ion modes under the following conditions: drying gas (nitrogen) flow rate = 10 L/min; nebulizer pressure = 30 psi; drying gas temperature = 350 °C; capillary voltage, negative ion mode = 2500 V and positive ion mode = 1500 V; fragmenter voltage = 100 V; mass range = 50–3000 Da.

Semipreparative HPLC and Fraction Collection. Semipreparative HPLC was conducted on an Agilent 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) fitted with a 25 cm × 10 mm i.d., reversed-phase C18 column (Latek, Eppelheim, Germany) similar to that used for analytical HPLC (*11*). Acetonitrile was used instead of methanol as the mobile phase with a flow rate of 3 mL/min. Peaks eluting from the column were collected on an Agilent HP 220 microplate sampler. Each purified fraction was pooled, and solvent was removed by lyophilization.

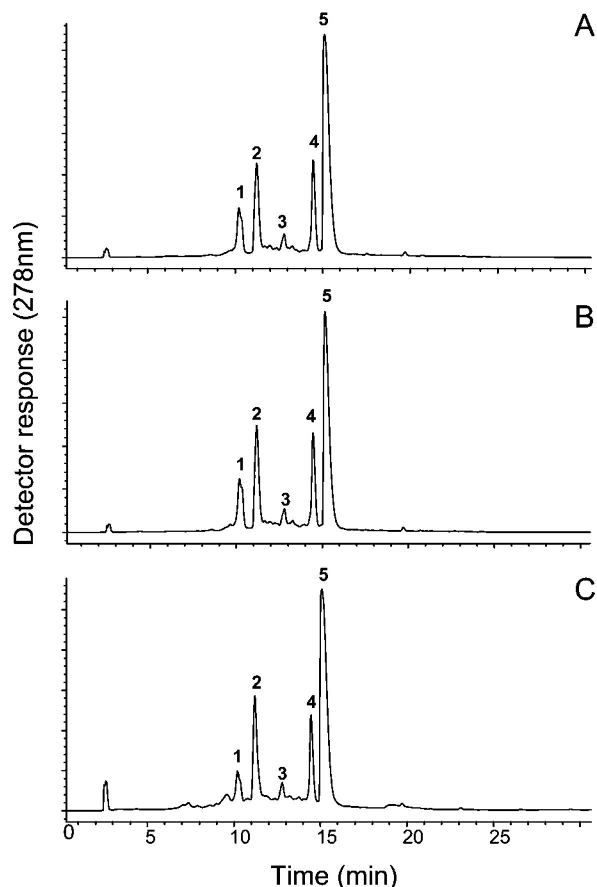

Figure 1. Analytical HPLC chromatograms of methanolic extracts of *C. nitida* spp. *alba* (**A**), *C. nitida* spp. *rubra* (**B**), and *C. acuminata* (**C**) at 278 nm. The peaks are as follows: procyanidin B_1 (1); (+)-catechin (2); procyanidin B_2 (3); (−)-epicatechin (4); caffeine (5).

Nanoelectrospray Ionization Mass Spectrometry (ESI-MS). Samples were dissolved in methanol, and ESI mass spectra were recorded on a MAT TSQ 7000 triple-quadrupole mass spectrometer (Finnigan, San Jose, CA) equipped with a nanoelectrospray source (EMBL, Heidelberg, Germany) using both the positive and negative ion modes. Argon was used as the collision gas at a nominal pressure of 2.5 mTorr. Samples were sprayed from gold-plated glass capillaries in-house prepared with a type 87-B microcapillary puller (Sutter Instruments, Novato, CA). The applied voltage was 400–700 V with a scan range of 20–2600 Da.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^1H and ^{13}C NMR spectra for all compounds (10–20 mg) were recorded in CD_3OD (99.8% D) on a Bruker AC-250 (at 5.87 T) spectrometer (Bruker Analytik, Rheinstetten, Germany) at a ^1H frequency of 250.133 MHz and a ^{13}C frequency of 62.89 MHz.

Hypoxanthine/Xanthine Oxidase Assay. To assess the total antioxidant potential due partly to the scavenging of reactive oxygen species and the inhibition of the enzyme xanthine oxidase, the hypoxanthine/xanthine oxidase assay system was utilized. In this assay, the extent of dihydroxyphenol (2,5-dihydroxybenzoic acid and 2,3-dihydroxybenzoic acid) produced by hydroxyl radical ($HO^\bullet$) attack on salicylic acid (*12*) is evaluated.

