Antioxidant and anti-inflammatory properties of *Ilex guayusa* tea preparations: a comparison to *Camellia sinensis* teas

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Abstract

Ilex guayusa tea preparations are now commercially available as Runa tea. Little is known regarding the antioxidant and anti-inflammatory bioactivities of this tea. The *I. guayusa* teas had a total polyphenolic content between 54.39 and 67.23 mg GAE/g dry mass and peroxyl radical scavenging capacities between 1773.41 and 2019 µmol TE/g dry mass, nearly half of that for the *Camellia sinensis* teas. The *I. guayusa* teas afforded 60-80% protection from oxidative stress in the Caco-2 cellular antioxidant assay, comparable to the *C. sinensis* teas. The anti-inflammatory activity in lipopolysaccharide-stimulated RAW 264.7 cells of *I. guayusa* teas was similarly comparable to the *C. sinensis* teas with nitric oxide production reduced by 10-30%. Major compounds identified by mass spectrometry were the phenolic mono- and dicaffeoylquinic acid derivatives. *I. guayusa* teas are a good source of dietary phenolic compounds with cellular antioxidant and anti-inflammatory properties.

Keywords: *Ilex guayusa,* Runa tea, antioxidant, anti-inflammatory, caffeoylquinic acid

1. Introduction

The habit of consuming tea is an ancient one, and archaeological discoveries suggest that it may be traced back as far as 500 000 years ago.[1] Black and green teas are beverages made from *Camellia sinensis* leaves manufactured by two different processes and are the most widely consumed beverages in the world after water. Different herbal teas are

consumed in different geographic locations and with varying degrees of popularity. Black tea makes up 76 – 78% of global consumption and is consumed in Europe, North America and North Africa, except Morocco. Green tea makes up 20-22% of global consumption and is popular in China, Japan, Korea and Morocco. Oolong tea only constitutes 2% of tea consumption and it is mainly consumed in China and Taiwan.[2]

Various health benefits have been found to be associated with the consumption of *C. sinensis* black and green teas and these have been reviewed by Dufresne and Farnworth[3] and McKay and Blumberg[4]. The health benefits of *C. sinensis* teas include antioxidant effects, anticancer, anti-diabetic and anti-inflammatory activities as well as cardiovascular protective effects. The consumption of *C. sinensis* tea has also been shown to have an impact on bone density, cognitive function and kidney stones. The bioactive compounds associated with the beneficial effects include the monomeric catechins found in green tea preparations and their dimers, called theaflavins, found in black tea preparations.[5,6]

Besides *C. sinensis* tea preparations, consumption of herbal teas prepared from the holly *Ilex* species is also popular, particularly in China and the Americas. The traditional uses, medicinal properties and chemical composition of *Ilex* species have been reviewed by Hao *et al*.[7] and Yi *et al*.[8] In China, large-leaved Kudingcha tea can be prepared from the leaves of *I. latifolia and I. kudingcha* to treat various ailments such as high fever, diarrhoea, swelling and pain.[7,8] In the Americas, herbal tea preparations from the leaves of caffeinated holly species such as *I. paraguariensis* (yerba mate), *I. vomitoria* (yaupon tea), *I. glabra* (Appalachian tea) and *I. guayusa* (Runa tea) are consumed for their stimulating effects.[8] Studies have been conducted on the health benefit properties of *I. paraguariensis* and *I. vomitoria* and include anti-inflammatory, antioxidant and anticancer activities.[9-12] Bioactive compounds identified in these two *Ilex* species include caffeoylquinic acids like chlorogenic acid as well as flavonols like quercetin and kaempferol and their glycosides.[9,11] Less is known regarding the bioactive compounds and health benefit properties of *I. guayusa.*

Tea preparations of *I. guayusa* are ritualistically consumed by Amazonian families as a stimulant to energize the body and mind due to its high caffeine content.[13] Teas prepared from the leaves of *I. guayusa* are now commercially available trading as Runa tea and is sold as tea bags or iced tea formulations. Studies have shown that *I. guayusa* tea reduces streptozocin-induced diabetes in mice, and some antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* has also been found.[14,15] Kapp Jr *et al.*[16] evaluated the toxicity of *I. guayusa* with a series of *in vitro* and *in vivo* tests and found no significant adverse effects related to the use of *I. guayusa*. Little information, however, can be found regarding the compounds found in Runa tea as well as its other potential health

benefit properties. To date, only studies by Kapp Jr *et al.*[16] and García-Ruiz *et al.*[17] have explored the composition and antioxidant activity of *I. guayusa* teas. This study aimed to build on this scientific knowledge of *I. guayusa* teas by further exploring the antioxidant and anti-inflammatory bioactivities of the teas under normal tea brewing conditions and comparing these bioactivities to that of the more popular and better characterized *C. sinensis* teas. *C. sinensis* teas have been shown to exert antioxidant protective, and anti-inflammatory effects in cellular, animal and clinical studies[18-22] and thus would serve as appropriate controls in assessing the bioactivity of *I. guayusa* teas.

2. Materials and methods

2.1. Tea samples and reagents

Runa tea samples prepared from the leaves of *I. guayusa* were obtained from the Runa tea factory in Ecuador and were fermented (IGB) and unfermented (IGG) teas. Lipton's black (LBT) and green (LGT) were purchased from supermarkets in New York, USA. Three different batches, each batch with a different lot number, were purchased, to achieve a variety of samples. All purchases were made during 2013-2014. All other reagents for antioxidant and anti-inflammatory studies were purchased from Sigma-Aldrich Company, Atlasville, South Africa.

2.2 Cell cultures

The human colon cancer cells Caco-2 were obtained from the American Type Culture Collection, Manassas, USA. The adult murine macrophage RAW 264.7 cell line was obtained from the European Collection of Authenticated Cell Cultures. All cell lines were maintained in Eagles Minimum Essential Medium supplemented with 10% fetal bovine serum and Penicillin/Streptomycin/Fungizone formulation (Highveld Biological Company, Johannesburg, South Africa) and maintained at 37°C, 5% CO_2.

