

KENYAN PURPLE TEA ANTHOCYANINS ABILITY TO CROSS THE BLOOD BRAIN BARRIER REINFORCING BRAIN ANTIOXIDANT CAPACITY IN MICE

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ABSTRACT

Studies on antioxidants as neuroprotective agents have been hampered by the impermeability of the blood brain barrier (BBB) to many compounds. However, previous studies have shown that a group of tea (*Camellia sinensis*) flavonoids, the catechins, are brain permeable and neuroprotective. Despite this remarkable observation, there exists no data on the bioavailability and pharmacological benefits of tea anthocyanins (ACNs) in the brain tissue. This study investigated the ability of Kenyan purple tea ACNs to cross the BBB and boost the brain antioxidant capacity. Mice were orally administered with purified and characterised Kenyan purple tea ACNs or a combination of Kenyan purple tea ACN's and coenzyme-Q₁₀, at a dose of 200 mg kg⁻¹ body weight in an experiment that lasted for 15 days. Twenty four hours post the last dosage of antioxidants, CO₂ was used to euthenise the mice. Then the brain was excised and used for various biochemical analyses. Kenyan purple tea ACNs significantly (P<0.05) raised brain Glutathione (GSH) levels, implying a boost in brain antioxidant capacity. Notably, ACN metabolites were detected in brain tissue of ACN fed mice. This is the first demonstration that Kenyan purple tea ACNs can cross the BBB, reinforcing the brain's antioxidant capacity. Hence, there is need to study ACNs as suitable candidates for dietary supplements that could support antioxidant capacity in the brain and have potential to provide neuroprotection in neurodegenerative conditions.

Key Words: Coenzyme-Q₁₀, Glutathione, neuroprotective

RÉSUMÉ

Les études sur les antioxydants comme agents neuroprotecteurs ont été handicapées par l'imperméabilité de la barrière de sang du cerveau (BBB) à plusieurs produits. Par ailleurs, les études antérieures ont montré qu'un groupe de flavonoïdes du thé (*Camellia sinensis*), les catéchines, permettent la perméabilité du cerveau et protègent les nerfs. Malgré cette observation importante, il n'ya pas de données sur la biodisponibilité et les vertus pharmacologiques des anthocyanines du thé (ACNs) dans le tissu du cerveau. Cette étude a évalué l'aptitude des anthocyanines du thé pourpre du Kenya de traverser le BBB et améliorer la capacité antioxydante du cerveau. Des rats étaient oralement administrés des anthocyanines du thé pourpre purifiés et caractérisés ou une combinaison des ACNs avec le coenzyme-Q₁₀, à une dose de 200 mg kg⁻¹ de poids vif dans un essai d'une durée de 15 jours. Vingt quatre heures après le dernier dosage d'antioxydants, le CO₂ était utilisé pour euthanasier les rats. Ensuite, le cerveau était excisé et soumis à diverses analyses biochimiques. Les ACNs ont augmenté significativement (P<0.05) les niveaux de Glutathione (GSH) du cerveau, ce qui implique une amélioration de la capacité antioxydante du cerveau. Remarquablement, les métabolites ACN étaient détectés dans le tissu des rats nourris aux ACNs. Ceci est la première démonstration que les anthocyanines (ACNs) du thé pourpre Kenyan peuvent franchir la barrière hémato-encéphalique (BHE), renforçant ainsi la capacité antioxydante du cerveau.

Ainsi, le besoin s'impose de les étudier comme des candidats appropriés pour les aliments de supplément qui pourraient renforcer la capacité antioxydante dans le cerveau et avoir le potentiel d'offrir la neuroprotection dans les conditions neurodégénératives.

Mots Clés: Coenzyme-Q₁₀, Glutathione, neuroprotecteur

INTRODUCTION

Tea (*Camellia sinensis*), an evergreen plant native to China, is one of the most commonly consumed beverages in the world; processed from the young tender leaves of the plant (Cabrera *et al.*, 2003). The chemical composition of tea is complex but includes polyphenols, amino acids, carbohydrates, proteins, chlorophyll, volatile compounds, minerals, trace elements and alkaloids such as caffeine, theophylline and theobromine. Among these, polyphenols constitute the main bioactive molecules in tea (Cabrera *et al.*, 2003).

