International Food Research Journal 24(2): 541-546 (April 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Kombucha for healthy living: evaluation of antioxidant potential and bioactive compounds

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Article history

Received: 24 January 2016
Received in revised form: 18 March 2016
Accepted: 26 April 2016

Keywords

Tea
Gallic acid
Catechins
HPLC
Quercetin
Bio-tea

Abstract

Tea is a beverage consumed by people of all walks of life. Kombucha is prepared by fermenting tea which causes alterations in the composition and concentration of the beneficial compounds. In order to know the changes that have occurred upon fermentation, both tea and Kombucha were subjected to various *in vitro* antioxidant analyses as well as HPLC analysis. The results of the *in vitro* antioxidant analyses indicate that tea scavenges free radicals mainly due to its hydrogen donating ability, while Kombucha acts mainly by electron donation, metal chelation and NO radial scavenging activities. The HPLC analysis of Kombucha showed an increase in concentrations of Epicatechin, Epigallocatechin gallate and Gallocatechin gallate as well as the presence of Gallocatechin which was absent in Black tea. All these results indicate that the beneficial effects of tea are enhanced upon fermentation to Kombucha, thus supporting the daily consumption of Kombucha.

Introduction

Tea is one of the most popular beverages and an important source of polyphenolic constituents. Polyphenols are present in all varieties of tea from *Camellia sinensis*; however, due to the process of fermentation in black tea, the types of polyphenols present are different. Many *in vitro* studies show that the polyphenols possess strong antioxidant and metal chelating properties and may therefore protect cells and tissue structures - lipids, proteins and DNA against reactive oxygen species (ROS) (Middleton *et al.*, 2000). Scientific study reveals that tea lowers the risk of cancer and coronary heart disease, improves oral health and also possesses antimicrobial health benefits and antioxidant properties (Ruxton, 2008).

Kombucha (Bio-tea) is a traditional fermentation of sweetened tea, carried out using a symbiosis of acetic acid bacteria and yeast species such as *Bacterium xylinum, Bacterium xylinoides, Bacterium gluconicum, Saccharomyces ludwigii, Saccharomyces apiculatus* varieties, *Schizosaccharomyces pombe, Acetobacter ketogenum, Torula varieties, Pichia fermantans* (Teoh *et al.*, 2004). During the fermentation process, bacteria and yeasts metabolize sucrose into a number of organic acids such as acetic acid and glucuronic acid, amino acids, antibiotics and a variety of micronutrients (Chu and Chen, 2006). Kombucha is said to improve resistance against cancer, prevent cardiovascular diseases, promote digestion, stimulate immunity and reduce inflammation (Dufresne and Farnworth, 2000). It also contains liver detoxifiers, antioxidants, polyphenols, probiotics and free-form amino acids (Jayabalan *et al.*, 2014).

The types of bioactive compounds as well as their concentrations vary depending on the solvent used for extraction. This is also observed during the preparation of tea (aqueous solvent), wherein, the components extracted and their concentrations differ significantly from studies undertaken using organic solvents. Also, Kombucha is prepared by fermenting tea, hence there is an ambiguity regarding its beneficial effects due to the presence of the tea proponents. Therefore, this study was designed to compare the *in vitro* antioxidant activities and analyze the constituents of aqueous decoction of tea before and after fermentation with Kombucha pellicle.

Materials and Methods

Chemicals

Linoleic acid, 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH), sodium nitroprusside, ascorbic acid were purchased from HiMedia laboratories Pvt. Ltd, Mumbai, India. 2-2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 6-hydroxy-2,5,7,8-

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tetramethylchromane-2-carboxylic acid (Trolox), 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma-Aldrich Inc., USA. All other routine chemicals were of AR grade.

Preparation of Kombucha

The tea decoction was prepared by adding 10% of commercial sucrose and 7.5g/L of tea leaves (Brooke Bond Red Label) to boiling water and allowed to simmer for 3 minutes. After cooling to 30ºC, the tea decoction was filtered into six clean glass bottles. Three were considered as black tea (triplicate) and to the rest a Kombucha pellicle each from previous culture was added. Incubation was carried out at room temperature under aerobic conditions for 7 days. After 7 days, the Kombucha obtained was filtered and sterilized before refrigeration.

Analysis of antioxidants

The total antioxidant capacity of tea and Kombucha was determined using the method of Prieto *et al.* (1999). Scavenging activity on DPPH was assessed according to the method reported by Blois (1958) against ascorbic acid standard. ABTS radical scavenging assay was carried out as per Re *et al.* (1999) using ammonium persulphate reagent. Modified Ferric ion reducing/antioxidant assay (FRAP) was carried out as described by Shanmugam *et al.* (2010). Nitric Oxide Radical (NO) scavenging assay was measured using the method of López-López *et al.*(2004) using Griess reagent. The ferrous ion chelation ability of the samples was measured using Decker and Welch (1990) method. The *in vitro* lipid peroxidation assay was carried out as per Liegeois *et al.* (2000) against Trolox standard.