2.3. Methods

2.3.1 LC-MS/MS evaluation of unfermented and fermented I. guayusa teas

Analysis of the unfermented and fermented *I. guayusa* tea samples was performed on a Waters Ultra performance liquid chromatography (UPLC) Acquity system fitted with a Waters Acquity photo diode array (PDA) detector and coupled to a Waters Synapt G2 mass spectrometer (Milford, Massachusetts, USA). The extract was analyzed with a Waters Acquity UPLC ethylene bridged hybrid (BEH) C18 column, 2.1 x 100 mm with 1.7 µm particle size coupled with a Waters Acquity UPLC BEH C18 pre-column, 2.1 x 5 mm with 1.7

µm particle size supplied by Microsep (Pty) Ltd, Johannesburg, South Africa. The column temperature was set at 40°C, and the solvents used were HPLC grade water with 2% acetic acid (solvent A) and HPLC grade acetonitrile (solvent B). Four µL of the extract was injected, and the flow rate was set at 0.350 mL/min. The chromatographic conditions were as follows: 0% to 19% solvent B over 24 minutes, 19% to 80% solvent B over 3 minutes, 80% to 2% solvent B over 0.1 minutes, 0% solvent B was maintained for 3 minutes followed by column re-equilibration. The mass spectrometer was operated in the both negative and positive ionization mode, and mass data were acquired from 50 to 1500 atomic mass units (amu). Conditions were as follows: capillary voltage 2.5 kV, sampling cone voltage 15 V, desolvation temperature 275°C, desolvation gas flow 650 L/hr and cone gas flow of 50 L/hr. A low energy function with trap collision energy of 4 V and a high energy function with trap collision energy that ramped from 15 to 60 V were used to acquire the mass data. Data were processed with Waters MassLynx Version 4.1 software.

2.3.2. Moisture content

To determine the dry mass of the tea material crucibles were dried overnight in a drying oven at 103°C and then cooled in a desiccator and weighed to determine the dry mass of the crucible. From each sample 2 g of ground leaves were placed in the dried crucibles. The tea and crucibles were then placed overnight in an oven at 103°C for drying. The next morning the crucibles containing the tea were weighed, and dry mass of the crucible and the dried tea was calculated using the following equation:

$$\textbf{Dry mass (DM)} = \frac{\textbf{(Dry mass of crucible + tea) – (Dry crucible mass) x 100}}{\textbf{Wet mass of tea (2g)}}$$

All data generated in this study is expressed as units per g (DM) tea.

2.3.3. Sample extraction

Water extractions were prepared as this is the conventional way in which tea is consumed. Water was added to flasks and allowed to reach 90°C, in a water bath, before each tea bag (LBT and LGT) or equivalent masses of IGD and IGG were in separate flasks. The final concentration of all tea leaves was 1 g/100 mL H_2O and a final 1% tea solution was prepared. A sample of tea was then removed at 2, 10, 30 and 60 minutes time intervals to evaluate the effect of brewing time on the antioxidant and anti-inflammatory activity.

2.3.4. Folin-Ciocalteu method

The antioxidant reducing activity was determined with the Folin-Ciocalteu (F-C) method in a microplate format as described by Serem and Bester.[23] To a 96-well plate, 10 µL of a 10% (v/v) tea solution of each sample was added to each well. This was followed by 50 µL of a 1:15 F-C reagent solution and 50 µL of a 7.5% (w/v) Na_2CO_3 solution. The samples were mixed and the absorption determined at 630 nm using a FLUOstar OPTIMA spectrophotometer (BMG labtechnologies, Offenburg, Germany). A 1 mg/mL gallic acid solution was diluted to 0 - 0.4 mg/mL to prepare a standard curve and calculate antioxidant reducing activity, expressed as mg gallic acid equivalents (GAE)/g DM tea.

2.3.5. ORAC assay

Procedures used were based on a modified method by Ou *et al.*[24] 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was used as a peroxyl radical generator and Trolox as standard and fluorescein as a fluorescent probe. A 190 µM fluorescein stock solution was made (3.2 mg fluorescein disodium in 50 mL phosphate buffered saline (PBS) which was then diluted four times with PBS. This was diluted to a final 133 nM concentration (140 µl dilute fluorescein in 5 ml PBS and 45 ml dH_20) to prepare a fluorescein working solution. A final experimental solution was then prepared by rapidly mixing 16 mL fluorescein working solution and 4 mL of 0.1 mM AAPH solution to achieve a final experimental solution. A concentration series of 0 – 0.8 mM was made from 1 mM Trolox. A 1% (v/v) tea extract in water, or 10 µL Trolox standard, was added to each well of a Greiner Bio-one 96-well opaque plate. A 200 µL volume of experimental solution was added, and samples were mixed well. The final assay contained 0.04 mM Trolox or 0.05% tea, 101.33 nM fluorescein disodium and 0.02 M AAPH in 0.137 M PBS. The microplate was placed into the plate reader and incubated at 37^0C. Fluorescence was measured at five minute intervals for four hours using a FLUOstar OPTIMA spectrophotometer (BMG Labtechnologies, Offenburg, Germany). The assay protocol was as follows: 0.0s measurement start time, 10 flashes per cycle, 300 seconds cycle time, 485 nm for the excitation filter and 520 nm for the emission filter. The final sample ORAC values were calculated with a net area under the decay curves (AUC). The following equation was used:

AUC = Sample AUC – Control AUC

Net AUC to represent antioxidant capacity was expressed in µmol Trolox equivalents (TE) per g DM tea.

2.3.6. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay

The cellular antioxidant activity was evaluated in the Caco-2 cell line. In a 96-well plate, Caco-2 cells were plated at a 2 x 10^4 cells/mL concentration, and the cells were cultured for a further 24 h period at 37°C in 5% CO_2. For the evaluation of cellular protection against oxidative damage 50 µL of a 75 µM solution of DCHF-DA was added to each well and incubated for 45 minutes at 37 °C. Following incubation, the medium was removed, and the wells washed once with PBS to remove excess DCHF-DA. To each well 50 µL of a 1% or 10% (v/v) tea solution was then added, followed by 50 µL of a 0.02 M AAPH solution. The samples were mixed, and the fluorescence was measured every two minutes for 60 minutes at Ex_{485} and Em_{520} using a FLUOstar OPTIMA fluorescence plate reader. The gradient of the change of fluorescence was measured, and the final results were reported as percentage protection (%P) relative to the control (no tea added) and calculated as follows:

$$\%P = 1 - [(gradient_{AAPH} - gradient_{AAPH + tea})] \times 100$$

2.3.7. Nitric oxide scavenging activity assay

A modified method of Kim *et al.*[25] was used to determine nitic oxide (NO) scavenging capacity of the tea samples. A 5 mM sodium nitroprusside (SNP) solution was prepared in PBS. To quantify the amount NO formed a standard solution of 0.1 mM $NaNO_3$ was used. A concentration series of 0 - 0.01mM of $NaNO_3$ was used to prepare a standard curve. To 20 µl of a 10% tea solution, 80 µL of the 5mM SNP solution was added.