For centuries, the Chinese have used tea beverage to treat a myriad of diseases (Sharangi, 2009). This pharmacological value of tea heavily relies on its antioxidative properties known to surpass that of major antioxidants such as vitamins C and E (Rice-Evans *et al.*, 1995) and other synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxytoluene (BHT) (Chen and Wan, 1994). The ability of tea to scavenge for free radicals is associated with the possession of a phenolic hydroxyl group attached to the flavan-3-ol structure of these compounds (Amie *et al.*, 2003). However, the importance of polyphenols in enhancing resistance to oxidative stress goes beyond simple radical scavenging activity. It is also due to amplified activity of most detoxifying enzymes such as glutathione peroxidase (GPx) and glutathione reductase (GR) (Mandel *et al.*, 2006). Indeed, as a result of their free radical quenching strengths, tea polyphenols have widely been credited with therapeutic action against free radical mediated diseases (Amie *et al.*, 2003).

Over the last few years, numerous epidemiological and clinical investigations have aroused increased interests in the use of tea polyphenols as neuroprotective agents. This is because previous studies have shown

phytochemicals present in tea such as epigallocatechin gallate (EGCG) and epicatechin (EC) metabolites formed after oral ingestion of EC by rats, can cross the blood brain barrier protecting nerve cells from reactive oxygen species (ROS)-induced cell death (Mandel *et al.*, 2006). Other neuroprotective properties associated with tea polyphenols include preventing loss of dopaminergic neurons and preservation of striatal levels of dopamine, decreased expression of neuronal nitric oxide synthase (nNOS), inhibition of pro-apoptotic genes and protection against beta-amyloid induced neurotoxicity (Zaveri, 2006). This strongly suggests that tea polyphenols have potential application in the treatment of neurodegenerative disorders such as Alzheimer's and Parkinson's disease.

However, compared to other flavonoid groups from tea such as the catechins, little is known about the bioavailability and pharmacological benefits of tea anthocyanins (ACN). This is, despite the fact that anthocyanins from other sources have been associated with a broad spectrum of health benefits including cardiovascular, neurological, urinary tract and ocular protection, as well as anti-carcinogenic, anti-diabetic, anti-aging, antioxidant and anti-inflammatory properties (Bagchi *et al.*, 2004).

It has been widely established that isolated individual antioxidants do not explain the observed health benefits of diets, implying that interactions between antioxidants may yield positive synergistic effects (Chu *et al.*, 2002). Indeed, synergistic effects of various ACNs have been demonstrated in black-current and wine grapes (Hosseini-Beheshti *et al.*, 2012). Therefore, the health benefits of a diet rich in phytochemicals is attributed to the complex mixture of phytochemicals present in it, an observation which clearly suggests that to improve their nutrition and health, consumers should take antioxidants from diverse sources. However, only

a very limited number of studies have investigated combinations of purified ACN extracts with other chemical components of food.

Intracellular synthesis that occurs in the inner mitochondrial membrane via the mevalonate pathway, is the major source of Co-Q₁₀, although small amounts can be obtained from the diet (Mancuso *et al.*, 2010). Co-Q₁₀ is an essential cofactor involved in mitochondrial oxidative phosphorylation and when reduced, it is a powerful antioxidant that prevents oxidative damage by free radicals including oxidation of lipids within the mitochondrial membrane (Matthews *et al.*, 1998). More importantly, Co-Q₁₀ crosses the BBB exerting a multitude of neuroprotective effects in the brain and protecting against pathophysiology associated with neurodegenerative disorders (Matthews *et al.*, 1998), hence the inclusion of this nutraceutical in this study.

This study aimed at evaluating the synergistic, additive or antagonistic types of interactions manifested by purified tea anthocyanin extracts and co-enzyme Q₁₀ (Co-Q₁₀). Co-Q₁₀, or ubiquinone, is an endogenously synthesized lipid, which shuttles electrons from complexes I and II to complex III (ubiquinol cytochrome c oxidase) of the electron transport chain.