Estimation of total polyphenols, total flavonoids and Gallic acid

Total polyphenol content in the various samples was analyzed using Folin-Ciocalteu reagent (Anesini *et al*., 2008). Colorimetric estimation of total flavonoids was carried out as per (Bukhari *et al*., 2008) against Quercetin standard. Concentration of Gallic acid was estimated by the method of Price and Butler (1977).

HPLC analysis

The tea and Kombucha samples were subjected to extraction using 70% methanol. 2 ml of the methanolic extract was purified through membrane filter of pore size 0.45µm into HPLC vials. 10 µl of this was injected into a Agilent 1200 HPLC ChemStation system equipped with Phenyl-bonded phase, Phenomex Luna 5µm Phenyl-Hexyl® column of dimensions 250mm x 4.6mm, fitted with Phenomex SecurityGuard® (4mm x 3mm Phenyl-Hexyl cartridge), used for its additional selectivity over reversed-phase materials. Separation of the various catechins, caffeine and its metabolites was carried out using a binary gradient system with respective mobile phases at a flow rate of 1ml/min. Mobile Phase A consisted of 9% v/v acetonitrile and 2% v/v acetic acid in water, while mobile phase B consisted of 80% v/v acetonitrile and 2% v/v acetic acid in water. All the chemicals were of HPLC grade. Detection was at 278nm using a UV detector and the column temperature was maintained at 35±0.5°C.

The individual component content w_C was calculated as follows,

$$w_C = \frac{(A_{sample} - A_{intercept}) \times F_{std} \times V_{sample} \times d \times 100}{S_{std} \times M_{sample} \times 10000 \times w_{DMsample}}$$

Where,

A_{sample} is the peak area of the individual component in the test sample

$A_{intercept}$ is the peak area at the point the standard calibration line intercepts the y-axis

S_{std} is the standard calibration line slope

F_{std} is the Relative Response Factor, measured with respect to caffeine for the individual component

V_{sample} is the sample extraction volume, in ml

d is the dilution factor

M_{sample} is the mass, in grams, of the sample

$w_{DMsample}$ is the dry matter content, expressed as a mass fraction in percent, of the test sample.

Results and Discussion

Tea is fermented to Kombucha by the action of the microorganisms present in the Kombucha pellicle, resulting in the alteration of the composition and concentration of the tea constituents. DPPH, FRAP, NO scavenging, ferrous ion chelation, ABTS radical scavenging and lipid peroxidation assays were carried out to study the various free radical scavenging abilities of the samples and are represented in Figure 1. Kombucha reduces ferric ions by donating electrons, leading to the neutralization of the free radicals (Yen and Chen, 1995). It is also able to neutralize DPPH radicals mainly by electron transfer and to a lesser extent by its hydrogen donating ability (Huang *et al*., 2005). Nitric oxide is a signaling molecule required for everyday functioning as it has a wide variety of biological functions. However, higher levels of nitric oxide react with superoxide to produce the damaging oxidant peroxynitrite (Solkolowska *et al.*, 2003). Similarly the ferrous ion chelation study is important

Table 1. Expression of total antioxidant activity, total polyphenols, total flavonoids and gallic acid in Black tea and Kombucha

Sample	Black tea	Kombucha
Total antioxidants (mg/dl)	589.4±4.6	600.0±2.6^{NS}
Total polyphenols (mg/dl)	64.0±0.7	67.0±0.5^{NS}
Total flavonoids (mg/dl)	143.592±1.2	156.92±1.3*
Gallic acid (mg/dl)	221.579±1.9	422.785±1.8***

Values are mean ± SD for 3 samples in each group. p-value calculated using student 't' test. Black tea Vs Kombucha: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS: Not Significant.

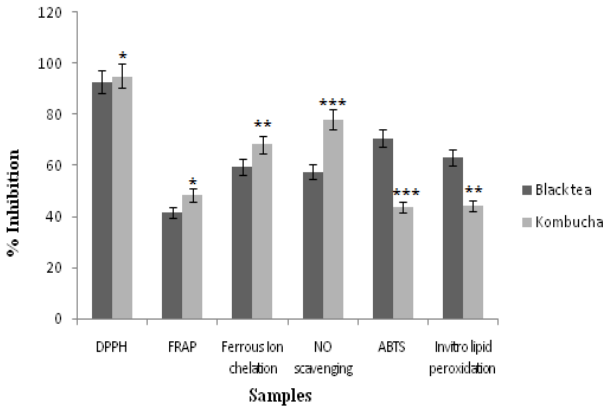

Figure 1. Free radical scavenging activity of tea and Kombucha

Values are mean ± SD for 3 samples in each group. p-value calculated using student 't' test. Black tea Vs Kombucha: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Results expressed as percentage radical scavenging activity