The assay involved the suspension (after drying under nitrogen) of different volumes of extract residues (0–500 μL) and purified compounds (0–4 mM) prepared in duplicate in 1.0 mL of phosphate assay buffer (pH 6.6). After addition of 5 μL of xanthine oxidase (20

Table 1. Analytical Data for Secondary Plant Metabolites Isolated from Kola Nuts

	secondary plant substance	mol formula	exact mass (M)	RT[a] (min)	HPLC-ESI-MS $[M-H]^-$	nano-ESI-MS[b] $[M-H]^-$	nano-ESI-MS[b] $[M+Na]^+$
I	procyanidin B_1	$C_{30}H_{26}O_{12}$	578.142	10.25	577.1[c]	576.9	601.2
II	(+)-catechin	$C_{15}H_{14}O_6$	290.079	11.23	289.1[c]	288.9	313.1
III	procyanidin B_2	$C_{30}H_{26}O_{12}$	578.142	13.28	577.1[c]	576.9	601.2
IV	(−)-epicatechin	$C_{15}H_{14}O_6$	290.079	14.46	289.1[c]	289.9	313.1
V	caffeine	$C_8H_{10}N_4O_2$	194.080	15.15	195.2[d]	192.9	217.2

[a] RT = retention time. [b] Negative and positive ion nano-ESI-MS after purification by semipreparative HPLC. [c] Online HPLC-ESI-MS with negative ion detection. [d] Online HPLC-ESI-MS with positive ion detection.

milliunits/1.09 mL), the tubes were incubated at 37 °C for 3 h, following which the reaction was terminated by addition of 5 μL of concentrated HCl. Where necessary, the reaction mixture was centrifuged at 10000 rpm for 5 min on a Fico Biofuge (Kendro Laboratory Products, Osterode, Germany), and 20 μL of the mixture was analyzed by analytical HPLC using the mobile phase and gradient conditions mentioned earlier. The hydroxylation of hypoxanthine and salicylic acid was monitored at 278 and 325 nm, respectively. The end products of the enzyme or free radical reactions were quantitated against standard curves measured at the same wavelength.

2-Deoxyguanosine Assay. To evaluate free radical scavenging capacity only of the extracts, the 2-deoxyguanosine assay model was adopted (*13*). The buffer system was similar to that of the hypoxanthine/xanthine oxidase system, except that salicylic acid was replaced with 2-deoxyguanosine (2 mM) and hypoxanthine was omitted. The generation of reactive oxygen species (ROS) was initiated by addition of ascorbic acid (500 μM). Residues (0–500 μL) and purified compounds (0–4 mM) prepared in duplicate were suspended (after drying under nitrogen) in assay buffer (1.0 mL) and incubated at 37 °C for 24 h. The assay of 8-oxo-2-deoxyguanosine resulting from ROS attack on 2-deoxyguanosine was analyzed using an isocratic system consisting of 5% methanol and 95% aqueous buffer (5 mM tetrabutylammonium hydroxide, adjusted to pH 4.3 with 6% formic acid). The UV detector was set at 293 nm.

DPPH Assay. The free radical scavenging capacity of the purified compounds was also determined using the DPPH$^\bullet$ discoloration method (*14*). The pure compounds were diluted in methanol, giving a range of 10–1000 μM. The dilutions (20 μL) were placed in a 96-well plate in duplicate. The reaction was initiated by addition of 180 μL of DPPH solution (20 μg/mL in methanol). The absorbance was read at 515 nm over 45 min on an universal microplate reader (Bio-Tek Instruments, Winooski, VT) compared to duplicate controls containing methanol (20 μL) only. The concentration of the DPPH$^\bullet$ radical was calculated against a standard curve (1–100 μg/mL) measured simultaneously.

FRAP Assay. The dilution of the different metabolites was identical to that described for the DPPH assay. Ten microliters of substance was incubated with 30 μL of water and 300 μL of FRAP reagent, consisting of 25 mL of acetate buffer (300 mM sodium acetate buffer, pH = 3.6), 2.5 mL of TPTZ (10 mM TPTZ in 40 mM HCl), and 2.5 mL of $FeCl_3$ solution (20 mM $FeCl_3 \cdot 6H_2O$ in water) at 37 °C. All reagents were freshly prepared and warmed to 37 °C before measurement. A calibration curve of ferrous sulfate (0.01–10 mM) was used, and the results are expressed in millimolar Fe^{2+} equivalents.

