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Analytical Methods

Changes in free-radical scavenging ability of kombucha tea during fermentation

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Abstract

Kombucha tea is a fermented tea beverage produced by fermenting sugared black tea with tea fungus (kombucha). Free-radical scavenging abilities of kombucha tea prepared from green tea (GTK), black tea (BTK) and tea waste material (TWK) along with pH, phenolic compounds and reducing power were investigated during fermentation period. Phenolic compounds, scavenging activity on DPPH radical, superoxide radical (xanthine–xanthine oxidase system) and inhibitory activity against hydroxyl radical mediated linoleic acid oxidation (ammonium thiocyanate assay) were increased during fermentation period, whereas pH, reducing power, hydroxyl radical scavenging ability (ascorbic acid–iron EDTA) and anti-lipid peroxidation ability (thiobarbituric assay) were decreased. From the present study, it is obvious that there might be some chances of structural modification of components in tea due to enzymes liberated by bacteria and yeast during kombucha fermentation which results in better scavenging performance on nitrogen and superoxide radicals, and poor scavenging performance on hydroxyl radicals.

Keywords: Kombucha; Free-radical scavengers; DPPH; Tea manufacture waste

1. Introduction

Tea contains polyphenols, flavonols (theaflavins and thearubigins), catechins, caffeine, catechin gallates, adenine, theobromine, theophylline, gallic acids, tannins, gallotannin, small amounts of aminophylline and a yellow volatile oil that is solid at ordinary temperatures and has strong aromatic odor and taste (Ho, Chen, Shi, Zhang, & Rosen, 1992; Tyler, Brady, & Robbers, 1988; Zi, 1993). Antioxidant activity is dependent on the structure of the free-radical scavenging compounds, the substituents present on the rings of flavonoids and the degree of polymerization. Although there is some debate as to whether the degree of polymerization increases the antioxidant capacity, it appears that epicatechin and epicatechin polymers are better antioxidants than the catechin and catechin polymers (Ricardo da Silva, Darmon, Fernandez, & Mitjavi, 1991; Saint-Cricq de Gaulejac, Provost, & Vivas, 1999). The structural criteria for the potent free-radical scavengers are that these should possess (i) a 3-hydroxy group on a unsaturated C ring or (ii) a 2,3-double bond with the 3-OH group and 4-one in the C ring or (iii) an *ortho*-OH substitution pattern in the B ring where the OH groups are not glycated (Rice-Evans, Miller, Bolwell, & Bramley, 1995; Rice-Evans, Miller, & Paganga, 1996). The major polyphenolic components, catechin and epicatechin fulfill the first and third structural criteria for being a good antioxidant.

Kombucha tea is sugared black tea fermented with a symbiotic association of acetic acid bacteria and yeasts

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forming "tea fungus" for about 14 days. Kombucha is composed of two portions: a floating cellulose pellicle layer and the sour liquid broth (Chen & Liu, 2000). This beverage has been consumed in Asia for over two millennia and is a popular beverage among traditional fermented foods across the world. The beverage has been claimed to be a prophylactic agent and to be beneficial to human health; however, this remains to be proved (Blanc, 1996). In 1951, an important population study conducted in Russia by the "Central Oncological Research Unit" and the "Russian academy of Sciences in Moscow" found that the daily consumption of kombucha was correlated with an extremely high resistance to cancer (Dufresne & Farnworth, 2000). The beneficial effects of kombucha tea are attributed to the presence of tea polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins, aminoacids, antibiotics and a variety of micronutrients produced during fermentation (Vijayaraghavan et al., 2000). The US Food and Drug Administration has evaluated the practices of several commercial producers of the starter (kombucha mushroom or tea fungus) and found no pathogenic organisms or other hygienic violations (CDC, 1996). This beverage has been reported to have medicinal effects against metabolic diseases, arthritis, indigestion and various types of cancer (Sreeramulu, Zhu, & Knol, 2000). Recent studies have suggested that kombucha tea prevents paracetamol induced hepatotoxicity (Pauline et al., 2001) and chromate(VI) induced oxidative stress in albino rats (Sai Ram et al., 2000). Oral administration of kombucha to rats exposed to pro-oxidation species also indicated the potent antioxidant properties of the fermented drink such as decrease of the degree of lipid oxidation and DNA fragmentation (Dipti et al., 2003; Sai Ram et al., 2000). Unbalanced oxidative stress had been known as an inducer of various diseases (Halliwell & Gutteridge, 2001). It is well-known that free-radicals are one of the causes of several diseases, such as Parkinson's disease, coronary heart disease and cancer. Tea production waste or tea manufacture waste is dry straw and fiber of tea leaves resulting from the black tea production process. Our previous study revealed the possibility of using tea waste material for manufacturing the kombucha tea beverage (Jayabalan, Marimuthu, & Swaminathan, 2007).