MATERIALS AND METHODS

Tea samples. Purple tea used to extract ACNs was obtained from the Tea Research Foundation of Kenya, Timbilil Estate in Kericho (latitude 0°22'S, longitude 35°21'E, altitude 2180 m.a.s.l). ACNs were extracted from the purple tea variety TRFK 306. Young tender shoots comprising of two leaves plus a bud were harvested, dried using a microwave and pulverised with a grinder into fine powder.

Extraction, purification and lyophilisation of anthocyanins. Extraction and purification of tea ACNs were carried out as described elsewhere (Kerio *et al.*, 2012). Prior to the lyophilisation of ACNs extract, methanol and formic acid were removed using a rotary evaporator at 35°C under vacuum and the residue was reconstituted with distilled water. Pre-freezing of the extract was done before being placed on the drying accessory.

A 200 ml sample of the ACN extract was each placed in dehydration flasks and rapidly frozen by spinning the round bottom flasks in a dry ice-acetone bath. Temperature and pressure of the lyophiliser were allowed to reach appropriate levels of -40 °C and 100x10⁻³ M Bar, respectively before freeze drying process was initiated. Lyophilisation was done using a Modulyo freeze dryer (Edwards, England) producing a free flowing powder that was weighed and stored in airtight containers at room temperature until use.

HPLC analysis of anthocyanins. Qualitative and quantitative analyses of the tea extract and anthocyanin profiles of purple tea variety TRFK/306 were carried out in triplicates by high performance liquid chromatography (HPLC) as described elsewhere (Kerio *et al.*, 2012).

Experimental animals. All experimental protocols and procedures involving use of mice as experimental animals adhered to rules and regulations approved by Institutional Animal Care and Use Committee (IACUC) of the Trypanosomiasis Research Centre of Kenya Agricultural Research Institute (KARI-TRC) Muguga, Kenya and Egerton University as well as the National Regulations of the Kenya Veterinary Association. A total of 15, eight-week old female adult healthy Swiss white mice, weighing between 21-30 g, were obtained from the TRC breeding colony and used in all experiments. The animals were housed in standard mice cages at a temperature of 21-28 °C. They were provided with *ad libitum* access to water and standard mice cubes (Unga Feeds Ltd Kenya), with wood-chippings provided as bedding material. All mice were treated with 0.02 ml of Ivermectin (Ivermectin®, Anupco, Suffolk, England) injected subcutaneously to each mouse to eradicate endoparasites and ectoparasites infestation.

After two weeks of acclimatisation, the mice were randomly selected and divided into two groups of five animals each. Appropriate controls were used for this experiment. Group one was supplemented with Kenyan purple tea ACNs; while group 2 was supplemented with a combination of Kenyan purple tea ACNs and Co-Q₁₀. Note that we did not have a group on Co-Q₁₀.

alone because the antioxidant abilities of Co-Q₁₀ are well known and this study was focused on determining if Co-Q₁₀ will boost the beneficial effects of ACNs.

The test antioxidants were administered orally at a dosage of 200 mg kg⁻¹ body weight for fourteen days, after every second day using a gavage needle. Twenty four hours after the last dosage of antioxidants; carbon dioxide was used to euthanize the mice after which the brain was excised, snap frozen in dry ice and stored in liquid nitrogen until analysis.

Packed cell volume (PCV) and body weight. At one week interval, blood was taken from each mouse by tail snip into 100 μ l microhaematocrit capillary tubes for PCV determination (Woo *et al.*, 1970). After blood collection, the capillary tubes were sealed with plasticin at one end and centrifuged in a haematocrit centrifuge (Hawksley H England) at 10,000 revolutions per minute (RPM) for 5 minutes. PCV was then read using a micro-haematocrit reader and expressed as a percentage of the total blood volume. Body weight of each mouse was determined every two days using the analytical electronic balance (Mettler PM34, DeltaRange®).

