ABSTRACT

Background: Dietary flavonoids may improve endothelial function and ultimately lead to beneficial cardiovascular effects.

Objective: The objective was to assess whether pure dietary flavonoids can modulate nitric oxide and endothelin-1 production and thereby improve endothelial function.

Design: A randomized, placebo-controlled, crossover trial in 12 healthy men was conducted to compare the acute effects of the oral administration of 200 mg quercetin, (−)-epicatechin, or epigallocatechin gallate on nitric oxide, endothelin-1, and oxidative stress after nitric oxide production was assessed via the measurement of plasma S-nitrosothiols and plasma and urinary nitrite and nitrate concentrations. The effects on oxidative stress were assessed by measuring plasma and urinary F2-isoprostanes. Plasma and urinary concentrations of quercetin, (−)-epicatechin, and epigallocatechin gallate were measured to establish the absorption of these flavonoids.

Results: Relative to water (control), quercetin and (−)-epicatechin resulted in a significant increase in plasma S-nitrosothiols, plasma nitrite, and urinary nitrate concentrations (P < 0.05), but not in plasma nitrate or urinary nitrite. Epigallocatechin gallate did not alter any of the measures of nitric oxide production. Quercetin and (−)-epicatechin resulted in a significant reduction in plasma endothelin-1 concentration (P < 0.05), but only quercetin significantly decreased the urinary endothelin-1 concentration. None of the 3 treatments significantly changed plasma or urinary F2-isoprostane concentrations. Significant increases in the circulating concentrations of the 3 flavonoids were observed (P < 0.05) after the corresponding treatment.

Conclusions: Dietary flavonoids, such as quercetin and (−)-epicatechin, can augment nitric oxide status and reduce endothelin-1 concentrations and may thereby improve endothelial function. Am J Clin Nutr 2008;88:1018–25.

INTRODUCTION

Endothelial dysfunction is a critical event in the pathogenesis of atherosclerosis and its clinical manifestations (1, 2). It accelerates the development of atherosclerosis and may be one of the earliest manifestations of this disease (3, 4). Therefore, endothelial function may serve as an indication for cardiovascular health and be used to evaluate new therapeutic strategies (5). The endothelium maintains vascular homeostasis and regulates vascular tone by balancing the production of vasodilators, most importantly nitric oxide (NO) (6), and vasoconstrictors, such as endothelin-1 (ET-1) (7). Because of its low concentration and short half-life (8), it is difficult to measure free NO in biological systems. Recent studies have reported that S-nitrosothiols, nitrite, and nitrate—all metabolites of NO—can be used as reliable measures of endogenous NO production (9, 10). ET-1 is a 21-amino acid vasoconstrictor peptide produced by endothelial cells, which has been identified as one of the strongest vasoconstrictors in human vasculature (11).

Flavonoids are ubiquitous in plant foods. Important dietary sources can include tea, red wine, apples, and cocoa (12, 13). Many flavonoids are potent antioxidants in in vitro systems (14). Epidemiologic studies have reported a reduced risk of cardiovascular disease in subjects with a high flavonoid intake (15). Flavonoid-rich tea (16), purple grape juice (17), and cocoa (18, 19) have all been found to improve endothelial function in acute and short-term intervention trials in humans (20). Improving endothelium-dependent vasodilation is believed to be one possible mechanism by which flavonoids may reduce cardiovascular risk (19). There are many hundreds of flavonoids in the human diet. However, it is likely that bioactivity relevant to endothelial function is limited to fewer compounds. We have studied 3 compounds: quercetin, (−)-epicatechin, and epigallocatechin gallate (EGCG). There is consistent evidence from population studies that flavonoids such as quercetin can reduce the risk of cardiovascular disease. The population data for flavan-3-ols, such as (−)-epicatechin and EGCG, are less consistent. However, in intervention studies, flavan-3-ol–rich foods and beverages, such as tea and cocoa, consistently improve endothelial function (19, 20). In addition, isolated (−)-epicatechin (21) and EGCG (22) have been found to acutely improve endothelial function in humans. Isolated (−)-epicatechin has also been shown to augment NO status (19, 21).