Many claimed beneficial effects of kombucha such as alleviation of inflammation and arthritis, cancer prevention and immunity enhancement may be associated to its antioxidant activities (Allen, 1998). Dufresne and Farnworth (2000) proposed that some curative effects of kombucha tea might come from fermentation process but the mechanism remained unclear. Kombucha was usually prepared statically at ambient temperature for up to 7–10 days but the roles of fermentation time were not seriously considered (Greenwalt, Steinkraus, & Ledford, 2000). It was therefore necessary to elucidate the relationship between the fermentation time and antioxidant activities of kombucha. Changes in free-radical scavenging abilities along with pH and total phenolic compounds of kombucha tea prepared from green tea (GTK), black tea (BTK) and tea waste material (TWK) during fermentation were determined in this study.

2. Materials and methods

Green tea and black tea used in this study were manufactured from *Camellia sinensis* (L) *O. Kuntze* at Parry Agro Industries Limited, Valparai, Tamil Nadu, India. Tea manufacture waste is a waste produced during the tea manufacturing process. Xanthine oxidase and xanthine were purchased from the Sigma Chemical Co. (St. Louis, USA). α,α-Diphenyl-β-picrylhydrazyl (DPPH), 2-(4-iodophenyl-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T), ammonium thiocyanate, trichloroacetic acid, thiobarbituric acid (TBA), Folin–Ciocalteau reagent, linoleic acid, sodium phosphate monobasic and sodium phosphate dibasic were purchased from HiMedia Pvt. Ltd. Mumbai, India. All the other chemicals and solvents were high-analytical grade ones. All the analysis were carried out in Parry Agro Industries Ltd., R&D Centre, Murugalli Bazaar, Valparai, Coimbatore (Dist.), Tamil Nadu, India which is a NABL (National Accreditation Board for Testing and Calibration Laboratories) accredited laboratory for both chemical and microbiological testing as per IS/ISO/IEC 17025:2005. "Institute Ethical Committee" clearance was obtained prior to our study to use rat liver in anti-lipid peroxidation study (588/02/A/CPCSEA; CPCSEA, 2003). Starter culture or tea fungal mat was collected from local people of Coimbatore, Tamil Nadu, India and was maintained in sugared black tea.

2.1. Preparation of kombucha tea

1.2% of black tea, green tea and tea manufacture waste were added to boiling water and allowed to infuse for about 5 min after which the infusions were filtered through sterile sieve. Sucrose (10%) was dissolved in hot tea and the preparation was left to cool to 22 °C. Tea (200 ml) was poured into 500 ml glass jars that had been previously sterilized at 121 °C for 20 min. The cooled tea was inoculated with 3% (w/v) of freshly grown tea fungus that had been cultured in the same medium for 14 days and 10% (v/v) of previously fermented liquid tea broth aseptically. The jar was carefully covered with a clean cloth and fastened properly. The fermentation was carried out in a dark incubator at 24 ± 3 °C for about 18 days. Sampling was performed periodically; each jar was sampled only once in order to avoid potential contamination. One green tea (GTK), black tea (BTK) and tea manufacture waste (TWK) glass jar per day were taken for the determination of pH, phenolic compounds, reducing power and free-radical scavenging abilities. The fermented tea was centrifuged at 10,000 rpm for 10 min and taken for the analysis. The pH of the samples was measured with an electronic pH meter (Orion model 290A).

