KOMBUCHA PROTECTS AGAINST ARSENIC-INDUCED PROTEIN PEROXIDATION IN RATS

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ABSTRACT
Kombucha, prepared by fermenting sugared-tea with symbiotic culture of acetic bacteria and yeasts belonging to the genera Saccharomycae is claimed to have numerous medicinal benefits some of which could partly be attributed to its antioxidant properties. However, the chemical composition of Kombucha is reported to vary with geographical location, suggesting geographical differences in its biological activities. The present study investigated the antioxidant activity of Kombucha produced locally by fermenting sugared–black tea for 2, 4, 8 and 12 weeks. In vitro antioxidant activity was assessed using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay and the ability to protect against arsenic-induced lipid and protein peroxidation was used to ascertain in vivo antioxidant activity. The Kombucha extracts exhibited good antioxidant activity compared to the standards both in vitro and in vivo.

INTRODUCTION
Kombucha, also known as tea fungus has been used both as beverage and a medicinal product (Jayabalan *et al.,* 2014) for several centuries. The tea, famously known as the "Godly Tsche" during the Chinese Qin Dynasty (221-206 BC), was referred to as a "beverage with magical powers enabling people to live forever" (Jayabalan *et al.,* 2014). It is prepared by fermenting sugared-tea (mainly black tea) with a symbiotic culture of acetic acid bacteria (*Acetobacter xylinum*, *Acetobacter xylinoides, Acetobacter aceti, Acetobacter pasteurianus)* and yeasts identified as *Schizosaccharomyces pombe, Saccharomycodes ludwigii, Kloeckera apiculata, Saccharomyces cerevisiae*, *Zygosaccharomyces bailii, Brettanomyces bruxellensis, Brettanomyces lambicus, Brettanomyces custersii* (Balentine, 1997; Jayabalan *et al.,* 2014; Liu *et al.,* 1996; Mayser *et al.,* 1995). However, the microbial composition tends to vary with geographical location (Teoh et *al.,* 2004).

Kombucha is composed of two portions, a floating cellulosic pellicle layer and a sour liquid broth (Fig. 1), which has a slightly sweet and sour taste similar to fermented apple cider (Chen and Liu, 2000; Dufresne and Farnworth, 2001; Jayabalan *et al.,* 2014). The name, Kombucha is reported to be associated with Doctor Kombu, who was said to have brought the tea from Korea to Japan in 414 A.D. to cure digestive problems of the Japanese Emperor (Dufresne and Farnworth, 2000). The tea was later introduced into European countries from China by the Portuguese and Dutch explorers as a medicinal herb (Dufresne and Farnworth, 2000). It then spread

throughout the Far East, Pacific, India, Russia, and Germany and eventually to the rest of Europe, to Africa and more recently across the entire globe as remedy for many ailments. In Ghana, the consumption of Kombucha became popular in the early 1980s (unpublished, oral communication). However, few years after its introduction, it was claimed to have received lots of criticism mainly from health practitioners for being toxic and causing adverse health effects in people. Consequently, the tea has since been used by few people in their closet. However, there seems to be a significant increase in the patronage of Kombucha across the country in recent times resulting in the selling of Kombucha capsules in the open market, health shops and drug stores.

Medicinally, Kombucha is reported to improve resistance against cancer, prevent cardiovascular diseases, promote digestion, stimulate immunity and reduce inflammation (Dufresne and Farnworth, 2000; Jayabalan *et al.,* 2014). These medicinal benefits of Kombucha could partly be due to its antioxidant properties since oral administration of Kombucha to rats exposed to pro-oxidation species caused a decrease in degree of lipid oxidation and DNA fragmentation (Dipti *et al.,* 2003; Sai Ram *et al.,* 2000). However, the chemical composition of Kombucha is reported to vary with geographical location (Teoh *et al.,* 2004), suggesting geographical differences in its biological activities (Jayabalan *et al.,* 2010). The present studies therefore investigated the antioxidant properties of a locally prepared Kombucha.