The reaction was measured every minute for 10 min at 593 nm. The reaction of all isolated secondary plant substances reached a steady-state level after 5 min. A linear regression curve was generated at the 5 min reaction time point for different concentrations of the isolated substances with the Microcal Origin 5.0 program. The EC_1 values were calculated from these regression curves, as the concentration of reducing agent (μM) giving an absorbance increase equivalent to 1.0 mM Fe^{2+}solution according to Pulido et al. (*15*).

Determination of IC_{50}. The amount of extracts and pure metabolites producing 50% inhibition of oxidation (IC_{50}) using the various *in vitro* assay systems were determined using the Table curve program (Jandel Scientific, Chicago, IL).

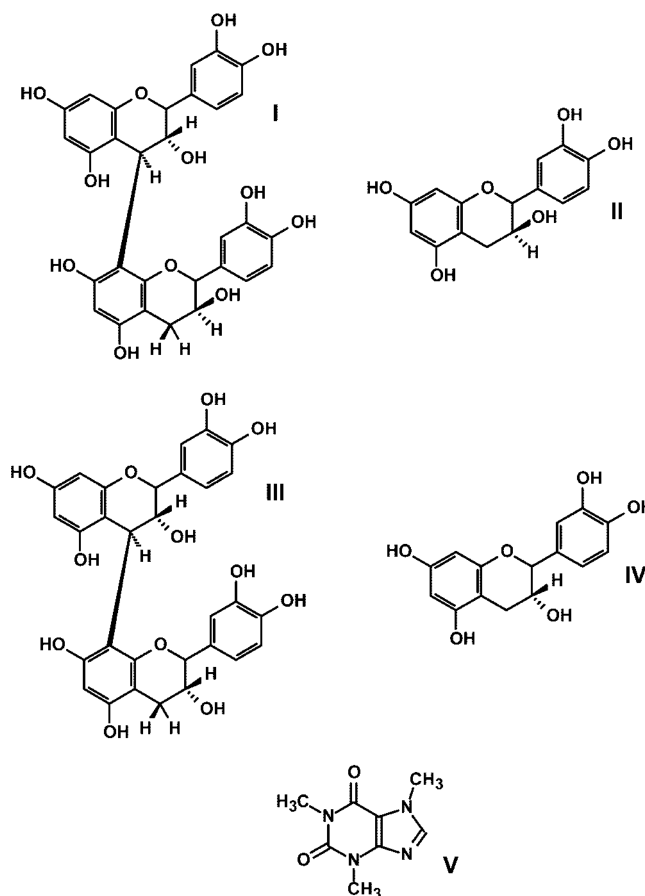

Figure 2. Structures of the various secondary plant metabolites detected in different varieties of kola nuts.

Table 2. Abundance of Secondary Plant Metabolites in *C. nitida* and *C. acuminata* As Determined by Analytical HPLC

no.	substance	RT (min)	*C. nitida alba* (g/kg)	*C. nitida rubra* (g/kg)	*C. acuminata* (g/kg)
I	procyanidin B_1	10.25	17.59	18.91	15.10
II	(+)-catechin	11.23	27.26	29.07	37.15
III	procyanidin B_2	13.28	7.63	6.80	9.61
IV	(−)-epicatechin	14.46	20.68	20.11	21.27
V	caffeine	15.15	21.26	17.85	23.65
total			94.42	92.74	106.78

RESULTS AND DISCUSSION

Determination of Structures. Preliminary structure determination of the major secondary plant substances in the extracts of kola nuts was achieved through comparison with authentic standards of their retention times by reversed-phase analytical HPLC (**Figure 1**) and ionization patterns by HPLC-ESI-MS (**Table 1**). The following major compounds were identified,