Brain sample preparation. Snap-frozen whole brains were homogenised on ice water (4 °C) in 0.5ml of 0.25 M sucrose, 5 mM Hepes-Tris, pH 7.4, with protease inhibitor cocktail to a final concentration of 10% (w/v). The homogenates were aliquoted into 1.5 ml microfuge tubes to avoid repeated freeze-thaw process and stored in liquid nitrogen until analysis

Glutathione assay. Glutathione assay was performed as described in a previous experiment (Rahman *et al.*, 2007), with slight modifications. A volume of 50 μ l of brain homogenates were mixed with 50 μ l solution containing sulphosalicylic acid (5% w/v) and 0.25 mM ethylene diamine tetra-acetic acid (EDTA) and the mixture centrifuged at 8000xg for 10 minutes at 4 °C. A volume of 200 μ mol l⁻¹ of GSH standard solution was prepared in 0.5% sulphosalicylic acid (SSA) and serial dilutions made using the same solution (0.5% SSA) to final concentrations of 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 μ mol l⁻¹.

Ellman's reagent (5,5'-Dithiobis (2-nitrobenzoic acid (DTNB)) was prepared by dissolving in 0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5 (KPE buffer) to a final concentration of 0.6 mg ml⁻¹. A volume of 25 μ l of each standard were loaded on a 96-well microtitre plate to wells B–H in column 1, 2 and 3 followed by 25 μ l of the sample to the remaining wells in triplicate. To each well, 100 μ l of freshly prepared DTNB was then added and the absorbance measured at 405nm at intervals of 30 seconds using a multi-detection microtitre plate reader (Bio-Tek Synergy HT).

HPLC for detection of anthocyanins. A volume of 600 μ l methanol/formic acid (99/1) was mixed with 600 μ l brain homogenate in a 1.5 ml microfuge tube and the mixture centrifuged at a speed of 5000 g for 10 minutes. One millilitre of the supernatant was then pipetted into a separate tube and HPLC analysis of the samples carried out as described in the earlier section, "High-performance liquid chromatography analysis of anthocyanins".

Data analysis. Data were analysed using Prism Graph pad version 5.0 and at a P<0.05. Significance of difference between means for PCV and glutathione was determined by one way ANOVA and Tukey post hoc test was performed to evaluate differences among group means.

RESULTS

Purple tea anthocyanin profile. Following lyophilisation of the anthocyanin extracts, a free flowing powder that was bright red in colour, with a characteristic smell of fresh berries was produced. ACN profiling of Kenyan purple tea revealed presence of anthocyanidins; cyanidin, peonidin, pelargonidin, delphinidin and malvidin (Fig. 1). The anthocyanidins profile revealed cyanidin as the most abundant (1755.60 μ g ml); while delphinidin was the least (122.85 μ g ml⁻¹) abundant (Table 1).

Retention time

Anthocyanin in brain tissue. ACN metabolites, vividly absent from animals not supplemented