MATERIALS AND METHODS

Preparation of Kombucha tea extracts

The Kombucha was prepared by dissolving 60 g of sucrose in 3 L of distilled water. This was then boiled and while still hot, was infused with three bags of Lipton (2g/bag) for three minutes. The sugared-tea was then transferred into 5 L bottles that had been previously sterilized at 121°C for 20 min and allowed to cool to room temperature. A mat of Kombucha from an already maturing culture was transferred unto the cooled sugared-tea and the container was covered with clean cheese cloth and fixed with rubber bands (Fig. 1). The set up was left to grow and ferment at room temperature (27° to 29°C) over periods of 0, 2, 4, 8 and 12 weeks. The extracts obtained were denoted unfermented tea, K-2, K-4, K-8 and K-12 respectively. At the end of each culture period, the extracts were filtered and freeze-dried using Martin Christ freeze-drying machine (Christ Gamma 1-16/2-16 LSC, Germany). The freeze-dried samples were weighed and immediately reconstituted into sterile distilled water, filtered under sterile conditions and stored at 4°C for further use.

Determination of in vitro antioxidant activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay was employed as previously described (Tettey *et al.,* 2014) with slight modification. To 1 ml of 0.5 g/ml methanolic DPPH was added 100 µl of 0.5 g/ml of each extract or the standard butylated hydroxytolouene (BHT). The reaction mixtures were incubated for 30 min in the dark and the reduction of DPPH free radicals was measured by reading the absorbance at 517 nm against a blank (80% methanol). The unfermented tea served as a control for the Kombucha extracts whilst the methanolic DPPH served as the control for the standard (BHT). The percentage DPPH scavenging activity was calculated as:

$$\{1 - (A_{sample} / A_{control})\} \times 100$$

where A_{sample} is the absorbance of the sample and $A_{control}$ is the absorbance of the control.

Determination of in vivo antioxidant activity

Based on the results of the DPPH assay (*in vitro* study), the antioxidant activity was assessed *in vivo* in arsenic-induced Sprague Dawley rats using the 2 and the 12 weeks old Kombucha extracts. Twenty-five male rats with average weight of 103 g were purchased from and housed in good ventilation and provided with standard feed and water *ad libitum* at the Animal Experimentation Department of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon. They were divided into 5 groups of 5 rats each. All

procedures in the present study conform to international animal care guidelines and the ethics committee of NMIMR and ethical approval was obtained from the Internal Review Board of NMIMR.

All the animals except those in group I, which served as negative control (control) were administered a single dose of 4 mg per kg body weight of sodium arsenate intraperitoneally (Haider and Najar, 2008). Forty-eight hours after induction, animals in Group II, which served as oxidative stress group (Ox-St) were given water. The treatment groups which comprised animals in Groups III (K-2) and IV (K-12) were respectively given 2 and 12 weeks old Kombucha whiles Group V animals were administered 200 mg per kg body weight ascorbic acid (Asc) by oral gavage for 10 days.

Mitochondria for the evaluation of the extent of lipid and protein peroxidation in the treatment and control groups were isolated from the liver of the animals by differential centrifugation using the refrigerated Beckman Model J2-21 centrifuge (Brand et al., 2003). The animals were sacrificed by cervical dislocation and the liver removed. Each liver was blotted dry, weighed and homogenised in ice-cold buffer A (250 mM sucrose, 20 mM Tris base, 1 mM EDTA, 20 mM $MgCl_2$ and 5 mM KCl, pH 7.4) to yield a 20% w/v liver homogenate using Dounce glass homogenisers with 12 passes. The homogenates were centrifuged at 787 rpm for 10 min to remove cell debris. The resulting supernatants were further centrifuged at 984 rpm for 10 min to remove residual cell debris. Mitochondria were then precipitated by centrifuging the resulting supernatants at 8,560 rpm for 10 min. The pellets were washed by re-suspension once in 10 ml ice-cold buffer A and then twice in 10 ml ice-cold buffer B (100 mM sucrose, 20mM Tris base and 1mM EDTA, pH 7.4) and pelleted at 8,560 rpm for 10 min. The final mitochondria pellets were re-suspended in 1 ml ice-cold buffer B, aliquoted into 1.5 ml Eppendorf tubes. All the centrifugations were carried out at 4°C. Mitochondrial protein concentrations were determined immediately after mitochondria isolation using UV/Vis NanoDrop 2000C spectrophotometer at a wavelength of 280 nm with bovine serum albumin (BSA) as protein standard (Desjardins *et al.,* 2009) and the samples stored at –20°C for further use.