2.2. Determination of the amount of total phenolic compounds

The amount of total phenolic compounds was determined by the method described by Singleton, Orthofer, and Lamuela-Raventos (1999). 0.1 ml of GTK, BTK and TWK were transferred to a 100 ml Erlenmeyer flask and the final volume was adjusted to 46 ml by addition of distilled water. Afterward, 1 ml of Folin–Ciocalteau reactive solution was added and incubated at room temperature for 3 min. 3 ml of 2% sodium carbonate solution was added and the mixture was shaken on a shaker for 2 h at room temperature. The absorbance was measured at 760 nm. Gallic acid was used as the standard for the calibration curve. The phenolic compound content was expressed as gallic acid equivalent (GAE, μg).

2.3. DPPH scavenging ability

Scavenging activity on DPPH was assessed according to the method reported by Blois (1958) with a slight modification. 100 μl of GTK, BTK and TWK were mixed with 1 ml of 0.1 mM DPPH in ethanol solution and 450 μl of 50 mM Tris–HCl buffer (pH 7.4). The solution was incubated at room temperature for 30 min and reduction of DPPH free-radicals was measured by reading the absorbance at 517 nm. Tube without tea solutions served as control. This activity is given as % DPPH radical scavenging calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(\text{control absorbance} - \text{sample absorbance})/\text{control absorbance}] \times 100 \quad (1)$$

2.4. Reducing power

This was carried out as described by Yildirim, Mavi, and Kara (2001). 20 μl GTK, BTK and TWK were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The reaction mixture was incubated at 50 °C for 30 min. Afterward 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 min at 3000 rpm. 2.5 ml of upper layer solution was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride. Absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.5. Inhibitory ability on hydroxyl radical mediated linoleic acid oxidation

The antioxidant activity was measured through ammonium thiocyanate assay (Lee, Kim, Jim, & Jang, 2002). Solution containing 100 μl of tea solutions, 200 μl of diluted linoleic acid (25 mg/ml 99% ethanol) and 400 μl of 50 mM phosphate buffer (pH 7.4) was mixed and incubated at 40 °C for 15 min. Aliquot (100 μl) from the reaction mixture was mixed with reaction solution containing 3 ml of 70% ethanol, 100 μl of ammonium thiocyanate (300 mg/ml distilled water) and 100 μl of ferrous chloride (2.45 mg/ml 3.5% hydrochloric acid). Final reaction solution was mixed and incubated at room temperature for 3 min. Absorbance was measured at 500 nm. Linoleic acid emulsion without tea solution was served as control. Inhibition of linoleic acid oxidation was calculated by using the following formula:

$$\text{Inhibition on hydroxyl radical mediated linoleic acid oxidation (\%)} = [(\text{control absorbance} - \text{sample absorbance})/\text{control absorbance}] \times 100 \quad (2)$$

2.6. Scavenging ability onto superoxide anions

Scavenging ability on superoxide radical ($O_2^{\cdot-}$) was assessed by the method described by Lee et al. (2002) with a slight modification. 100 μl of GTK, BTK and TWK were mixed with 1.7 ml of 0.94 mmol EDTA containing 0.05 mmol xanthine and 0.025 mmol 2-(4-iodophenyl-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T). The reaction mixture was incubated at room temperature for 3 min. 250 μl of xanthine oxidase (80 U/l) was added to the reaction mixture and incubated for 20 min at room temperature. Absorbance was measured at 505 nm with control tubes having all the reaction compounds except the tea solutions. Scavenging of superoxide anions was calculated by using the following formula:

$$\text{Superoxide radical scavenging activity (\%)} = [(\text{control absorbance} - \text{sample absorbance})/\text{control absorbance}] \times 100 \quad (3)$$

2.7. Hydroxyl radical scavenging ability

The hydroxyl radical scavenging ability was determined according to the method of Klein, Cohen, and Cederbaum (1981). 100 μl of GTK, BTK and TWK were taken in test tubes and evaporated to dryness. 1 ml of an iron EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA (0.018%) and 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added to the test tubes and the reaction was initiated by adding 0.5 ml of 0.22% ascorbic acid. Test tubes were capped tightly and heated on a water bath at 80–90 °C for 15 min. The reaction was terminated by the addition of 1 ml of ice-cold trichloroacetic acid (17.5% w/v). 3 ml of Nash reagent (75.0 g of ammonium acetate, 3 ml glacial acetic acid and 2 ml of acetyl acetone were mixed and made up to 1 l with distilled water) was added to all of the tubes and left at room temperature for 15 min for color development. The intensity of the yellow color formed

was measured spectrophotometrically at 412 nm against reagent black. The percentage hydroxyl radical scavenging is calculated by the formula

$$\text{Hydroxyl radical scavenging activity (\%)} = [1 - (\text{absorbance of sample}/\text{absorbance of blank})] \times 100 \quad (4)$$