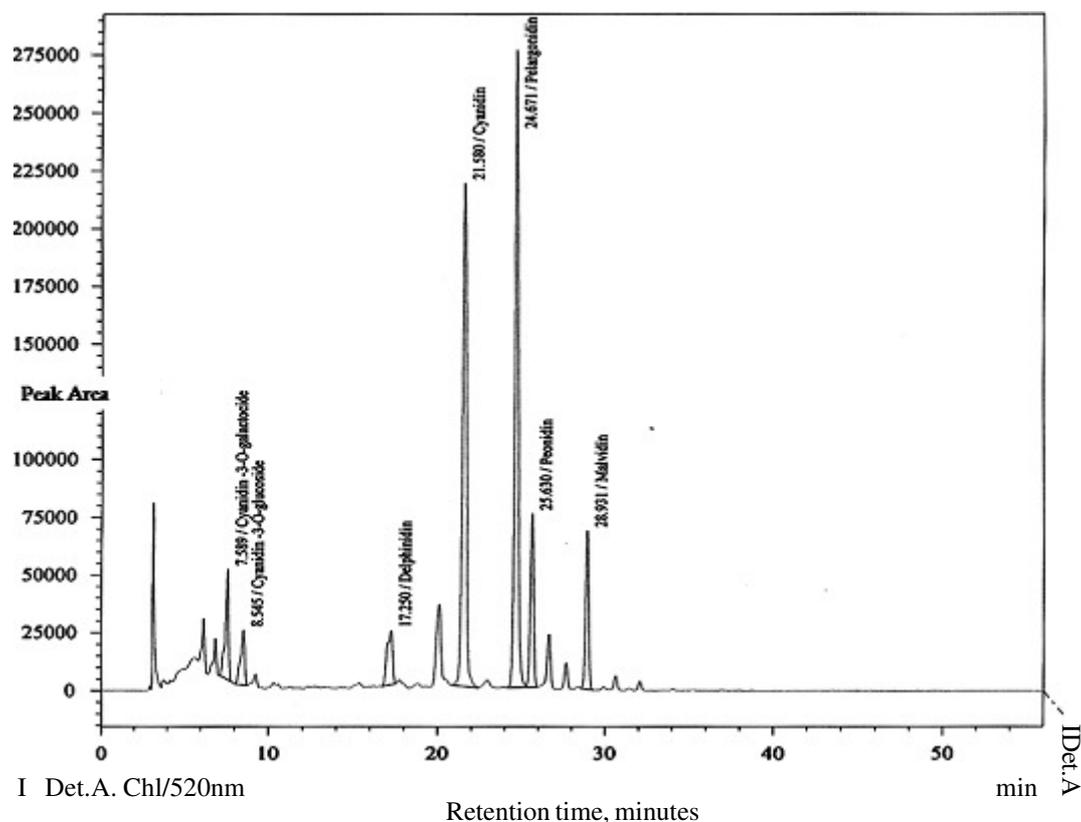


Figure 1. A representative HPLC chromatogram of un-aerated (green) tea from a Kenyan purple tea variety clone TRFK 306.

TABLE 1. Concentration of anthocyanins in non-aerated (green) tea derived from Kenyan purple leaf colored variety TRFK 306 by HPLC

Individual anthocyanins/ anthocyanidins	Concentrations ($\mu\text{g ml}^{-1}$)
Cyanidin-3-O-Galactoside	139.25
Cyanidin-3-O-Glucoside	50.26
Delphinidin	122.85
Cyanidin	1755.60
Pelargonidin	840.08
Peonidin	371.36
Malvidin	304.83
Total anthocyanin content	3584.23

with ACNs and having very close retention times to individual intact anthocyanins (Table 2), were detected in brain tissue of animals from the various anthocyanins groups (Fig. 2).

Tea anthocyanins and coenzyme- Q_{10} in mice

Clinical symptoms and survival. Animals supplemented with ACNs and Co- Q_{10} were marked with hyperactivity from the onset of Co- Q_{10} supplementation to the last day of the experiment. No clinical signs were detectable in the ACNs only groups, signifying that the tea polyphenols were well tolerated in the experimental animals. One animal supplemented with both test antioxidants died 9 days post start of antioxidant administration.

Packed cell volume and body weight. PCV levels and body weight baseline data are presented in Table 3. There was a gradual increase in PCV levels of the untreated animals throughout the experiment rising from 55.2 ± 0.49 to $58.4 \pm 2.50\%$. Experimental animals supplemented with ACNs only and ACNs and Co- Q_{10} had a rather steady

TABLE 2. Retention times in minutes of the detected metabolites against intact individual anthocyanins

Chromatogram B- Retention times in minutes of the detected metabolites	Possible metabolites of anthocyanin
23.321	Cyanidin-21.580
25.227	Pelargonidin-24.671
27.114	Peonidin-25.630
	Malvidin-28.931
Chromatogram C- Retention times in minutes of the detected metabolites	Possible metabolites of anthocyanin
15.334	Delphinidin-17.250
21.272	Cyanidin-21.580
28.287	Malvidin-28.931