Our study investigated the acute effects of quercetin, (−)-epicatechin, and EGCG on NO status and ET-1 production, which have ultimate implications for endothelial function. We

1 From the School of Medicine and Pharmacology (WML, JMH, JMP, IBP, and KDC) and the School of Biomedical, Biomolecular and Chemical Sciences (WML and AJM), University of Western Australia, Perth, Western Australia.

2 Supported by grants from the National Heart Foundation of Australia and the National Health and Medical Research Council (Australia).

3 Reprints not available. Address correspondence to KD Croft, School of Medicine and Pharmacology, Royal Perth Hospital Unit, Medical Research Foundation Building, Level 4, 50 Murray Street, Perth, Western Australia. E-mail: kevin.croft@uwa.edu.au.

Received March 3, 2008.

Accepted for publication June 30, 2008.

Wai Mun Loke, Jonathan M Hodgson, Julie M Proudfoot, Allan J McKinley, Ian B Puddey, and Kevin D Croft

Pure dietary flavonoids quercetin and (−)-epicatechin augment nitric oxide products and reduce endothelin-1 acutely in healthy men1–3
also investigated whether these compounds induce changes in oxidative stress, which might also be a determinant of any effects on endothelial function.

**SUBJECTS AND METHODS**

**Chemicals and materials**

N-Ethylmaleimide, sulfanilamide (SFA), potassium iodide, copper(II) sulfate, \[^{15}\text{N}\]sodium nitrite, \[^{15}\text{N}\]sodium nitrate, 2,3,4,5,6-pentafluorophenyl bromide (PFPBr), 1-hydroxy-2-naphthoic acid, \(\beta\)-glucuronidase, \(N,O\)-bis(trimethylisilyl)trifluoroacetamide (BSTFA), pyridine, toluene, quercetin, and (−)-epicatechin were purchased from Sigma Aldrich (St Louis, MO); acetone, acetonitrile, hydrochloric acid, glacial acetic acid, and sulfuric acid were purchased from Univar (WA, Australia); methanol and ethanol were purchased from Mallinckrodt (NJ); NOA antifoam agent was purchased from GE Sievers (CO); 3′-O-methyl- (−)-epicatechin was purchased from Nacalai Tesque Inc (Kyoto, Japan); and 3′-O-methylquercetin was purchased from Advanced Technology & Industrial Co, Ltd (Kln, Hong Kong).

**Subjects**

Twelve healthy male subjects participated in the study. The study was approved by and performed under the guidelines of the Human Ethics Committee of the University of Western Australia, and informed consent was obtained from each of the subjects before commencement of the study. All subjects were healthy and had no evidence of chronic disease. None of the subjects consumed >20 g alcohol/d or were taking other medications, antioxidants, or vitamin supplements. The study group had a mean (± SEM) age of 43.2 ± 4.3 y, a mean (± SEM) body weight of 76.8 ± 2.3 kg, and a mean body mass index (BMI; in kg/m\(^2\)) of 25.1 ± 0.8. All subjects had normal blood pressure (mean systolic blood pressure of 123 ± 2 mm Hg and mean diastolic blood pressure of 78 ± 2 mm Hg). The mean concentrations of serum total, LDL, and HDL cholesterol and triglycerides in the subjects were 4.92 ± 0.28, 2.74 ± 0.31, 1.48 ± 0.24, and 1.65 ± 0.45 mmol/L, respectively.

**Experimental design**

The acute effects of 3 common dietary flavonoid aglycones—quercetin, (−)-epicatechin, and EGCG on plasma and urinary NO metabolites were assessed and compared with a placebo treatment (water only). A total of 4 clinic visits were conducted in the morning 1 wk apart on the same day of the week and at the same time of the day. Subjects received each of the following 4 treatments in random order: 300 mL water (control), 200 mg quercetin dissolved in 300 mL water, 200 mg (−)-epicatechin dissolved in 300 mL water, and 200 mg EGCG dissolved in 300 mL of water. These amounts were chosen as they represent a phase was extracted with toluene and the organic extract (0.5 mL) was injected in the splitless mode, and the oven temperature was held at 70 °C for 1 min, then increased to 160 °C at a rate of 20 °C/min and finally to 280 °C at a rate of 30 °C/min. Helium (92.5 kPa and flow rate 0.7 mL/min) was used as the carrier and methane as the reagent gas for negative-ion chemical ionization. Samples (1.0 µL) were injected in the splitless mode, and the oven temperature was held at 70 °C for 1 min, then increased to 160 °C at a rate of 20 °C/min and finally to 280 °C at a rate of 30 °C/min. Helium (92.5 kPa and flow rate 0.7 mL/min) was used as the carrier and methane as the reagent gas for negative-ion chemical ionization. Peak identification was based on retention time and mass spectra compared with \[^{15}\text{N}\]labeled authentic standards (sodium \[^{15}\text{N}\]nitrite and sodium \[^{15}\text{N}\]nitrate). Quantification was performed by using calibration curves obtained from authentic standards and labeled standards.