2.8. Measurement of anti-lipid peroxidation by thiobarbituric acid (TBA) assay

The lipid peroxidation was measured by the method of Halliwell and Gutteridge (1999). Liver homogenate was prepared by grinding fresh normal albino rat liver using phosphate buffer saline, pH 7.4 (10% w/v). The homogenate was centrifuged at 3000 rpm for 15 min and clear supernatant was taken for analysis. 100 μl of GTK, BTK and TWK were taken in test tubes and evaporated to dryness. 1 ml of 0.15 M potassium chloride and 0.5 ml of rat liver homogenate were added. Peroxidation was initiated by adding 100 μl of 0.2 mM ferric chloride. The tubes were incubated at 37 °C for 30 min. The reaction was stopped by adding 2 ml of ice-cold hydrochloric acid (0.25 N) containing 15% trichloroacetic acid, 0.38% thiobarbituric acid and 0.5% butylated hydroxyl toluene. The reaction mixture was annealed at 80 °C for 1 h. The samples were cooled and centrifuged and the absorbance of the supernatants was measured at 532 nm. A similar experiment was performed in the absence of tea solutions to determine the amount of lipid peroxidation obtained in the presence of inducing agents, which served as control. The percentage of anti-lipid peroxidation was calculated by using the following formula:

$$\text{Anti-lipid peroxidation activity (\%)} = [(\text{control absorbance} - \text{sample absorbance})/\text{control absorbance}] \times 100 \quad (5)$$

All analyses were carried out in triplicate. Each datum represents the mean of three different experiments in each of which two measurements were made. SPSS 13.0 software was used for statistical calculations. Values of $P < 0.01$ were considered to be significant.

3. Results and discussion

Tea decoction, either prepared with black tea or green tea leaf exhibited good antioxidant activity. Though kombucha possessed some curative effects such as reduction of atherosclerosis, arthritis and inflammation which might be related to its antioxidant activity compared to black tea broth (Allen, 1998), they have not been scientifically validated. Recent studies demonstrated that kombucha had in vivo antioxidant activities, but the cause of this remained unclear. Therefore, various free-radical generating systems were used to evaluate the changes in free-radical scavenging abilities along with pH, total phenolic compounds and reducing power of kombucha tea broth during fermentation.

3.1. pH

The pH of kombucha tea was decreased with fermentation time (Fig. 1). It was decreased rapidly from 5.0 to 3.0 within 3 days of fermentation and then it was continued to decrease slightly up to 18 days. Due to increased concentration of organic acids produced during the fermentation process by bacteria and yeasts in the tea fungus consortium, the pH value decreased from 5.0 to 3.0. Apparently the fermentation broth possessed some buffer capacity. During the fermentation process carbon dioxide is released at first slowly and much faster after 2–3 days. The obtained water solution of carbon dioxide dissociates and produces the amphiprotic hydrocarbonate anion (HCO_3^-), which easily reacts with hydrogen ions (H^+) from organic acids, preventing further changes in the H^+ concentration and contributing to a buffer character of the system. This will be the valid reason for slight decrease in pH after 3 days. These observations are in agreement with the findings of other studies (Chen & Liu, 2000; Reiss, 1994; Sreeramulu et al., 2000).

3.2. Reducing power

Results of reducing power assay showed differential ability of GTK, BTK and TWK (Fig. 1). GTK showed maximum reducing power (0.6) on 15th day whereas BTK and TWK showed maximum reducing power on 9th day of fermentation. A decrease in reducing power was observed on 3rd day in BTK and TWK. Throughout the fermentation period, temporal increase and decrease in reducing power ability was observed in all the substrates studied. Reducing power of a compound is related to electron transfer ability of that compound. Therefore, the reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity (Meir, Kanner, Akiri, & Hadas, 1995). No correlation found between phenolic compounds and reducing power ability of GTK, BTK and TWK.