Lipid peroxidation was determined by measuring levels of malondialdehyde (MDA) using the thiobarbituric acid (TBA) method (Draper and Hadley, 1990). A reaction mixture was prepared by adding 1.5 ml of 5% aqueous trichloroacetic acid (TCA) and 0.5 ml of methanolic BHT (0.5 g/L) to 1 ml of each of the thawed mitochondria sample and heated in a capped tube for 30 min in a boiling water bath to release protein-bound MDA. To avoid adsorption of TBA-MDA complex into insoluble protein, any solid particulate material observed after cooling to room temperature was removed by centrifugation at 984 rpm for 10 min (Centurion Model K240 microfuge). An equal volume of saturated solution of TBA was added to 1 ml of the supernatant and heated in boiling water for additional 30 min. The amount of MDA produced was measured by reading the absorbance at 532 nm against a reagent blank containing 1 ml of TBA and 0.5 ml of methanolic BHT. A reference blank for each sample was prepared just as the reaction mixture above, except that the TBA was replaced with an equal volume of 5% TCA. The concentration of MDA was calculated as:

$$MDA_{Abs} = Reaction_{Abs} - Reference_{Abs}$$

$$\text{Concentration of MDA} = MDA_{Abs} / \varepsilon$$

where

MDA_{Abs} = Actual Absorbance of MDA;
$Reaction_{Abs}$ = Absorbance of reaction mixture;
$Reference_{Abs}$ = Absorbance of reference blank;
ε = Molar extinction coefficient of MDA
($14.9 \times 10^4\ M^{-1}\ cm^{-1}$)

Protein peroxidation was determined by measuring the amount of protein carbonyls (Levine *et al.,* 1990). Protein carbonyls were precipitated by adding three drops of 20% w/v TCA to 1 ml of each of the isolated mitochondria in a 1.5 ml centrifuge tubes. These were reacted with 0.5 ml

of 10 mM of 2, 4-dinitrophenyl-hydrazine (DNPH) in 2 M HCl and the mixture allowed to stand at room temperature for 1 h, with vortexing every 15 min. The mixture was centrifuged at 10,823 rpm (Centurion Model K240 microfuge) for 3 min and the supernatant discarded. The pellets were washed 3 times with 1 ml ethanol-ethyl acetate mixture (1:1) to remove free reagent, allowing the sample to stand for 10 min each time. The pellets were redissolved in 0.6 ml of 6.0 M guanidine solution, placed in a water bath at 37°C for 15 min, and centrifuged at 10,823 rpm for 3 min to remove any insoluble material. The absorbance was read at 375 nm. The protein carbonyl concentration was then estimated as:

Concentration of protein carbonyl = Absorbance/ε

Where ε

= Molar extinction coefficient of protein carbonyl ($2.2 \times 10^4\ M^{-1}\ cm^{-1}$)

Statistical analysis
Each experiment was conducted at least three times and the data reported as Mean ± S. E. M. Comparisons between means were performed and significance was evaluated by one-factor analysis of variance (ANOVA) using Microsoft Excel. Probability value of $p < 0.05$ was used as the criteria for significant differences.

RESULTS

In vitro antioxidant activity
As illustrated in Table 1, all the Kombucha extracts showed strong DPPH scanvenging activities, which were comparable to the standard butylated hydroxytoluene (BHT). The DPPH scanvenging activity of the extracts were independent of the period of fermentation (Table 1).

In vivo antioxidant activity
To ascertain the *in vivo* antioxidant effect of the Kombucha, the ability of the 2 and 12 weeks old extracts to protect against arsenic-induced oxidative stress were assessed in male Sprague Dawley rats. Figures 2 and 3 respectively show the results of the protective effect of the Kombucha extracts against arsenic-induced lipid and protein peroxidations compared to the standard ascorbic acid (Vit. C). As shown in figure 2, the administration of a single dose of 4 mg per kg body

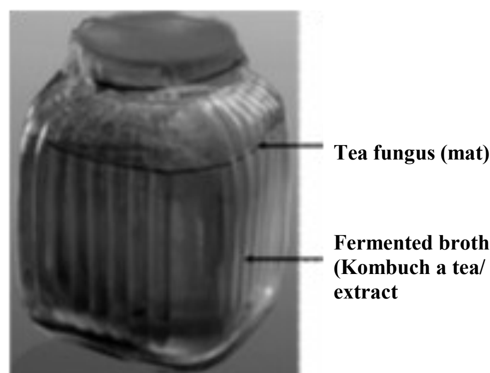

Figure 1: **A set up of Kombucha fermented from sugared-black tea**

Table 1: DPPH scavenging activity

Kombucha tea/Standard	DPPH scavenging activity (%)
BHT	64.0 ± 4.0
K-2	70.0 ± 10.0
K-4	65.0 ± 15.0
K-8	70.0 ± 10.0
K-12	68.0 ± 10.0