**Measurement of endothelin-1**

The acute effects of quercetin, (−)-epicatechin, and EGCG on systemic ET-1 production were investigated by measuring its concentration in the initial and 5-h urine samples by using a commercially available ET-1 (human) enzyme immunoassay kit (Assay Design, GA). ET-1 concentrations were adjusted for creatinine concentrations. Peak identification was based on retention time and mass spectra compared with \[^{15}\text{N}\]labeled authentic standards (sodium \[^{15}\text{N}\]nitrite and sodium \[^{15}\text{N}\]nitrate). Quantification was performed by using calibration curves obtained from authentic standards and labeled standards.

**Systemic oxidative stress**

Plasma and urinary \(\text{F}_{2}\) isoprostanes, a well-established marker of systemic oxidative stress, were determined by GC-MS according to a previously described method (25).

**Absorption of quercetin, (−)-epicatechin, and EGCG**

Quercetin and (−)-epicatechin are present in plasma and urine as glucuronides and sulfates and in their methylated forms with
very small amounts present in their free forms (26, 27). Absorption of quercetin, (−)-epicatechin, and EGCG were determined by measuring the amounts of free quercetin, 3′-O-methylquercetin, (−)-epicatechin, 3′-O-methyl(−)-epicatechin, and EGCG after the hydrolysis of conjugates in the baseline plasma and urine, 2-h plasma, and 5-h urine samples by using a previously reported GC-MS method (28). Briefly, 1-hydroxy-2-naphthoic acid (50 ng, internal standard) was added to plasma or urine (750 μL) and acidified to pH 4.8 with dilute hydrochloric acid. Thirty microliters of β-glucuronidase with sulfatase activity (3000 units of glucuronidase activity and 1500 units of sulfatase activity) was added, mixed, and incubated at 37 °C for 24 h with occasional mixing. Samples were then extracted twice with ethyl acetate (1 mL), dried under nitrogen, and derivatized with BSTFA (100 μL) and pyridine (50 μL) at 40 °C for 60 min. The trimethylsilyl (TMS) derivatives were analyzed on an Agilent 6890 gas chromatograph coupled to a 5973 mass spectrometer with the use of a cross-linked methyl silicone column (25 m × 0.20 mm, 0.33-mm film thickness, HP5-MS). Aliquots (1.0 μL) were injected in the splitless mode. The column temperature was held at 150 °C for 1 min and then increased to 300 °C at a rate of 20 °C/min and to 320 °C at a rate of 30 °C/min. Helium (0.7 mL/min) was used as the carrier gas. Peak identification was based on retention time, and the mass spectra were compared with authentic standards (quercetin, 3′-O-methylquercetin, (−)-epicatechin, 3′-O-methyl(−)-epicatechin, and EGCG). Quantification was performed by using calibration curves obtained from authentic standards and internal standard.

Statistical analysis

Statistical analyses were performed by using SAS version 9.0 (SAS Institute Inc, Cary, NC) or SPSS version 11.5 (SPSS Inc, Chicago, IL). Data are presented as means ± SEMs. The baseline-adjusted between-group differences were analyzed with random effects models with PROC MIXED (SAS) with Tukey’s adjustment for multiple comparisons. In these models, subject was treated as the random effect, and treatment, period,
Acute treatment with quercetin and (−)-epicatechin significantly reduced the plasma concentrations of ET-1 (P < 0.001 and P < 0.05 for quercetin and (−)-epicatechin, respectively, compared with water control) 2 h after ingestion (Figure 3A). Only quercetin produced a significant acute reduction in urinary ET-1 concentrations (P < 0.005 compared with water control) over the 5 h after oral ingestion (Figure 3B). There was no significant reduction in ET-1 concentrations with (−)-epicatechin treatment when compared with either the water control or baseline. EGCG had no effect on ET-1 production (Figure 3).