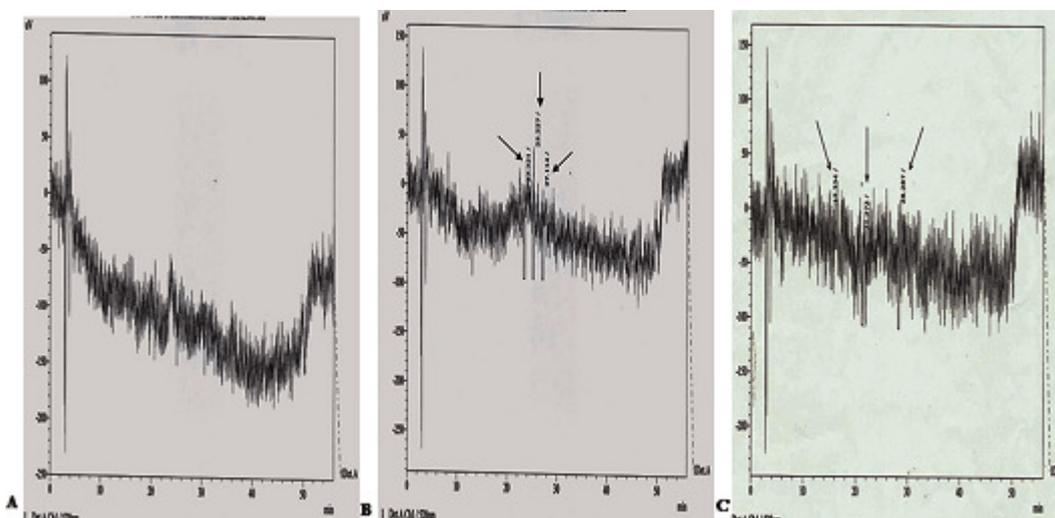


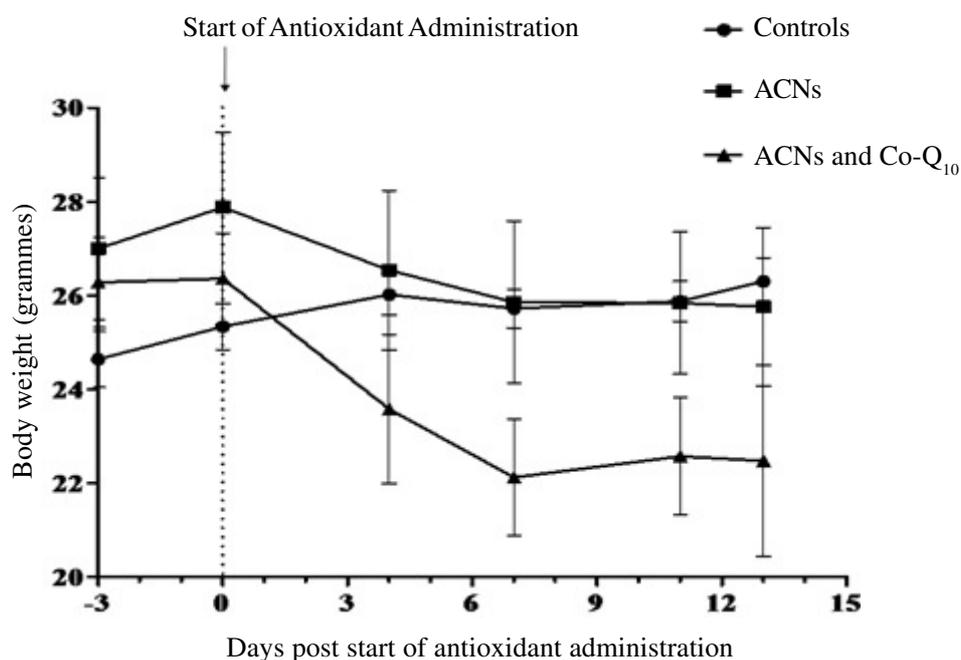
Figure 2. Representative HPLC chromatogram of brain homogenates from animal not supplemented with antioxidants (A), animal supplemented with ACN's and Co-Q₁₀ (B) and animal supplemented with ACN's only (C). Chromatograms B and C show presence of possible ACN's metabolites in the brain tissue indicated by arrows.