because ferrous ions interact with hydrogen peroxide in biological systems and can lead to the formation of highly reactive hydroxyl radicals (Robu *et al*., 2012). Kombucha scavenges NO radicals 1.4 times more efficiently than tea and also exhibits a higher metal chelating ability (Figure 1). ABTS radical is soluble in both aqueous and organic solvents and is not affected by ionic strength. Thus it can be used to determine both the hydrophilic as well as the lipophilic antioxidant capacities of various compounds (Awika *et al*., 2003) over a wide range of pH (Prior *et al*., 2005). Tea has higher ABTS radical scavenging ability than Kombucha as it mainly consists of hydrogen donating antioxidants. The *in vitro* peroxidation of lipids is measured by the oxidation of linoleic acid in the presence AAPH which generates a constant rate of conjugated dienes (Liegeois *et al*., 2000). Both tea and Kombucha provide protection against lipid peroxidation. Due to the presence of hydrogen donating antioxidants, tea constituents scavenge aqueous phase radicals and act as chain breaking antioxidants for the elimination of lipid peroxyl radicals as observed in previous studies (Hirayama *et al*., 1997; Kumamoto and Sonda, 1998). The above results indicate that Kombucha has a higher radical scavenging ability than Black tea.

Flavonoids are one of the most diverse and widespread groups of natural compounds and possess a broad spectrum of chemical and biological activities including radical scavenging properties. The concentration of flavonoids (as detected using quercetin) shows an increasing trend while gallic acid increased significantly upon fermentation to Kombucha (Table 1). Quercetin is the most abundant dietary flavonol and is a potent antioxidant because it has all the right structural features for free radical scavenging activity (Buhler and Miranda, 2000). When quercetin comes across a free radical, it donates a proton thereby itself becoming a radical which is too low in energy to be reactive (Mariani *et al*., 2008). This antioxidant activity of quercetin is attributed to the presence of the catechol group in the B ring (Figure 3) and the hydroxyl group at the third position in the AC ring (Heijnen *et al*., 2002), while the inhibitory activity of gallic acid (Figure 3) has been attributed to its o-dihydroxy group (Kroes *et al*., 1992).

The retention time of the mixed catechin standard, Black tea and Kombucha are expressed in Figures 2(A-C). The HPLC result obtained by calculation (Table 2) indicate an increase in the concentrations of Epicatechin (EC), Epigallocatechin gallate (EGCG), and Gallocatechin gallate (GCG) upon fermentation of Black tea to Kombucha. The metabolic activities of the microorganisms present in the Kombucha pellicle are responsible for these changes as well as the formation of Gallocatechin (GC), which was absent in Black tea. Catechins efficiently scavenge peroxyl radicals, NO, superoxide and hydroxyl radicals, DPPH radical, carbon-center free radicals, singlet oxygen, lipid free radicals, and peroxynitrite by preventing the nitration of tyrosine (Zhao *et al*., 2001). They also chelate copper (II) and iron (III) ions to form inactive complexes thereby preventing the generation of potentially damaging free radicals (Seeram and Nair, 2002). Catechins also exert their antioxidant effects by the ultra rapid electron transfer to ROS-induced radical sites on DNA (Anderson *et al*., 2001) and scavenge free radicals resulting in the formation of stable semiquinone free radicals, thus, preventing the deaminating ability of free radicals (Sutherland *et al*., 2006). In addition to this, reaction with free radicals result in the formation of a dimerized catechin product which has better superoxide scavenging and iron-chelating

Table 2. The principal antioxidant components of Black tea and Kombucha

Parameter	Black tea	Kombucha
Catechin (C)	Nil	Nil
Epicatechin (EC)	0.294±0.003	0.389±0.003***
Epigallocatechin (EGC)	0.87±004	0.862±0.002^{NS}
Epigallocatechin gallate (EGCG)	0.486±0.004	0.532±0.004**
Epicatechin gallate (ECG)	0.196±0.002	0.055±0.002***
Gallocatechin (GC)	Nil	0.229±0.002
Gallocatechin gallate (GCG)	0.026±0.002	0.185±0.003***

Values are mean ± SD for 3 samples in each group. p-value calculated using student 't' test. Black tea Vs Kombucha: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS: Not Significant. All results are expressed in mg/dl.