The Trolox standard and the tea samples were incubated with the SNP for 1 hour at room temperature in the dark. The levels of NO were determined with the Griess reagent as follows: 100 µL of a 1% sulfanilamide (SA) and 0.1% N-(1-naphthyl)ethylenediamine (NED) solution prepared in 2.5% phosphoric acid was added. The samples were mixed, and the absorbance was measured at 570 nm, and NO scavenging activity was expressed as µmol TE/mg DM tea.

2.3.8. Nitric oxide production assay

The RAW 264.7 cells were plated in a 96-well plate at a cell density of 2x 10^4 cells per well. After 24 hours, the cells were stimulated by adding to the media, 10 µL of 10 µg/mL lipopolysaccharide (LPS) solution prepared in cell culture media and 10 µL of a 200 U/mL of interferon gamma (IFN-γ) diluted in PBS. To each well, 10 µL of the 10% tea samples were added. To the positive control wells (100% NO production) (PC), dH_2O was added instead of the sample while the negative controls (NC) contained no sample, LPS and IFN-γ. The ability of each tea sample to either scavenge and/or reduce NO production was determined

after 24 hours. To 50 µL of the medium removed from the well, 100 µL of Griess reagent prepared as described (1% SA and 0.1% NED in 2.5% phosphoric acid) was added. The absorbance was measured at 570 nm and the percentage of NO inhibition was calculated as follows:

$$\% \text{ Change in NO levels} = 100 \times [(Abs_{TS} - Abs_{NC})/(Abs_{PC} - Abs_{NC})]$$

2.3.9. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay for cell viability

Following the sampling of the cell culture media for NO determinations, 5 µL of a 1mg/mL MTT solution was added. After 3 hours of incubation at 37°C in 5% CO_2, the media was removed and the plates were allowed to dry before the formazan product that had formed was extracted with shaking for 10 minutes with 100 µL of a 25% dimethyl sulfoxide (DMSO) solution. The absorbance was then measured at 570 nm, and the cell viability was expressed as a percentage of the control (untreated).