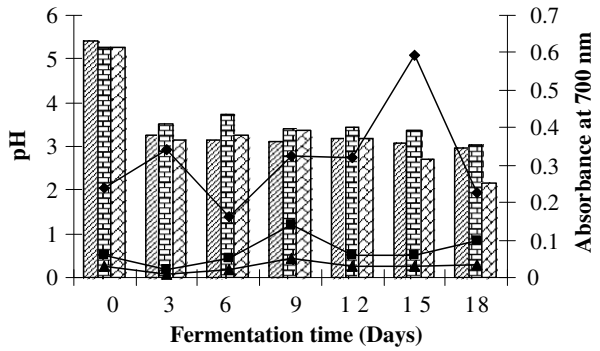

Fig. 1. Changes in pH (bars) and reducing power (symbols) during kombucha fermentation {▨, —◆— = GTK; ▤, —■— = BTK; ▨, —▲— = TWK}. Data are expressed as mean ± SD of $n = 3$ samples.

3.3. Total phenolic compounds

Changes in concentration of total phenolic compounds in GTK, BTK and TWK during fermentation are shown in Fig. 2. Total phenolic compounds were progressively increased with fermentation time. Phenolic compounds are called high-level antioxidants because of their ability to scavenge free-radical and active oxygen species such as singlet oxygen, superoxide free-radicals and hydroxyl radicals. Complex phenolic compounds in GTK, BTK and TWK might be subjected to degradation in acidic environment of kombucha and by the enzymes liberated by bacteria and yeast in tea fungus consortium. In our previous work, we observed the degradation of epicatechin isomers during kombucha fermentation (Jayabalan et al., 2007). Friedman and Jurgens (2000) reported the resistance pattern of (−)-catechin and (−)-epigallocatechin to pH in the range of 3–11. Zhu, Zhang, Tsang, Huang, and Chen (1997) noticed that flavonoids and proanthocyanidins have higher stability under acidic conditions. It has been reported that theaflavin is unstable in alkaline solution, while it was stable in acidic solutions (Jhoo et al., 2005). Duenas, Hernandez, and Estrella (2007) demonstrated that bioactive polyphenolic compounds of lentils were modified due to exogenous application of enzymes like phytase, α-galactosidase and tannase. They have also demonstrated the increased antioxidant activity of enzyme treated lentils. So, there are many chances for the enzymes liberated by bacteria and yeast during kombucha fermentation will be the reason for the degradation of complex polyphenols to small molecules which in turn results in the increase of total phenolic compounds.

3.4. DPPH scavenging ability

The DPPH scavenging abilities of GTK, BTK and TWK were increased up to 8%, 15% and 19%, respectively, during fermentation (Fig. 2). Maximum increase was observed at 3rd day of fermentation with a remarkable decrease on 6th day in GTK, BTK and TWK. After 6th day, the DPPH scavenging ability was continued to increase up to 18th day. Among the three substrates tried, green tea was found to be the best substrate which showed higher DPPH scavenging ability (88%) on 18th day of fermentation. Recent studies demonstrated that kombucha had in vivo antioxidant activities, but the cause of this remained unclear. The increased potential against DPPH radical might explain the phenomena that kombucha feeding significantly reversed the chromate(IV) or lead induced oxidative injury in rats (Dipti et al., 2003; Sai Ram et al., 2000). It is generally believed that polyphenols and catechins are mainly responsible for antioxidant actions. Phenolic compounds could easily donate hydroxyl hydrogen due to resonance stabilization (Fessenden & Fessenden, 1994). There was a statistically significant correlation between the amount of phenolic compounds and DPPH scavenging abilities of black tea ($r = 0.718$, $P < 0.01$).