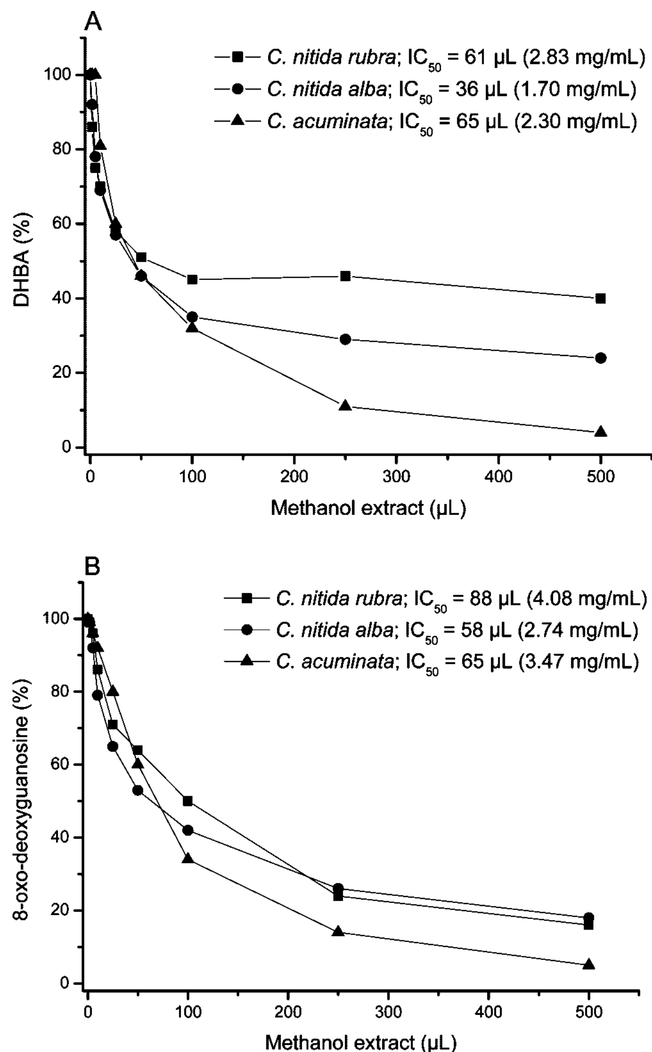

Figure 3. Antioxidant capacity of methanolic extracts from different varieties of kola nuts using (**A**) the HPLC-based hypoxanthine/xanthine oxidase assay system and (**B**) the 2-deoxyguanosine HPLC-based assay system.

namely, procyanidin B_1 (**I**), (+)-catechin (**II**), procyanidin B_2 (**III**), (−)-epicatechin (**IV**), and caffeine (**V**), and their structures (**Figure 2**) were confirmed after purification by column chromatography and semipreparative HPLC by nano-ESI-MS-MS (**Table 1**) and ^{1}H NMR (data not shown).

Content of Phenolic Antioxidants. The total amount (range, 93–107 g/kg) and proportions of phenolic compounds in methanolic extracts of the three species of kola nuts were very similar (**Table 2**). On average, the major and minor components were (+)-catechin (32%) and procyanidin B_2 (8%), respectively.

Antioxidant Capacity of Extracts. Extracts of kola nut varieties displayed discernible antioxidant capacity (**Figure 3**) in both of the HPLC-based model systems. *C. nitida alba* exhibited the stronger antioxidant capacity with IC_{50} values of 1.70 and 2.74 mg/mL in the hypoxanthine/xanthine oxidase and the 2-deoxyguanosine model systems, respectively.

Antioxidant Capacity of Purified Compounds in the HPLC-Based Antioxidant Assays. Of the individual compounds purified by semipreparative HPLC, the higher antioxidant capacity (**Table 3**) was affected by caffeine in both the hypoxanthine/xanthine oxidase (IC_{50} = 0.43 mg/mL, 2.22 mM) and 2-deoxyguanosine (IC_{50} = 0.84 mg/ mL, 4.33 mM) assays. Caffeine was more effective than Trolox in the hypoxanthine/xanthine oxidase assay, but the reverse was true in the 2-deoxyguanosine assay. The reason is that, in the 2-deoxyguanosine assay, ascorbic acid regenerates Trolox from the Trolox radical after donation of a proton to hydroxyl radicals. On a molar basis the catechin isomers (+)-catechin and (−)-epicatechin were approximately two to three times less effective in scavenging the hydroxyl radical in the hypoxanthine/xanthine oxidase assay than caffeine and were virtually ineffective in the 2-deoxyguanosine assay. Of concern is that the purified procyanidin B dimers, while having no effect in the 2-deoxyguanosine assay, behaved as prooxidants in the hypoxanthine/xanthine oxidase assay.

Antioxidant Capacity of Purified Compounds in the Colorimetric Antioxidant Assays. The antioxidant capacities of the individual secondary metabolites (**Table 3**) in the colorimetric antioxidant assays were very different from the HPLC-based assays. In these assays, the catechins and procyanidins behaved as potent antioxidants, generally comparable to ellagic acid but more effective than ascorbic acid and Trolox. However, caffeine displayed virtually no antioxidant capacity in these systems, which apart from the control substances ellagic acid and uric acid was the most potent in the HPLC-based assays. The HPLC-based and colorimetric assays are ostensibly quite distinct from one another, in that the former are designed to quench $HO^•$ radicals that are generated *in vivo*, while the latter are related to sequestration of ROS ($DPPH^•$) and reduction capacity (FRAP) *in vitro*. Given that the HPLC-based assays