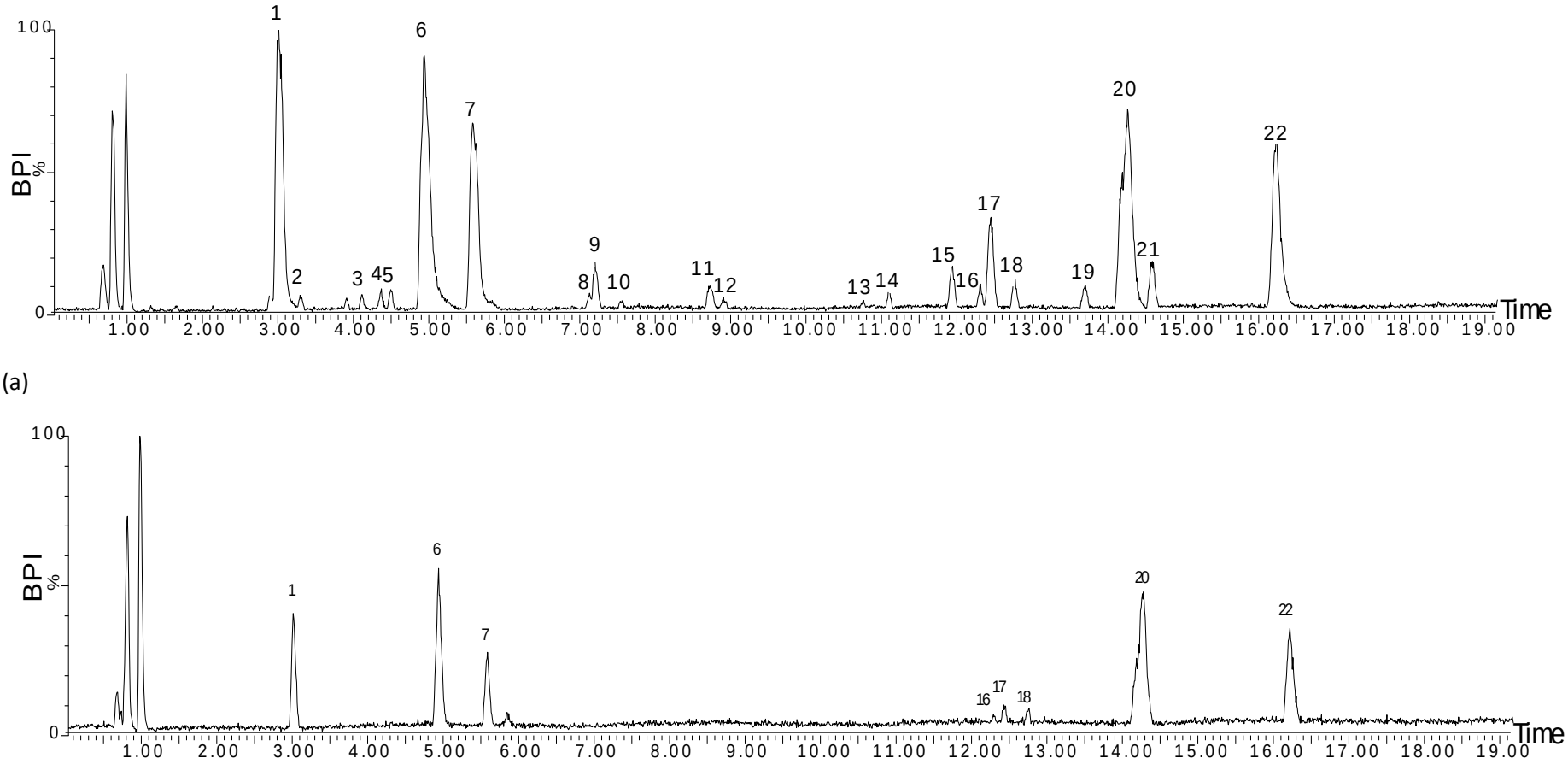
2.4. Data management and statistics

For each commercial tea type, three different lot numbers were used. Only two factory *I. guayusa* batches were available for the green and black *I. guayusa* tea. Each sample was analysed three times in triplicate. The results are expressed as mean ± standard error of the mean (SEM). Data were statistically evaluated using the t-Test assuming unequal variance with samples as independent variables and the values determined as dependent variables. Pearson's product moment correlation coefficients to determine the linear dependence of two assays were also calculated.

3. Results and discussion

3.1 LC-MS/MS evaluation of unfermented and fermented I. guayusa teas

Mass spectrometry analysis of unfermented and fermented *I. guayusa* teas in the negative ionization mode, revealed that the teas are rich in phenolic compounds particularly caffeoylquinic acid derivatives at 353 *m/z* for retention times (RT) of 3.01, 4.50, 4.94, 5.58 and 7.20 minutes and the dicaffeoylquinic acids derivatives at 515 *m/z* for RT of 14.26 and 16.23 minutes (Fig. 1 and Table 1). Flavonol glycosides of quercetin and kaempferol were also tentatively identified at 447 *m/z*, 463 *m/z,* 593 *m/z* and 609 *m/z*. Only the unfermented tea IGG contained the caffeic acid glucosides (341*m/z*), coumaroylquinic acids (337 *m/z*) and feruloylquinic acids (367 *m/z*) as well as the quercetin rutinosides (609 *m/z*).



(a)

(b)

Fig. 1 Base peak chromatograms of *I. guayusa* (a) unfermented and (b) fermented teas obtained in negative ionization mode. Number of peaks refers to compounds tentatively identified in Table 1.

Table 1. Tentative identification of compounds in *I. guayusa* tea samples.

Compound	RT (min)	Accurate Mass [M-H]-	Proposed formula	Error (ppm)	Fragment ions	λ max (nm)	Proposed compound	Tea sample
1	3.01	353.0873	C16H17O9	-1.7	191, 179, 135	322, 296sh	Caffeoylquinic acid	Both
2	3.29	341.0873	C15H17O9	-1.2	179, 161	324, 296sh	Caffeic acid glucoside	Unfermented
3	4.11	341.0873	C15H17O9	0.6	179, 161	324, 290sh	Caffeic acid glucoside	Unfermented
4	4.37	337.0923	C16H17O8	-3.6	191, 163	310	Coumaroylquinic acid	Unfermented
5	4.5	353.0873	C16H17O9	-2.5	191, 179, 135	316, 290sh	Caffeoylquinic acid	Unfermented
6	4.94	353.0873	C16H17O9	-1.1	707, 191, 179	322, 296sh	Caffeoylquinic acid	Both
7	5.58	353.0873	C16H17O9	-1.1	707, 191, 179	324, 296sh	Caffeoylquinic acid	Both
8	7.13	337.0923	C16H17O8	-1.2	191, 163	310	Coumaroylquinic acid	Unfermented
9	7.2	353.0873	C16H17O9	-1.7	191, 179, 135	314	Caffeoylquinic acid	Unfermented
10	7.56	337.0923	C16H17O8	-0.9	191, 163	311	Coumaroylquinic acid	Unfermented
11	8.72	367.1029	C17H19O9	3	191, 134	324, 296sh	Feruloylquinic acid	Unfermented
12	8.91	367.1029	C17H19O9	4.9	191, 134	324, 296sh	Feruloylquinic acid	Unfermented
13	10.76	609.1456	C27H29O16	-0.8	301, 300, 271	253, 343	Quercetin rutinoside	Unfermented
14	11.09	609.1456	C27H29O16	2.3	301, 300, 271	254, 349	Quercetin rutinoside	Unfermented
15	11.93	463.0877	C21H19O12	2.6	301, 300, 271	254, 349	Quercetin glycoside	Unfermented
16	12.31	593.1506	C27H29O15	-2	285	264, 340	Kaempferol rhamnoside	Both
17	12.45	463.0877	C21H19O12	0	301, 300, 271	254, 351	Quercetin glycoside	Both
18	12.76	593.1506	C27H29O15	-0.8	285, 255, 227	264, 346	Kaempferol rhamnoside	Both
19	13.71	447.0927	C21H19O11	2	342, 285, 255	264, 342	Kaempferol glycoside	Unfermented
20	14.26	515.119	C25H23O12	0.8	353, 191, 179	325, 296sh	Dicaffeoylquinic acid	Both
21	14.59	447.0927	C21H19O11	0.2	285, 255	264, 334	Kaempferol glycoside	Both
22	16.23	515.119	C25H23O12	2.3	353, 191, 179	326, 296sh	Dicaffeoylquinic acid	Both

Compounds were tentatively identified according to their mass fragmentation patterns and previous literature on *Ilex* species.[9,17,26] Although the unfermented tea was more complex, the fermented tea showed similar bioactivities suggesting that caffeoylquinic acid derivatives are the major determinants of the antioxidant and anti-inflammatory activity observed here for the tea preparations. These types of compounds have previously shown antioxidant and anti-inflammatory activity.[27] Caffeine at 195 *m/z* was found as the unique peak in the positive ionization mode in both tea samples (not shown). The caffeine content was found to be similar to that observed in *C. sinensis* green and black tea samples ranging from 2.9 to 3.2% m/m versus 2.6 to 3.1% m/m for the *C. sinensis* teas (not shown). Extracts prepared from *I. guayusa* have previously been shown to contain caffeine as well as other methylxanthine derivatives like theobromine and theophylline.[28] The composition of the *I. guayusa* teas shown here was similar to that previously reported by García-Ruiz *et al*.[17] that evaluated the composition of fresh and processed *I. guayusa* leaves extracted with a methanol/water solution. García-Ruiz *et al*.[17] also showed the presence of carotenoids α-carotene, β-carotene, lutein, violaxanthin and neoxanthin in *I. guayusa* leaves extracted with a hexane/acetone solution. Kapp Jr *et al.*[16] found trace amounts of catechin, epicatechin, epicatechin gallate, epigallocatechin and epigallocatechin gallate (EGCG) in *I. guayusa* leaves brewed with water. The coumaroylquinic acid and kaempferol rhamnoside isomers were the only new compounds tentatively identified in this study. The *I. guayusa* teas were also found to be similar in chemical composition as other caffeinated holly species namely *I. paraguariensis* and *I. vomitoria*. These *Ilex* species like the *I. guayusa* teas have in previous studies been shown to contain mono-, di- and tricaffeoylquinic acids as well as flavonol glycosides.[9,26]