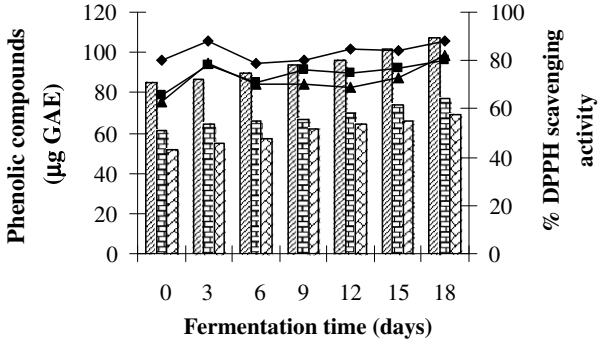

Fig. 2. Changes in phenolic compounds (bars) and DPPH scavenging ability (symbols) during kombucha fermentation {▨, —●— = GTK; ▦, —■— = BTK; ▩, —▲— = TWK}. Data are expressed as mean ± SD of $n = 3$ samples.

3.5. Inhibitory ability on hydroxyl radical mediated linoleic acid oxidation

In the present study, the antioxidant activities of GTK, BTK and TWK were determined by using the thiocyanate method in that amount of peroxides formed in emulsion during incubation is determined spectrophotometrically by measuring the absorbance at 500 nm. High absorbance is an indication of high concentration of formed peroxides. Therefore, low absorbance indicates high antioxidant activity. Initially GTK, BTK and TWK (0 day) showed the inhibitory ratio against linoleic acid peroxidation by 38.46%, 34.4% and 33.3%, respectively. Kombucha fermented after 18 days showed a strengthened potential averagely up to 65.3% (Fig. 3). There was a remarkable decrease in inhibitory potential against linoleic acid peroxidation on 3rd day of fermentation in GTK, BTK and TWK, but an increase on 9th and 15th day was observed in all substrates tested. Among the three substrates studied, GTK performed better ability, which was similar to those observed in DPPH scavenging ability (Fig. 3). Statistical significant correlation was observed between phenolic compounds and inhibitory ability of linoleic acid oxidation of GTK ($r = 0.896$, $P < 0.01$), TWK ($r = 0.847$, $P < 0.01$) and BTK ($r = 0.699$, $P < 0.01$). These results also corresponded to the studies that oral administration of kombucha to rats challenged with pro-oxidation species such as chromate(VI) and lead or hepatotoxic drug, paracetamol significantly decreased the malondialdehyde content implicated in lipid peroxidation (Dipti et al., 2003; Pauline et al., 2001; Sai Ram et al., 2000).

3.6. Scavenging ability onto superoxide anions

In cellular oxidation reactions, superoxide radicals are normally formed and their effects can be magnified because they produce other kinds of cell-damaging free-radicals and oxidizing agents. Moreover, xanthine oxidase is one of the main enzymatic sources of reactive oxygen species

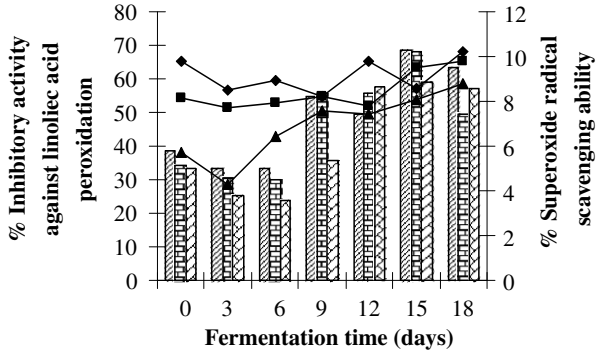

Fig. 3. Changes in inhibitory ability against hydroxyl radical mediated linoleic acid oxidation (bars) and superoxide radical scavenging ability (symbols) during kombucha fermentation {▨, —◆— = GTK; ▦, —■— = BTK; ▨, —▲— = TWK}. Data are expressed as mean ± SD of $n = 3$ samples.

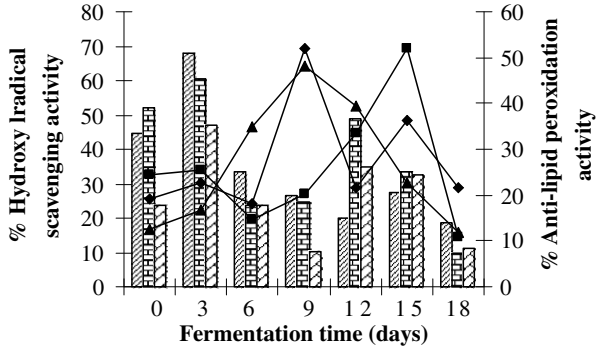

Fig. 4. Changes in hydroxyl radical scavenging ability (bars) and anti-lipid peroxidation ability (symbols) during kombucha fermentation {▨, —◆— = GTK; ▦, —■— = BTK; ▨, —▲— = TWK}. Data are expressed as mean ± SD of $n = 3$ samples.