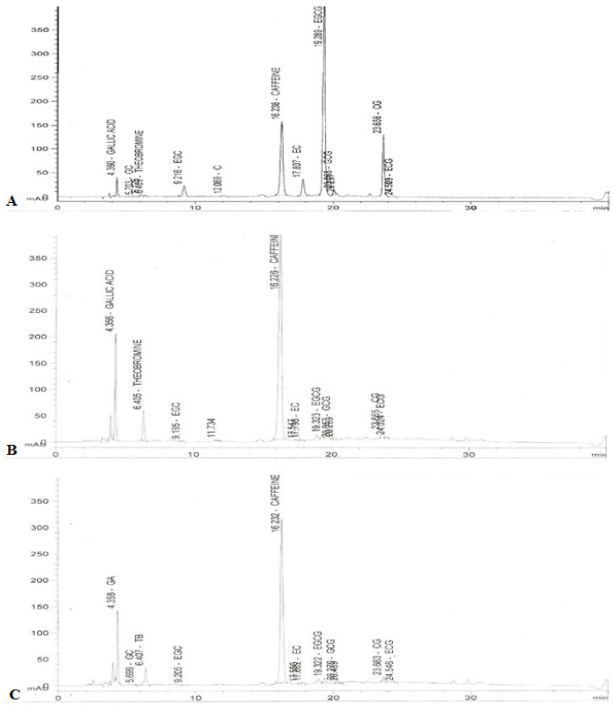

Figure 2 (A, B, C). HPLC graphs showing retention time of mixed catechins (standard), Black tea and Kombucha respectively

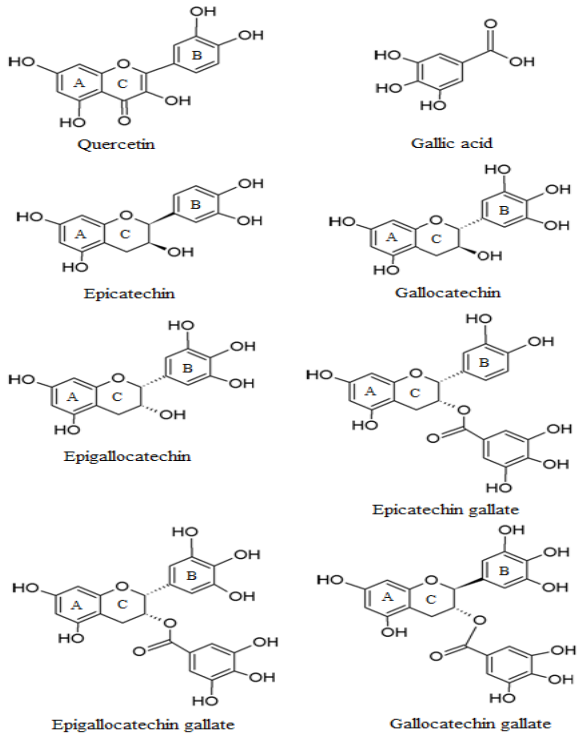

Figure 3. Structures of some bioactive compounds of Kombucha

potential (Yoshino *et al*., 1999). The free radical scavenging activity by catechins relates directly to the chemical structure of each compound, namely, the gallate moiety esterified at the 3 position of the C ring (Figure 3), the catechol group (3,4-dihydroxyl groups) on the B ring and the hydroxyl groups at the 5 and 7 positions on the A ring (Sutherland *et al*., 2006). The A ring of catechins is oxidized and decarboxylated on reaction with hydrogen peroxide thus adding to their antioxidant potential (Zhu *et al*., 2000). The galloylated catechins (EGCG and ECG) have higher phospholipid/water partition coefficients which increases their solubility (Caturla *et al*., 2003). Catechins also have high number of hydroxyl groups which makes them more effective in scavenging free radicals (Zhao *et al*., 2001). Thus, the increase in concentration and composition of the constituents in Kombucha are responsible for its various antioxidant activities.

Conclusion

The consumption of food rich in antioxidant phytochemicals such as flavonoids and other polyphenols is known to be advantageous for our health as they protect the human body from free radicals, thereby retarding the progress of many chronic diseases. The present study indicates that the antioxidant activity of Kombucha is mainly due to its electron donating, metal chelating ability and also to a lesser extent its hydrogen donating ability. However, additional secondary molecular interactions could also be responsible for some of the health benefits of Kombucha. The metabolic conversion of tea constituents during fermentation by microbial enzymes may contribute towards the

increase in antioxidant activity of Kombucha when compared to tea. This suggests that Kombucha has a greater potential as a therapeutic agent than tea for the prevention of diseases and also establishes a firm phytochemical basis for its therapeutic properties. Further, the bioactive compounds of Kombucha need not be directly responsible for the health benefits as they are digested, absorbed, and metabolized by the body. This will subsequently increase their versatility as potential therapeutic interventions. Kombucha is also a rich source of probiotics which adds to its health benefits. Further, *in vivo* radical scavenging activity of Kombucha can be investigated to support its *in vitro* antioxidant potential.

Acknowledgement

The authors are grateful to Bangalore Test House, an ISO 9001 certified and National Accredition Board for Testing and Calibration Laboratories (NABL) Accredited Laboratory for carrying out the HPLC analysis.

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