3.2. Antioxidant activity

3.2.1 Biochemical assays: F-C and ORAC assays

The F-C method has been established as the standardized method in the process of quantifying polyphenol content and executing quality control in food products and dietary supplements and is the ISO approved method for quantifying polyphenols in tea.[29,30] It is essentially a reducing capacity assay based on electron transfer.[31] For all tea samples, there was a rapid extraction of polyphenols from 0-10 minutes and later from 10 - 60 minutes the reducing capacity was constant (Table 2). LGT was found to contain the highest reducing capacity with a range of 94.9 ± 6.3 - 198.4 ± 4.1 mg GAE/g DM for water extraction times of 2 – 60 minutes. This was followed by LBT with a range of 72.71 – 108.33 mg GAE/g DM and lastly the *I. guayusa* teas with 22.13 – 67.23 mg GAE/g DM for the same time intervals. A slightly higher F-C reducing capacity was observed for the unfermented *I. guayusa* tea IGG

Table 2. Effect of brewing time on the antioxidant activity of *I. guayusa* teas.

F-C (mg GAE/g DM)	2 min	10 min	30 min	60 min
IGB	**22.13 ± 7.94$^{a;1}$**	**44.64 ± 9.56$^{ab;1}$**	**51.56 ± 9.21$^{ab;1}$**	**54.39 ± 8.2$^{;1}$**
IGG	**30.10 ± 8.01$^{a;1}$**	**55.79 ± 12.17$^{a;1}$**	**64.82 ± 13.07$^{a;1}$**	**67.23 ± 11.15$^{a;1}$**
LGT	94.90 ± 6.32$^{a;2}$	158.43 ± 4.58$^{b;3}$	182.38 ± 5.18$^{c;3}$	198.36 ± 4.11$^{c;3}$
LBT	72.71 ± 0.45$^{a;2}$	105.67 ± 3.22$^{b;2}$	112.25 ± 4.14$^{b;2}$	108.33 ± 0.68$^{b;2}$
ORAC (µmol TE/g)	2 min	10 min	30 min	60 min
IGB	**798.08 ± 109.85$^{a;2}$**	**1779.84 ± 228.15$^{b;1}$**	**2180.44 ± 244.40$^{b;1}$**	**2019.00 ± 229.11$^{b;1}$**
IGG	**246.96 ± 155.01$^{a;1}$**	**1161.43 ± 479.28$^{ab;1}$**	**1659.63 ± 425.45$^{b;1}$**	**1773.41 ± 357.31$^{b;1}$**
LGT	2800.59 ± 132.55$^{a;4}$	4383.54 ± 252.73$^{b;2}$	5029.16 ± 218.51$^{b;2}$	4897.92 ± 220.88$^{b;2}$
LBT	1941.44 ± 97.02$^{a;3}$	3585.70 ± 83.65$^{b;2}$	4319.49 ± 217.79$^{bc;2}$	4056.72 ± 217.87$^{c;2}$

Runa tea samples fermented (IGB) and unfermented (IGG) highlighted in bold. LGT = Lipton's green tea and LBT = Lipton's black tea. Data is an average of three independent experiments ± SEM. Different letters across rows (a-d) indicates statistical significance between extraction times ($p < 0.05$). Different numbers down columns (1-4) indicates statistical significance between tea samples ($p < 0.05$).

versus the fermented tea IGB. Higher F-C reducing capacity has also been observed for the LGT versus the LBT. The F-C reducing capacity for the *I. guayusa* teas were shown to be significantly lower than both LGT and LBT ($p < 0.05$), as shown in Table 2.

The ORAC assay is the direct measurement of the hydrophilic and lipophilic chain-breaking antioxidant capacity against peroxyl radicals, generated by AAPH.[31] As observed with the F-C reducing capacity assay, a significant increase in the radical scavenging capacity of all tea samples was observed between the 2 and 10 minute extraction times. After that, only LBT and IGB showed a significant difference in scavenging capacity between 10 and 60 minutes. The ORAC for the *I. guayusa* teas ranged from 264.96 ± 155.01 to 1773.41 ± 357.31 µmol TE/g DM and 798.08 ± 109.85 to 2019.00 ± 229.11 µmol TE/g DM for unfermented and fermented samples, respectively. For all extraction times, both *I. guayusa* tea samples had lower ORAC values compared with LGT and LBT ($p < 0.05$). With regard to the control tea samples LGT and LBT, there is no statistical difference between the ORAC values at the various extraction times, as shown in Table 2.