Values are Means ± SEM of 3 independent assays. K-2, K-4, K-8 and K-12 represent Kombucha prepared by fermentation for 2, 4, 8 and 12 weeks. There are no significant difference between treatment groups

weight of sodium arsenate intraperitoneally caused significant increase in the level of mitochondrial protein peroxidation. That is, the levels of protein carbonyls were significantly (30%) higher in the oxidatively stressed group (Ox-St) compared to the non-oxidatively stressed group (control). However, the administration of the Kombucha extracts produced by 2 and 12 weeks fermentation and the ascorbic acid significantly reduced this arsenic-induced protein peroxidation. That is, the levels of protein carbonyls were significantly lower in the groups treated with the 2 weeks old Kombucha (K-2), 12 weeks old Kombucha (K-12) and the ascorbic acid (Vit. C) than the Ox-St group and were comparable to the control (Fig. 2).

On the other hand, in the case of lipid peroxidation, the sodium arsenate only slightly increased lipid peroxidation. The levels of MDA was slightly higher in the oxidatively stressed group (Ox-St) compared to the non-oxidatively stressed control (control) (Fig. 3). Whilst the K-12 weeks and the ascorbic acid reduced the levels of MDA to basal non-oxidative stress levels, the K-2 however, significantly decreased MDA levels below (40%) the basal level (Fig. 3).

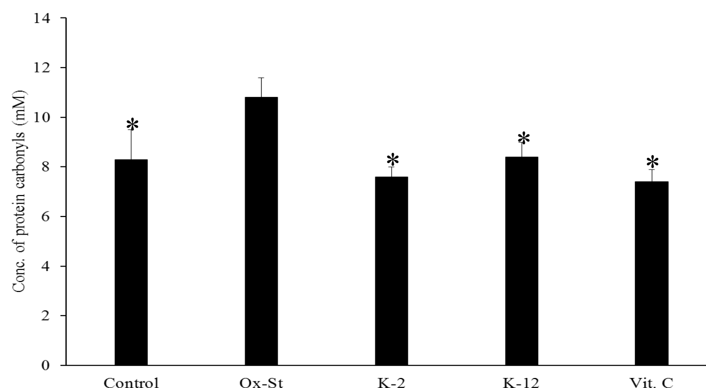

Figure 2: The protective effect of *Kombucha* against arsenic-induced protein peroxidation in Sprague Dawley rat liver mitochondria. The error bars represent Standard Error of the Means (SEM) of n = 5. *Significant compared to the oxidatively stressed (Ox-St) group ($p < 0.5$). K-2 and K-12 represent Kombucha prepared by 2 and 12 weeks fermentation.

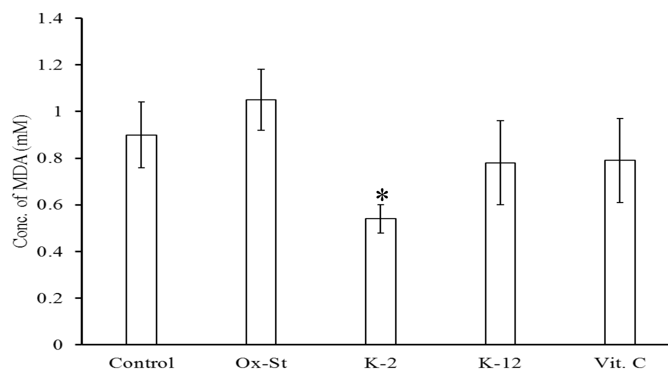

Figure 3: Effect of Kombucha against arsenic-induced lipid peroxidation in Sprague Dawley rats liver mitocondria. The error bars represent Standard Error of the Means (SEM) of n = 5. *Significant compared to the others ($p< 0.5$). K-2 and K-12 represent Kombucha prepared by 2 and 12 weeks fermentation.

DISCUSSION

The present studies investigated the antioxidant property of a locally prepared Kombucha. Free radicals, which are either reactive oxygen species (ROS) or nitrogen species (RNS) damage macromolecules such as proteins, DNA and lipids leading to overall damage to cells and eventually cell death (Halliwell and Gutteridge, 1985). The damage to cells caused by free radicals is believed to underlie the development of more than hundred diseases and disorders including cancer, diabetes, aging and many neurodegenerative diseases (Ames *et al.,* 1993; Goh *et al.,* 2012). Exogenous antioxidants from fruits, herbs, vegetables and medicinal plants or foods supplement the body's endogenous antioxidant defence mechanism and protect the body against the dilapidating effect of free radicals (Kunwar and Priyadarsini, 2011).