in vivo. Hypoxanthine–xanthine oxidase generates superoxide radicals, which reduce I.N.T. to yield pink colour. Kombucha tea prepared from three different substrates time dependently inhibited the I.N.T. reduction induced by hypoxanthine–xanthine oxidase (Fig. 3). Like phenolic compounds, DPPH scavenging ability and inhibitory ability against linoleic acid peroxidation, superoxide radical scavenging ability was also increased in GTK, BTK and TWK after 18 days fermentation. When compared to other free-radical scavenging abilities studied, only slight increase of superoxide radical scavenging ability was observed in GTK, BTK and TWK. Statistically significant correlation was observed between phenolic compounds and superoxide radical scavenging ability in BTK ($r = 0.828$, $P < 0.01$) and in TWK ($r = 0.902$, $P < 0.01$).

3.7. Hydroxyl radical scavenging ability

Hydroxyl radical scavenging ability was estimated by generating the hydroxyl radicals using ascorbic acid–iron EDTA. Hydroxyl radicals formed by the oxidation react with DMSO to yield formaldehyde, which provides a convenient method for their detection by treatment with Nash reagent. The scavenging effect of infusions from green tea, black tea and tea waste on hydroxyl radicals were 44.8%, 52.3% and 23.8%, respectively, on 0 day of fermentation. Scavenging effect was decreased while fermentation continues up to 18 days (Fig. 4). After 18 days, the scavenging effect on hydroxyl radical reached 18.5% in GTK, 10% in BTK and 11.2% in TWK. Maximum scavenging effect on hydroxyl radicals was observed on 3rd day of fermentation in GTK (67.7%), BTK (60.5%) and TWK (46.9%). There is no correlation between phenolic compounds and scavenging ability on hydroxyl radicals. Hydroxyl radical is an extremely reactive species formed in biological systems and has been implicated as highly damaging in free-radical pathology, capable of damaging almost every molecule found in living cells (Hochestein & Atallah, 1988). This radical has the capacity to join nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity. In addition, hydroxyl radical is considered to be one of the quick initiators of the lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids (Kappus, 1991). The ability of kombucha tea to quench hydroxyl radicals seems to directly relate to the prevention of propagation of the process of lipid peroxidation, and it seems to be a good scavenger of active oxygen species, thus reducing the rate of chain reaction.

3.8. Anti-lipid peroxidation

Like hydroxyl radical scavenging ability, anti-lipid peroxidation ability of GTK, BTK and TWK was also decreased after 18 days fermentation (Fig. 4). Maximum activity was exhibited on 9th day in GTK (51.84%) and TWK (48.15%) whereas BTK showed maximum activity (51.8%) on 15th day of fermentation. Amorati, Pedulli, Cabrine, Zambonin, and Landi (2006) reported that phenolic acids or esters behaved as weak inhibitors of peroxidation in acidic pH whereas with increasing pH, their antioxidant activity increased substantially. No significant correlation was found between phenolic compounds and anti-lipid peroxidation activity. Shi, Dalal, and Jain (1991) reported scavenging activity on hydroxyl radicals of caffeine and attributed the alleged anticarcinogenic properties of caffeine to this activity. In complex lipid systems, where several different antioxidant and prooxidant actions occur simultaneously, it is obviously more difficult to observe the effect of a single factor than in simplified radical scavenging models. In lipid oxidation models, peroxyl-radical scavenging and metal inactivation properties are very important mechanistic factors, but the polarity of the compound and the physical state of the lipid system also affect the behavior of antioxidants. In addition, synergism, that is, the ability of antioxidant compounds to reinforce each other, can have a significant effect on the antioxidant response (Frankel, 1998). These may be valid reasons for the reduction in anti-lipid peroxidation ability of kombucha tea during fermentation.