Systemic oxidative stress

The acute effects of the 3 flavonoids on systemic oxidative stress were determined by measuring plasma and urinary F2-isoprostane concentrations before and after the treatments. None of the treatments significantly affected acute plasma or urinary F2-isoprostane concentrations (Table 1).

**Quercetin, (−)-epicatechin, and EGCG absorption**

The absorption of quercetin and (−)-epicatechin was investigated by measuring total quercetin and (−)-epicatechin concentrations in the circulation 2 h after ingestion and the amounts excreted 5 h after ingestion. The total flavonoid concentration was calculated as the sum of the flavonoid and its 3'-O-methyl-derivatives after enzymatic hydrolysis with glucuronidase and sulfatase. The mean (± SEM) baseline circulating concentrations of total quercetin and total (−)-epicatechin were 0.84 ± 0.39 and 0.70 ± 0.34 μmol/L, respectively. Acute treatment with quercetin and (−)-epicatechin significantly increased (P < 0.001) the total circulating concentration of each flavonoid (3.54 ± 1.57 μmol/L for quercetin and 3.57 ± 1.21 μmol/L for (−)-epicatechin) (Figure 4, A and B). There were significant increases in the circulating concentrations of quercetin (P < 0.001), 3'-O-methyl-querceytin (P < 0.05), (−)-epicatechin (P < 0.001), and 3'-O-methyl-(−)-epicatechin (P < 0.005) 2 h after the flavonoid ingestion when compared with baseline concentrations.

The baseline concentrations of total quercetin and total (−)-epicatechin present in the urine were 0.61 ± 0.15 and 0.50 ± 0.28 μmol/mmol creatinine, respectively. After acute treatment with quercetin and (−)-epicatechin, the total amount of each flavonoid excreted over the 5-h period significantly increased (P < 0.05) compared with baseline.

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Plasma F2-isoprostanes</th>
<th>Urinary F2-isoprostanes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 2 h</td>
<td>Baseline 5 h</td>
</tr>
<tr>
<td><strong>pg/mL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>509 ± 33</td>
<td>205 ± 40</td>
</tr>
<tr>
<td>200 mg</td>
<td>502 ± 37</td>
<td>248 ± 62</td>
</tr>
<tr>
<td>EGCG, 200 mg</td>
<td>494 ± 20</td>
<td>227 ± 69</td>
</tr>
<tr>
<td><strong>pg/mmol creatinine</strong></td>
<td></td>
<td></td>
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<tr>
<td>Water</td>
<td>544 ± 40</td>
<td>226 ± 40</td>
</tr>
<tr>
<td>Quercetin, 200 mg</td>
<td>531 ± 38</td>
<td>235 ± 51</td>
</tr>
<tr>
<td>EC, 200 mg</td>
<td>483 ± 27</td>
<td>216 ± 57</td>
</tr>
<tr>
<td>EGCG, 200 mg</td>
<td>488 ± 26</td>
<td>243 ± 66</td>
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</table>

1 All values are x ± SEM; n = 12. There were no significant differences at baseline and no significant difference in plasma or urinary F2-isoprostanes between the treatment groups and the water group.
DISCUSSION

We showed that acute treatment with quercetin and (−)-epicatechin, but not EGCG, augmented endogenous NO (S-nitrosothiols, nitrite, and nitrate). Quercetin also reduced ET-1 production. These molecules may therefore improve endothelial function in healthy human subjects. NO reacts with the free thiol groups in proteins under physiologic conditions to form S-nitrosothiols (9). These S-nitrosothiols possess NO-like effects, such as vasodilatation and platelet inhibition (29), but have half-lives in hours (9) and have the physiologic benefit of being resistant to superoxide, unlike free NO (30). The comparably higher concentrations of S-nitrosothiols in the human circulation (≈7 μmol/L; S-nitrosothiols compared with ≈3 nmol free NO/L in human plasma) suggest that plasma S-nitrosothiols may serve as a reservoir for NO, effectively buffering its concentration and thereby maintaining NO homeostasis (31). Free NO can also undergo oxidation in human plasma to form mainly nitrate as well as nitrite (32). Recent in vivo studies have reported that circulating nitrite, rather than nitrate, reflects endothelium-dependent NO synthesis in humans and mammals (33, 34).