Table 3. Antioxidant Capacity of Individual Secondary Plant Metabolites Isolated from Kola Nuts in Comparison to Control Substances[a]

	IC_{50}				
secondary plant substance	scavenging of ROS in HX/XO assay, µg/mL (mM)	inhibition of xanthine oxidase in HX/XO assay, µg/mL (mM)	inhibition of 8-oxo-dG formation in 2-doG assay, µg/mL (mM)	DPPH assay, µg/mL (µM)	FRAP assay (EC_1), µg/mL (µM)
procyanidin B_1 (epicatechin/catechin)[b]	prooxidant	none	>2000	6.14 (10.62)	4.66 (8.06)
(+)-catechin	1010 (3.48)	none	>2000	2.19 (7.55)	15.14 (52.21)
procyanidin B_2 (epicatechin/epicatechin)[c]	prooxidant	none	>2000	4.05 (7.01)	5.58 (9.65)
(−)-epicatechin	1260 (4.34)	none	>2000	2.15 (7.41)	12.85 (44.31)
caffeine	430 (2.22)	none	840 (4.33)	>1000 (5.0 mM)	>1000 (5.0 mM)
ellagic acid (control)	3 (0.01)	2 (0.01)	>2000	3.96 (13.11)	4.1 (13.58)
Trolox (control)	574 (2.30)	none	230 (0.92)	16.49 (65.96)	15.5 (62.0)
ascorbic acid (control)[d]	prooxidant	none	prooxidant[e]	8.93 (50.74)	14.4 (81.82)
uric acid (control)	361 (2.15)	none	310 (1.85)	1.91 (11.38)	1.83 (11.15)

[a] Mean of two experiments. [b] At 1 mM (578 µg/mL) increase in DHBA = 46%. [c] At 1 mM (578 µg/mL) increase in DHBA = 57%. [d] At 500 µM (88 µg/mL) increase in DHBA = 239%. [e] At 500 µM (88 µg/mL) gives 120 µM 8-oxo-dG (ascorbic acid used here as a generator of ROS).

better reflect the *in vivo* situation, estimation of efficacy based on bioavailability in humans was assessed using the data generated in the hypoxanthine/xanthine oxidase assay. Reported maximal values for plasma (–)-epicatechin are 6.0 μM (*16*) while 29 μM is required to scavenge 1 μM $HO^{\bullet}$ radical. However, for caffeine with reported maximal plasma values of 50 μM (*17*) only 15 μM is required to scavenge 1 μM $HO^{\bullet}$ radical. The data for caffeine compare favorably with those for uric acid, with reported maximal plasma values of 450 μM (*18*). Similar to caffeine, 14 μM uric acid is required to scavenge 1 μM $HO^{\bullet}$ radical. Because uric acid is the most ubiquitous and potent antioxidant substance in human biofluids, the data generated in this study indicate that caffeine, due to its similar properties, may also have an important role to play in cancer chemoprevention because it is more bioavailable and efficacious than polyphenolic compounds.

The procyanidins, also detected in these varieties of kola nuts, are polymeric flavonols synthesized as one of several branch products of the flavonoid pathway. They are present in plants as complex mixtures of polymers with an average degree of polymerization between 2 and 11 (*19*). Our data, however, showing the prooxidant effect of the procyanidin B dimers in the hypoxanthine/xanthine oxidase HPLC-based assay indicate that caution is required here in the recommendation of these substances as potential anticancer-mediating agents.

Finally, this study, which compared various *in vitro* assays for the evaluation of the antioxidant capacities of individual secondary plant metabolites isolated from kola nuts, has raised some interesting questions. All isolated metabolites gave favorable results compared to reference compounds when tested in systems using free radicals ($DPPH^{\bullet}$), which are not generated *in situ*, and reducing power (FRAP), but in the HPLC-based assays which evaluate the effects of quenching the hydroxyl radical (generated in abundance *in vivo*) only, caffeine, of the secondary plant metabolites isolated from kola nut extracts, had significant effects. These data lend support to the recent concept (*20*) that among secondary plant metabolites of plants, it is possible that the beneficial chemopreventive effects of high plant intake may also be due to compounds which are essentially nonphenolic in nature (e.g., alkaloids in tea and coffee) but are highly bioavailable, which is not always the case in terms of some polyphenol classes (e.g., flavonoids). These findings may have far-reaching implications in cancer chemoprevention in countries such as Nigeria, where kola nuts are widely consumed, in that the potential modulatory effects elicited by these particular secondary plant metabolites, especially caffeine, may be similar to that of green tea consumption in Asia.

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