In the present study, scavenging abilities of kombucha tea on hydroxyl radicals as determined by hydroxyl radical scavenging assay and anti-lipid peroxidation assay were decreased during fermentation time with a temporal increase. Scavenging properties of kombucha tea are directly depending on the components produced during fermentation time and also on the tea constituents. The compound which is responsible for the maximum scavenging ability might get transformed into less potential scavenging compounds with structural modifications due to enzymes liberated by bacteria and yeast in the tea fungus consortium. The antioxidant activities of the individual phenolic compounds may depend on structural factors, such as the number of phenolic hydroxyl or methoxyl groups, flavone hydroxyl, keto groups, free carboxylic groups, and other structural features. Dihydroxylation in both rings and in the 3-position in catechin, myricetin, quercein and epicatechin is required for antioxidant activity as reported in various lipid systems (Pratt & Hudson, 1990). Dziedzic and Hudson (1983) found that steric hindrance of phenolic hydroxyl groups such as by the addition of methoxyl groups could enhance antioxidant activity. Glycosylation resulted in lower antioxidant activity for quercetin, cyanidin, pelargonidin and peonidin. Addition of a sugar moiety decreased the activity of the aglycon, and the addition of a second moiety decreased activity further, probably due to steric hindrance by addition of sugar moieties. Tsuda et al. (1994) found that cyanidin had greater antioxidant activity than cyanidin 3-glycoside in linoleic acid system. Pratt and Hudson (1990) noted that 3-glycosides of flavonoids can possess the same or sometimes less activity than their corresponding aglycons. With these informations, it is clear that there might be some chances of structural modification of components in tea during kombucha fermentation which results in better scavenging performance on nitrogen and superoxide radicals, and poor scavenging performance on hydroxyl radicals.

Kombucha tea showed different scavenging abilities on hydroxyl radicals as determined by three model systems (ammonium thiocyanate assay, hydroxyl radical scavenging assay using ascorbic acid–iron EDTA and anti-lipid peroxidation by TBA assay). Inhibitory ability of kombucha tea on hydroxyl radical mediated linoleic acid oxidation (ammonium thiocyanate assay) was increased during fermentation time, whereas scavenging ability on hydroxyl radical (ascorbic acid–iron EDTA) and anti-lipid peroxidation ability (TBA assay) were decreased. Generally, compounds exhibiting good antioxidant activity by one method had good antioxidant activity by the other methods and like wise for compounds with low activity. But there were some notable exceptions. Using the β-carotene bleaching and DPPH scavenging methods, ellagic acid and α-tocopherol had high antioxidant activity, but not with linoleic acid emulsion model. Rutin had low antioxidant activity with β-carotene bleaching model, but not with other methods. Poor solubility in aqueous solutions and steric hindrance of compounds may have contributed to the observed differences (Fukumoto & Mazza, 2000). The discrepancies noted in the present study may also have been due to the types of reactions occurring in each assay. Because methods of measuring antioxidant activity are extremely dependent on the conditions used and the substrates or products monitored, all methods did not give the same results for activity. Franker (1993) and Warner (1997) reviewing limitations for antioxidant activity assays, suggested activity be measured by using more than one method, measuring primary and secondary oxidation products, and using tests that measure specific substrates or products. These differences could lead to variations in the measurement of antioxidant activity.

4. Conclusion

The present study demonstrated that kombucha tea prepared from green tea, black tea and tea waste material have excellent antioxidant activities. It is interesting to note and worthy to further investigate the potential effectiveness or usage of kombucha tea prepared from tea waste material in preventing diseases caused by the over production of radicals. Kombucha exhibited increased free-radical scavenging activities during fermentation. The extent of the activity depend upon the fermentation time, type of tea material and the normal microbiota of kombucha culture, which in turn determined the forms of their metabolites. Although free-radical scavenging properties of kombucha showed the time-dependent profiles, prolonged fermentation was not recommended because of accumulation of organic acids, which might reach harmful levels for direct consumption. The identification of extracellular key enzymes responsible for the structural modification of components during kombucha fermentation and potent metabolites responsible for the free-radical scavenging abilities are necessary to elucidate the metabolic pathway during kombucha fermentation. Metabolic manipulations may be one of the effective methods to elevate the antioxidant activities and fermentation efficiency of kombucha. To study the antioxidant mechanisms by potential antioxidant components, the fractionation of kombucha tea and further identification are in progress.

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