The F-C and ORAC determinations observed here were in agreement with those previously reported for methanol/water (70:30) extracts of *I. guayusa* teas[17] and the results of this study suggest that maximal extraction of the antioxidant phenolics of *I. guayusa* teas can be achieved under normal tea brewing conditions. With both biochemical type assays, the antioxidant activity of the *I. guayusa* teas was shown to have significantly lower activity than the *C. sinensis* teas ($p < 0.05$). Similarly, in studies comparing the reducing capacity as well as the ORAC of *C. sinensis* teas to that of similar holly species *I. paraguariensis* and *I. vomitoria*, the *C. sinensis* teas were found to have better antioxidant activity.[32,33]

Several structural factors have been found to influence the antioxidant activity of polyphenolic compounds and include the number of hydroxyl groups on the benzene ring, the position of the hydroxyl groups as well as substituent groups.[34] Green tea is rich in the catechin type polyphenolic compounds, in particular, EGCG. Studies have shown EGCG to be a powerful antioxidant compound with its three hydroxyl groups on the B-ring together with an added galloyl group on the C-ring.[35] The *I. guayusa* teas, however, were found to contain caffeoylquinic acid derivatives. Only the two ortho hydroxyl groups of the caffeoyl group are likely to be involved in antioxidant reactions and the more caffeoyl groups esterified to the quinic acid group, the higher the antioxidant activity.[36] In comparison studies of the antioxidant activity of chlorogenic acid versus catechins, the catechins have been found to have better antioxidant activity.[35,37] Another factor to consider, is the relative abundance of the antioxidant compounds extracted in each tea sample. This is observed with the antioxidant activity of green versus black *C. sinensis* teas. With both biochemical

antioxidant assays, the black tea was found to have lower activity than the green tea. Studies have shown, however, that the theaflavins found in black tea are as effective antioxidant compounds as the catechins found in green tea.[38]

3.2.2 Cellular antioxidant activity

Chemical assays, such as the F-C and ORAC assays fail to take into consideration processes such as cellular absorption, distribution, metabolism and excretion. For these reasons, the in *vitro* cellular antioxidant activity of each tea sample was determined to include some of these parameters and provide some physiologically relevant antioxidant information. The cellular antioxidant activity of the tea samples was evaluated in the Caco-2 human colon cancer cells with the DCFH-DA probe that upon exposure to reactive oxygen species generated by AAPH, is oxidized and converted to fluorescent DCF.[39] As observed with the F-C and ORAC assays, extraction time was a major factor in the measured amount of cellular antioxidant activity. Small increases in antioxidant activity were observed for IGB between the different time points increasing from 56.43% at 2 minutes, 66.74% at 10 minutes, 62.71% at 30 minutes and 71.04% at 60 minutes (Fig. 2). For IGG, on the other hand, there was a significant increase in antioxidant activity from 32.35% at 2 minutes to 71.74% at 10 minutes after which little change in antioxidant activity was observed, 73.43% at 30 minutes and 75.67% at 60 minutes. Similarly, control tea samples LBT and LGT also showed a significant increase in antioxidant activity between 2 and 10 minute extraction times, and for LGT there was a significant continuous increase in activity over time. After 2 minutes extraction time, the cellular protective rankings of tea samples were as follows: LGT > IGB > LBT > IGG. LGT and IGB were found to be statistically similar and have significantly higher cellular protective effects compared with other tea types. At 10 and 30 minutes extraction time, the rankings remained the same. At the 60 minutes extraction time, LGT had significantly higher cellular protective effects than all other tea types followed by LBT which was similar to IGG.

Interestingly with this assay, the *I. guayusa* teas were found to be statistically comparable with that of the *C. sinensis* black tea regarding antioxidant protective effect and were even comparable to the *C. sinensis* green tea at the 2 and 10 minute extraction times. The antioxidant compounds in the *I. guayusa* teas appeared to be as effective as the catechins and theaflavins found in the *C. sinensis* teas in gaining access to the cell membrane and scavenging the lipid peroxyl radicals that form. With the longer extraction times, it is likely that higher concentrations of the catechins were extracted from the *C. sinensis* green tea versus the caffeoylquinic acids from the *I. guayusa* teas. Also, the catechins in particular EGCG have been shown in some studies to afford better protection against lipid peroxidation

than caffeoylquinic acids, and this is due to the differences in the number of hydroxyl groups in their chemical structures.[40,41]