The Kombucha extracts were first screened for antioxidant activity using DPPH assay. The DPPH assay is widely used for screening antioxidant activities of natural products because of its sensitivity in detecting active compounds at micromolar concentrations (Kratchanova *et al.,* 2010). Being a nitrogen-centred free radical, any compound that can scavenge a significant amount of DPPH may be useful in reducing the levels of reactive nitrogen species in living cells. In the present study, all the Kombucha extracts showed strong DPPH scavenging activities, which were comparable to the standard butylated hydroxytoluene (BHT) and independent of the period of fermentation (Table 1). Although tea polyphenols are known to have antioxidant activity (Hakim *et al.,* 2003), the antioxidant activity of the Kombucha in the present study is not solely due to the constituent polyphenols. This is because the percentage antioxidant activity of the Kombucha was estimated from the difference between the activity of the Kombucha and the unfermented tea (control) (see methodology). This indicates that the Kombucha tea has higher antioxidant property than the unfermented tea, thus suggesting that the fermentation process increases the antioxidant capacity. This is consistent with findings from Jayabalan and others (2008), who reported that the fermentation of sugared tea increases the DPPH radical scavenging activity. In contrast however, we did not find that DPPH scavenging activity varied with the fermentation duration over a 12 week period. This may be due to differences in the symbiotic composition as compared to the study by Jayabalan and others (2008) and dynamic changes of the microbial colonies in the fermentation culture.

"Apart from the inherent production of ROS by the mitochondria (Murphy, 2009), other substances such as pesticides, heavy metals and metalloids cause oxidative stress through the induction of ROS production or inhibition of the endogenous antioxidant defence system (Dinis-Oliveira *et al.,* 2008; Shi *et al.,* 2004; Valko*et al.,* 2005)."

Arsenic, a metalloid causes oxidative stress by inhibiting the production of glutathione, a potent endogenous antioxidant defence system (Miller *et al.,* 2002). Arsenic compounds are often found in food, water and air coming from sources such as mine wastes, mineral debris, wood preservatives, insecticides and some agrochemicals (Haider and Najar, 2008) and pose serious health hazards (Jomova *et al.,* 2011).

In the present study, to ascertain the *in vivo* antioxidant effect of the Kombucha, the ability of the 2 and 12 weeks old extracts to protect against arsenic-induced oxidative stress were assessed in male Sprague Dawley rats. Figures 2 and 3 respectively show the results of the protective effect of the Kombucha extracts against arsenic-induced lipid and protein peroxidations compared to the standard ascorbic acid (Vit. C). The administration of the Kombucha extracts produced by 2 and 12 weeks fermentation and the ascorbic acid significantly reduced protein peroxidation. The levels of protein carbonyls were significantly lower in the groups treated with the 2 weeks old Kombucha (K-2), 12 weeks old Kombucha (K-12) and the ascorbic acid (Vit. C) than the Ox-St group and were comparable to the control (Fig. 2).

On the other hand, in the case of lipid peroxida-

tion, the levels of MDA was slightly higher in the oxidatively stressed group (Ox-St) compared to the non-oxidatively stressed control (control) (Fig. 3). Whilst the tea fermented for 12 weeks and the ascorbic acid reduced the levels of MDA to basal non-oxidative stress levels, the 2 weeks fermented Kombucha however, significantly decreased MDA levels below (40%) the basal level (Fig. 3).

The overall *in vivo* antioxidant activity observed in the present study agrees with previous findings, which showed that Kombucha reduces total blood free radicals in rats induced by trichloroethylene (Gharib, 2009). The observed difference in the extent of the arsenic-induced protein and lipid peroxidation could be due to the differences in reaction pathways involved in the synthesis of the MDA compared to the protein carbonyl adducts and is consistent with previous findings, which reported higher protein peroxidation than lipid peroxidation in mice underexpressing uncoupling protein 3 (Brand *et al.,* 2002).

CONCLUSION

In conclusion, the Kombucha tea produced by 2 weeks fermentation showed a relatively stronger antioxidant activity both *in vitro* and *in vivo* compared to the controls. These findings support the anecdotal evidence and other reports that the consumption of Kombucha helps in controlling many ailments since most ROS production are implicated in many diseases. It provides the scientific evidence that the locally produced Kombucha has antioxidant properties.

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