increase in PCV, rising from 53.4 ± 1.03 and $53.4 \pm 1.36\%$, three days prior to antioxidant administration to 58.6 ± 1.08 and $59.25 \pm 2.03\%$ on the sixth day after the start of antioxidant administration, respectively. This was followed by a steady decrease in PCV levels to the last day of the experiment, reaching 53.2 ± 1.93 and $55 \pm 0.71\%$ in ACN's only and ACN's and Co-Q₁₀ groups, respectively. However, these fluctuations did not portray a statistical significant difference ($P > 0.05$) between the different groups analysed. Mice receiving both antioxidants showed a significant decrease in mean body weight changes during the experimental period ($P < 0.05$)

(Fig. 3). The decrease in weight commenced immediately after the start of antioxidant administration, falling from 26.76 ± 0.77 to 23.30 ± 1.67 g by the seventh day post start of antioxidant administration (DPSAA) after which a marginal increase was observed reaching 23.45 ± 1.82 g. Animals supplemented with ACN's lost weight consistently from the first DPSAA to the last day of the experiment, dropping from 27.88 ± 1.60 to 25.76 ± 1.69 g. Untreated animals registered an unsteady, but gradual increase in mean body weight rising from 24.64 ± 0.59 to 26.3 ± 0.50 g by the end of the experimental period. However, no significant differences in mean body

TABLE 3. Changes in PCV levels of mice supplemented with ACNs, ACNs and Co-Q₁₀ or water only

Days post start of antioxidant administration	PCV levels in %		
	Controls	ACNs	ACNs and Co-Q ₁₀
-3	55.2±0.49	53.4±1.03	53.40±1.36
6	57.4±0.51	58.6±1.08	59.25±2.03
12	58.4±2.50	53.2±1.93	55.00±0.71

Figure 3. Changes in body weight of mice supplemented with ACNs, ACNs and Co-Q₁₀ or water only.

weight were recorded between untreated animals and animals supplemented with ACNs ($P>0.05$).

Anthocyanins and Co-Q₁₀ versus glutathione levels. The level of GSH in brain tissue of mice supplemented with either one or both antioxidant supplements is presented in Figure 4. Supplementing experimental animals with Kenyan purple tea ACNs significantly boosted the levels of endogenous total GSH levels in the brain tissue ($2.22\pm 0.18 \mu\text{M}$) compared with the controls ($1.27\pm 0.12 \mu\text{M}$) ($P=0.0006$). However, when both test antioxidants were provided, there was a decline in total GSH levels to amounts below even the level recorded in healthy animals not supplemented with antioxidants ($0.97\pm 0.06 \mu\text{M}$).

DISCUSSION

Polyphenols and other phytochemicals in plants must cross the BBB to be able to exert their beneficial effects in the central nervous system (CNS). Otherwise, the multitude of health benefits associated with these flavonoids, such as strong antioxidant and anti-inflammatory properties, would be excluded from the brain and the CNS in general. Our research clearly demonstrates that ACNs from the Kenyan purple tea cross the BBB and exert physiological effects by boosting antioxidant capacity in the brain. The presence of ACNs in this pivotal organ was confirmed by the detection of ACN metabolites in the ACN fed animals. These metabolites were absent in