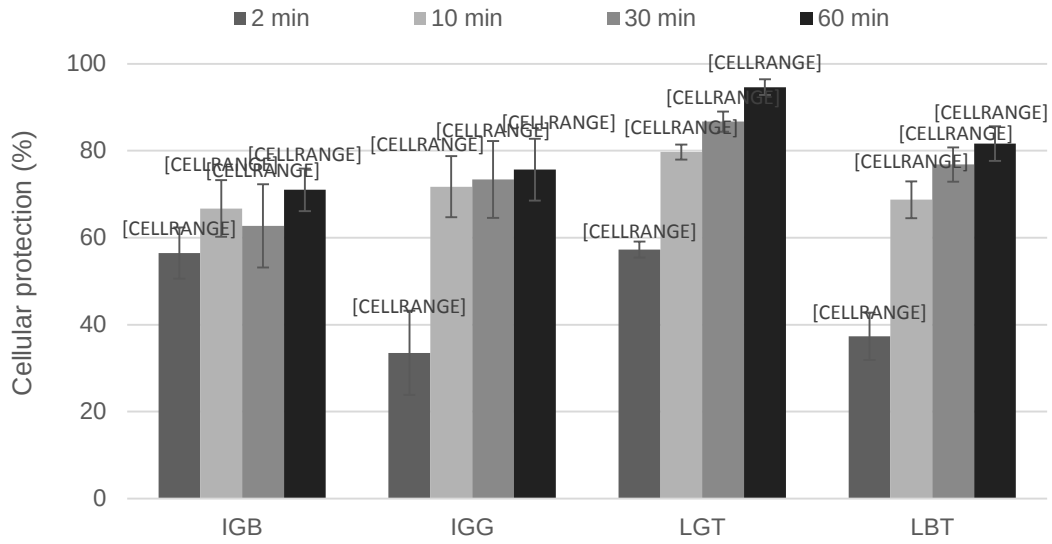


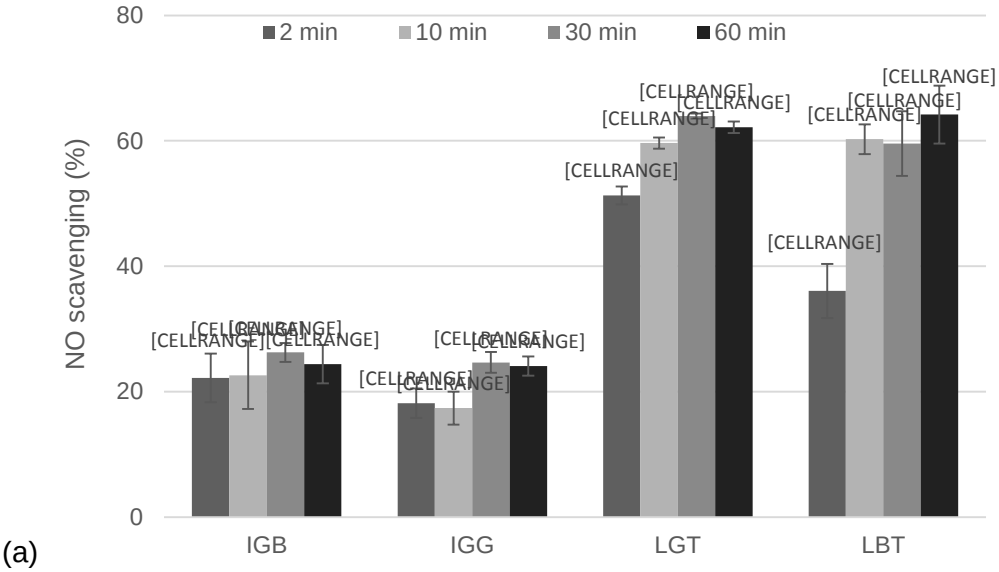
Fig. 2 Cellular protection against AAPH induced oxidative damage for *I. guayusa* tea samples versus that of control teas LGT and LBT in the Caco-2 cell line. *The histograms represent means from three independent experiments ± SEM. Different letters (a-c) within each tea type indicate statistical differences between extraction times (p < 0.05). Different numbers (1-3) across all four tea types and all four-time points, indicate statistical significance differences between tea samples at each time point (p < 0.05).*

For all tea types, the correlation between F-C and ORAC was 0.978-0.995, F-C and DCFH-DA was 0.849-0.998 and for ORAC and DCFH-DA was 0.751-0.980 with the lowest correlation observed for IGB. These correlations indicate essentially that with increasing extraction time there was a corresponding increase in antioxidant activity. Several studies have shown that the major increases in antioxidant activity of tea samples occur between the 1 minute extraction time and 5 minute extraction time where after only small increases or no change in antioxidant activity is observed.[42-44] This is similar to what we observed in our study where, with the antioxidant assays, the biggest change in activity was observed between the 2 and 10 minute infusion times. The infusion time, however, was observed to have less of an effect on the *I. guayusa* teas versus the *C. sinensis* teas suggesting the relative abundance of the bioactive compounds is greater in the *C. sinensis* teas versus the *I. guayusa* teas. With longer extraction times, more of these bioactive compounds can be extracted from the *C. sinensis* teas while with the *I. guayusa* teas the bioactive compounds may have already been exhaustively extracted after the first 10 minutes.

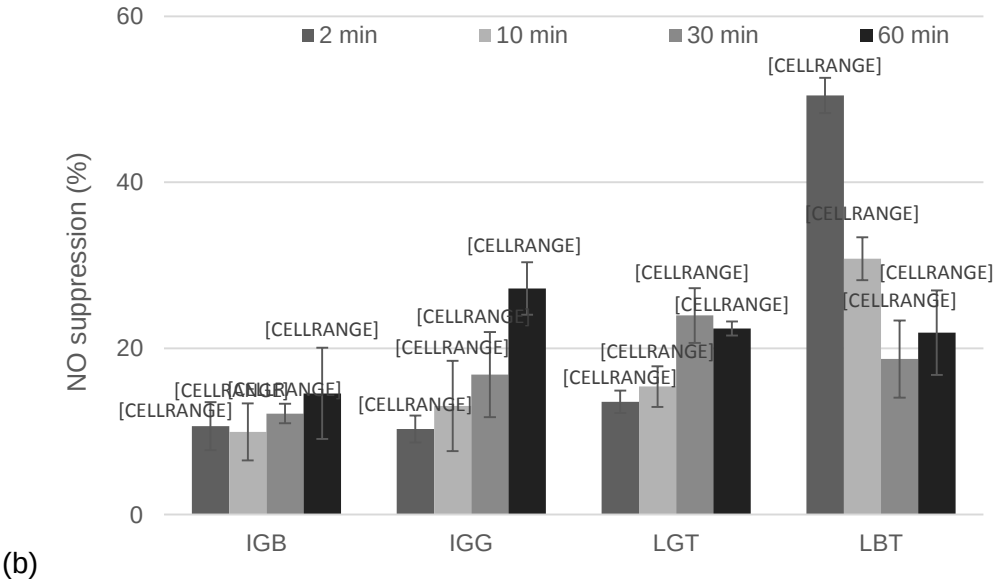
3.3. Anti-inflammatory activity

The anti-inflammatory activity of the *I. guayusa* teas was evaluated in terms of ability to scavenge NO as well as the ability to suppress cellular NO production. NO plays important roles in cell signaling events and is involved the body's defense mechanisms, regulation of blood pressure and neurotransmission.[45] However, during pathological conditions such as persistent infections and excessive inflammation, the large amount of NO that is produced can react with oxygen to form highly reactive and destructive nitrogen species like peroxynitrite. The direct NO scavenging activity of the tea samples was determined with the SNP assay where NO as nitrite was quantified with the Griess reagent. Both *I. guayusa* teas were capable of scavenging NO, however, this activity was found to be significantly lower than that of control tea samples LGT and LBT ($p < 0.05$) with scavenging capacities ranging from 18.16% to 24.38% for the *I. guayusa* teas versus 36.05% to 64.19% for the *C. sinensis* teas (Fig. 3a). There was an increase in the NO scavenging capacity of each tea with increased extraction time, with the exception being the scavenging activity of IGB, which remained unchanged over the different extraction times. Increases in NO scavenging capacity was observed for IGG between 10 and 30 minutes, LBT between 2 and 10 minutes while for LGT increases in activity were observed for the 2, 10 and 30 minute extraction times where after the activity remained unchanged.