Numerous studies have shown that acute and repetitive consumption of flavonoid-rich foods for up to 4 wk can improve endothelial function in both subjects with coronary artery disease and healthy volunteers (17, 35). Flavonoids are presumed to be the active constituents. However, to date, there is little direct evidence that flavonoids are the bioactive molecules responsible. We have shown that oral administration of pure dietary flavonoids, quercetin, and (−)-epicatechin augment NO status in healthy men. This is shown by the significant elevation of circulating S-nitrosothiol and nitrite concentrations (Figures 1 and 3).
interference with the expression and activity of eNOS (42), indicating that diminished ET-1 concentrations may be accompanied by elevated NO bioavailability. It was reported that NO inhibits ET-1 production through the suppression of nuclear factor κB (43). There seems to be an inverse relation between NO and ET-1, which may serve to modulate endothelial function in the vasculature. Quercetin was shown to decrease ET-1 production in thrombin-stimulated cultured human umbilical vein endothelial cells in a dose-dependent manner with an IC₅₀ of 1.54 μmol/L (44). Red wine polyphenols were recently shown to prevent vascular oxidative stress by inhibiting NADPH oxidase activity and/or by reducing ET-1 release in rats (45). Because the mechanism by which flavonoids affect ET-1 production is still not well understood, the comparatively smaller increase in NO status by (−)-epicatechin than by quercetin is consistent with the observed effects on ET-1.

Surprisingly, EGCG did not show the same augmentation of NO products as quercetin and (−)-epicatechin (Figure 1 and 2). EGCG has been widely assumed to be the vasoactive flavonoid present in green tea, which offers vascular protection against cardiovascular diseases (46). EGCG was shown to mediate NO-dependent vasodilation in rat aortic rings (47) and was found to work primarily by the rapid activation of eNOS and an increase in eNOS activity, independent of an altered ENOS protein content (48) However, it must be noted that these studies reported the effects of EGCG at nonphysiologic concentrations. We carried out experiments to ascertaining whether EGCG had degraded during the process of dissolution and found that EGCG does degrade with time (up to 45% in 30 min) in the aqueous mixture prepared for this study (200 mg/300 mL water). However, ≥95% of the prescribed 200-mg dose was present in the aqueous mixture at the time of consumption (1–2 min after dissolution) (data not shown). EGCG was present at much lower concentrations (0.1 ± 0.01 μmol/L) in the circulation than quercetin (3.54 ± 1.57 μmol/L) and (−)-epicatechin (3.57 ± 1.21 μmol/L) after acute treatment (Figure 4). Similar circulating concentrations of EGCG were reported in a recent study in which a 300-mg dose of EGCG acutely improved brachial artery flow-mediated dilation measured by vascular ultrasound in humans with coronary artery disease (22). If improved endothelial function is brought about by EGCG, the compound is likely to have exerted its effects through mechanisms other than those mediated by NO or ET-1.

Oral administration of quercetin, (−)-epicatechin, and EGCG (200 mg) did not acutely affect plasma and urinary F₂-isoprostanes (Table 1). There is a growing body of evidence from controlled human trials casting doubt as to whether flavonoids can act as antioxidants in vivo. Whereas plasma (−)-epicatechin and EGCG concentrations were increased after consumption of dark chocolate and black tea, they neither improved plasma antioxidant capacity nor reduced urinary 8-isoprostane concentrations (49–51). In another trial in which subjects consumed onions, significantly elevated plasma quercetin concentrations did not result in any significant effects on plasma F₂-isoprostanes concentrations (52). Because oxidative stress is implicated in the development of cardiovascular diseases, one of the main properties thought to explain the effects of flavonoids is their antioxidant property (53). However, recent studies carried out in this area should be interpreted with caution because the native unmodified forms of flavonoids found in the diet were used in in

![Graph A](image1)

**FIGURE 5.** Linear correlation between changes in plasma S-nitrosothiols and plasma concentrations of quercetin (A) and (−)-epicatechin (B).