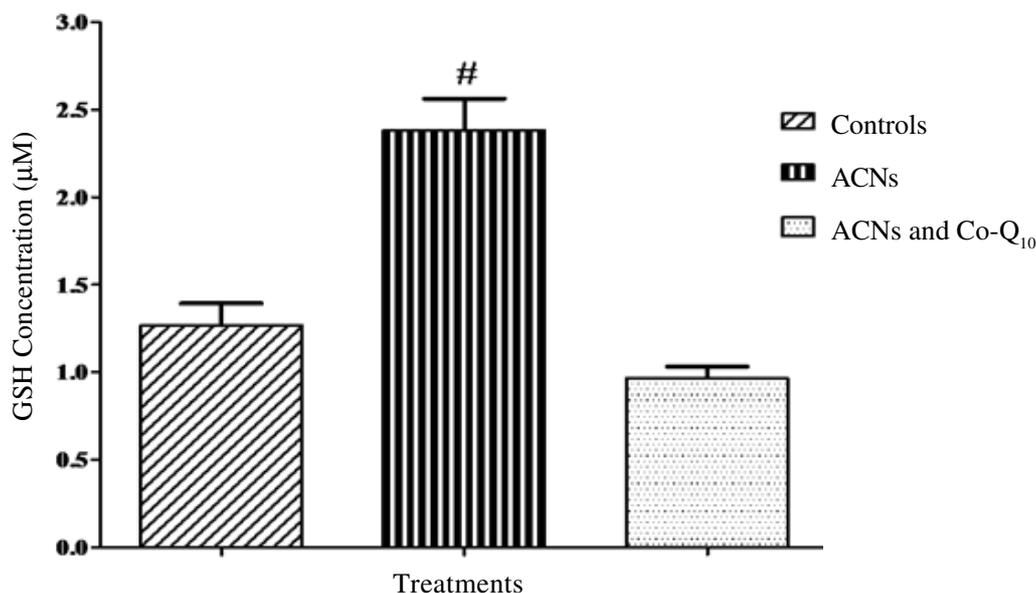


Figure 4. Total brain GSH levels in mice supplemented with ACNs, ACNs and Co-Q₁₀ or water only. [#]P<0.05, statistically significant versus untreated group.

the placebo group that was not fed the purple tea. However, we were not able to detect intact ACNs from the brain homogenates of the ACN fed mice. Considering that approximately two-thirds of ACNs are highly biotransformed and end up as methylated and glucuronidated metabolites (Kay, 2006), absence of such intact ACN's in the brain homogenates of our ACN fed mice was not surprising.

Our results also indicate that oral intake of tea ACNs markedly increases brain GSH levels. Indeed, ACNs from a wide array of sources have been shown in several instances to up-regulate endogenous antioxidant levels. A previous study working with the same variety of purple tea reported an increase in cellular GSH content in cells exposed to ACNs (Kerio *et al.*, 2011). Red mixed berry juice rich in ACNs has also been shown to decrease oxidative DNA damage, increase GSH levels and glutathione status in healthy human volunteers (Weisel *et al.*, 2006). The mechanisms by which ACNs are thought to exert these effects include quenching of ROS, chelating of metals known to participate in reactions that result in the vicious formation of reactive moieties and by the activation of antioxidant response element (ARE) upstream of

genes that are involved in antioxidation and detoxification (Shih *et al.*, 2007).

Our results on PCV, body weight and brain glutathione strongly suggest negative interactions between Kenyan purple tea ACNs and Co-Q₁₀. An antagonistic effect between the two antioxidants was unexpected as it was thought that the nutraceuticals would have a more pronounced synergistic effect in combination rather than in isolation. However, ACNs and other substances known to lower cholesterol levels or prevent its absorption are expected to have adverse effects on non sterol compounds such as Co-Q₁₀ (Bliznakov *et al.*, 1998). The negative interactions have also been observed while employing other lipid lowering substances such as HMG-CoA competitive inhibitors known as statins (Bliznakov *et al.*, 1998). Indeed, several authors have reported the ability of Co-Q₁₀ to reverse the detrimental side effects associated with statins including muscle myopathy and rhabdomyolysis (Langsjoen and Langsjoen, 2003). Further studies will be necessary to determine whether indeed purple tea ACNs could nullify the beneficial effects of Co-Q₁₀ supplements or vice versa.

CONCLUSION

Data from our study provide compelling evidence that Kenyan purple tea ACNs are able to cross the BBB and exert their physiological effects in this organ by up-regulating endogenous antioxidant reserves. We, therefore, recommend the study of this nutraceutical as a suitable candidate for consideration as dietary supplements to modulate conditions associated with oxidative stress in the brain such as Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis (ALS) and multiple sclerosis.

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