NO like the peroxyl radical is also a radical species having an unpaired electron on the nitrogen atom.[45] Thus, to scavenge and neutralize NO, antioxidant compounds as with peroxyl radicals, need to transfer both electrons and hydrogen atoms. Subsequently, the structural rules such as the number of hydroxyl groups as observed with the other biochemical antioxidant assays, likely apply to the differences observed in the activity of the *I. guayusa* teas versus that of the *C. sinensis* teas. Green tea and its major catechin compounds have also been shown in previous studies to scavenge NO radicals in a dose-dependent fashion, and at concentrations of 1 to 5 µg/mL, a green tea extract was shown to scavenge NO by 15.3% to 34.6%.[46] In a study comparing the NO scavenging capacity of various herbal teas to that of green tea, green tea was found to have better activity and this activity was correlated to the higher total phenolic content found in green tea.[47] Preparations of *I. paraguariensis* have also been shown to be scavengers of NO by scavenging 30 to 70% nitric oxide depending on the brand.[48] The lower activity for the *I. guayusa* teas found here versus that previously reported for the *I. paraguariensis* preparations is likely due to the preparation of the samples. In this study, the teas were prepared by extracting 1g of leaves with 100 mL of water while in the study of Colpo *et al*.[48] the *I. paraguariensis* preparations were prepared by extracting 85g of leaves with 70 mL water.



(a)



(b)

Fig. 3 Anti-inflammatory activity related to (a) NO scavenging and (b) NO production activity of *I. guayusa* teas IGB and IGG versus control tea samples LGT and LBT. *The histograms represent means from three independent experiments ± SEM. Different letters (a-c) within each tea type indicate statistical differences between extraction times ($p < 0.05$). Different numbers (1-3) across all four tea types and all four-time points, indicate statistical significance differences between tea samples at each time point ($p < 0.05$).*

The ability of the tea samples to suppress NO production in a cellular environment was assessed in the RAW 264.7 cell line that when exposed to LPS and INF-γ induces NO formation. The capacity of IGG and IGB to suppress NO production ranged from 10.30% to 27.21% for IGG and 10.65% to 14.59% for IGB for the different extraction time points (Fig. 3b). IGG showed significantly higher activity ($p < 0.05$) than IGB at 60 minutes by

suppressing NO production by 27.21% versus only 14.59% for IGB. In comparison with the control tea samples, the *I. guayusa* teas were only significantly lower ($p < 0.05$) than LBT at 2 and 10 minutes. At 30 and 60 minutes, the inhibition of NO production of all teas was relatively similar. An increase in inhibitory activity could be seen with increasing brewing times for IGG, IGB and LGT. Interestingly, LBT showed significant activity at the 2 minute extraction time with 50.46% followed by decreases in activity to 30.81% and 18.73% at the 10 and 30 minute extraction times, respectively. As no decrease in antioxidant activity over the different brewing times was observed with any of the antioxidant assays, the loss of activity observed here with the NO suppressing activity of LBT may indicate polymerization of the theaflavins during the longer brewing times. This would result in the formation of large complexes that may have limited absorption across the cell membrane. This, however, can only be confirmed with future studies. Theaflavins have been shown to form complexes with caffeine.[49] Only for LGT a correlation of 0.873 was found between NO scavenging and the suppression of NO production by 264.7 RAW cells. For all other tea types, the correlation was poor indicating that the beneficial effects of these tea types were not only direct NO scavenging but related to the targeting of other inflammatory pathways resulting in the reduction of NO production.

The effects of tea extracts on RAW 264.7 cell viability was evaluated to determine whether a specific effect on NO production was being observed by the tea samples or if the reduction observed in NO production was due to cytotoxicity. Following exposure, no toxicity as a result of tea exposure or LPS and INF-γ exposure was observed (results not shown). These results indicate that the tea samples used in the study are not toxic to the RAW 264.7 cell line and that any reduction in NO production is not as a result of toxicity and/or cell death.

As with the antioxidant activity, the caffeoylquinic acids are again likely responsible for the observed inhibition of NO production by the *I. guayusa* teas. Caffeoylquinic acids like chlorogenic acid as well as 3,5-dicaffeoylquinic acid have been shown in previous studies to suppress NO production by RAW 264.7 following LPS stimulation.[50,51] The suppression of NO production by these compounds was found to be associated with a decrease in the expression of the nuclear factor-kappa B (NF-κB) that is activated upon LPS stimulation and in turn induces the expression of iNOS and pro-inflammatory cytokines. Similarly, EGCG, theaflavins and thearubigins have also been shown to suppress NO production by LPS-stimulated RAW 264.7 cells through the down-regulation of NF-κB.[52] The down-regulation of NF-κB was found to be associated with the inhibition of the inhibitor of κB (IκB) kinase (IKK) that is responsible for degrading IκB resulting in the translocation of NF-κB to the nucleus. Theaflavin-3,3'-digallate was found to be a stronger inhibitor of IKK than EGCG.

4. Conclusion

This study provides additional evidence for the antioxidant activity of *I. guayusa* tea preparations and demonstrates for the first time the potential anti-inflammatory activity of these teas. The *I. guayusa* teas were shown to be rich in mono- and di-substituted caffeoylquinic acid derivatives and these compounds are likely responsible for the observed antioxidant and anti-inflammatory activity. In a study by Swanston-Flatt *et al.*[14], an extract of *I. guayusa* was found to prevent hyperglycaemia development in streptozotocin-induced diabetic mice. In this study, caffeoylquinic acids were found to be the major components of *I. guayusa* extracts and it is likely that the observed anti-diabetic activity found previously for the *I. guayusa* extract is due to the presence of the caffeoylquinic acids. Chlorogenic acid, in particular, has been shown to enhance insulin sensitivity and glucose tolerance and inhibit gluconeogenesis in diabetic mice.[53] Other beneficial anti-diabetic effects of *I. guayusa* preparations may also be attributed to the antioxidant and anti-inflammatory activity found here in this present study. Inflammation and oxidative stress have both been implicated in the pathogenesis of diabetes and its associated complications.[54]

Although *C. sinensis* teas were found to have better activity possibly due to differences in the chemical nature of the bioactive compounds as well as their relative abundance in each tea preparation, *I. guayusa* tea preparations present themselves as additional sources of dietary phenolic compounds with health benefits.

Acknowledgements

We would like to thank RUNA LLC in New York for kindly providing us with the Runa tea samples.

Conflicts of interest

All authors declare no conflict of interest

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