2). The changes in plasma S-nitrosothiol concentrations were also shown to be significantly correlated with the changes in plasma quercetin and (−)-epicatechin concentrations (Figure 5). Our results confirm that flavonoids, such as quercetin and (−)-epicatechin, do indeed influence NO status in humans as reported in recent in vitro experiments (36) and animal studies (37). Quercetin may have increased NO production by increasing endothelial NOS (eNOS) activity (37, 38) or by enhancing the bioavailability of endothelium-derived NO (39). (−)-Epicatechin has been shown to elevate NO in endothelial cells in vitro via the inhibition of NADPH oxidase (36). Schroeter et al (21) reported that oral administration of pure (−)-epicatechin to humans closely emulated the acute vascular effects of flavonol-rich cocoa. Quercetin and (−)-epicatechin may also act as antioxidants by reducing nitrites and nitrates to free NO (40).

Quercetin treatment showed a significant reduction in systemic ET-1 production in this study while (−)-epicatechin has shown a significant decrease in plasma but not in the urine (Figure 3, A and B). ET-1 has been demonstrated to be associated with increased oxidative stress and endothelial dysfunction in humans. ET-1 stimulates superoxide production and vasoconstriction through the activation of NADPH oxidase and uncoupled NOS in the rat aorta (41). It also reduces NO bioavailability via the production of NO products as quercetin and (−)-epicatechin (Figure 1 and 2). EGCG has been widely assumed to be the vasoactive flavonoid present in green tea, which offers vascular protection against cardiovascular diseases (46). EGCG was shown to mediate NO-dependent vasodilation in rat aortic rings (47) and was found to work primarily by the rapid activation of eNOS and an increase in eNOS activity, independent of an altered ENOS protein content (48) However, it must be noted that these studies reported the effects of EGCG at nonphysiologic concentrations. We carried out experiments to ascertain whether EGCG had degraded during the process of dissolution and found that EGCG does degrade with time (up to 45% in 30 min) in the aqueous mixture prepared for this study (200 mg/300 mL water). However, ≥95% of the prescribed 200-mg dose was present in the aqueous mixture at the time of consumption (1–2 min after dissolution) (data not shown). EGCG was present at much lower concentrations (0.1 ± 0.01 μmol/L) in the circulation than quercetin (3.54 ± 1.57 μmol/L) and (−)-epicatechin (3.57 ± 1.21 μmol/L) after acute treatment (Figure 4). Similar circulating concentrations of EGCG were reported in a recent study in which a 300-mg dose of EGCG acutely improved brachial artery flow-mediated dilation measured by vascular ultrasound in humans with coronary artery disease (22). If improved endothelial function is brought about by EGCG, the compound is likely to have exerted its effects through mechanisms other than those mediated by NO or ET-1.

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vitro experiments instead of the metabolites found in vivo. We recently showed that structural modification of flavonoids, such as quercetin, by metabolic transformation, is likely to have a profound effect on biological activity (54). There is also the issue of bioavailability; plasma concentrations of flavonoids can reach between 2 and 5 μmol/L after supplementation with flavonol-rich foods (such as onions or apples) or various flavonoid-glycosides at doses of 50–200 mg equivalents (55). Because the metabolism of flavonoids is likely to influence bioactivity, it is interesting to note that metabolites possessing the 3'-O-methyl function have increased activity as inhibitors of NADPH oxidase (56).

Overall, our study suggests that pure dietary flavonoids, such as quercetin and (−)-epicatechin, can improve endothelial function acutely by modulating the circulating concentrations of vasoactive NO products and ET-1. These effects are possibly exerted via the inhibition of NADPH oxidase and the activation of eNOS. Similar studies, using pure dietary flavonoids, should be performed over a longer trial period to investigate their chronic effects.

We thank Noelene Atkins for expert nursing assistance. WML thanks the University of Western Australia for an International Research Fees Scholarship.

The authors’ responsibilities were as follows—WML: conducted most of the experimental work and drafted manuscript; JMH: helped design the study, analyzed the data, and helped revise the manuscript; JMP: performed some experiments and helped revise the manuscript; AIM: provided experimental advice and helped revise the manuscript; IBP: helped design the study and revise the manuscript; and KDC: supervised the project and helped design the study and revise the manuscript. None of the authors had a conflict of interest.

REFERENCES


