T. Srihari et al /J. Pharm. Sci. & Res. Vol.4(11), 2012, 1978 - 1981

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Changes in Free Radical Scavenging Activity of Kombucha during Fermentation

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Abstract
Kombucha is a homemade fermented tea that has been traditionally consumed in China for over 2200 years, and is now being used in many other countries. The biochemical basis of the health benefits of kombucha remains largely unknown. The present study was focused on changes in the total antioxidant activity, total phenolic assay and titratable acid content of the kombucha. In addition, the important organic acids and water-soluble vitamins present in kombucha were estimated by using RP-HPLC. Kombucha exhibits good antioxidant and free radical scavenging activity, besides the presence of phenolic compounds, malic acid, tartaric acid, acetic acid, B-complex vitamins and found increased concentration with increased fermentation time. It is concluded that the desired quality or composition of kombucha can be obtained through the proper control of fermentation time.
Keywords: *Antioxidant activity, B-complex vitamins, HPLC, Kombucha, Organic acids.*

INTRODUCTION

Tea is grown in about 30 countries and is the most widely consumed beverage in the world, next to water [1]. Epidemiological studies suggest a protective effect of tea consumption on human cancer. Polyphenolic compounds (catechins) present in tea are capable of affording protection against various types of cancer. Theaflavin and thearubigins are polyphenolic derivatives present in black tea. The protective effects of tea polyphenolic compounds against various types of cancer were reviewed by several authors [1-4]. Brewed tea is also found to contain significant levels of the catechins and flavanoids. Thus, brewed tea is major dietary source for this potentially important group of compounds. Catechins are one of the few groups of flavanoid compounds, possess a significant degree of bioavailability [5].

Kombucha tea is sugared black tea fermented with a symbiotic association of acetic acid bacteria and yeasts forming 'tea fungus' for about 18 days. Kombucha tea is composed of two portions: a floating cellulose pellicle layer and the sour liquid broth [6]. 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 beneficial to human health; however, this remains to be proved [7].

Many claimed health beneficial effects of kombucha such as alleviation of inflammation and arthritis, cancer prevention and immunity enhancement may be associated to its antioxidant activities and these effects are attributed to the presence of polyphenols, certain organic acids too, produced during fermentation [8]. Dufresne and Farnworth (2000) proposed that some curative effects of kombucha tea might come from fermentation process but the mechanism remained unclear [9]. Kombucha was usually prepared statically at ambient temperature for upto 7-10 days but the roles of fermentation time were not seriously considered [10]. Kombucha is therefore necessary to elucidate the relationship between the fermentation time and antioxidant activities of kombucha. Changes in free-radical scavenging abilities, total phenolic compounds assay, titratable acidity for acid content of kombucha tea from black tea during fermentation. Furthermore, HPLC analysis of organic acids, vitamins was carried out in this study.

MATERIALS AND METHODS

Chemicals

2,2′-Azino-bis-(-3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), potassium persulphate, were purchased from the Sigma Chemical Company (St. Louis, MO, USA). Sodium carbonate, Folin-Ciocalteau reagents were from Qualigens Chemicals (Mumbai, India).

Starter Culture

The kombucha baby mat with its mother liquor was obtained and authenticated by a quality maintaining web based supplier 'Kombucha Exchange' worldwide group through one of its members, from Khammam, A.P., India, and was maintained in sugared black tea under aseptic conditions.

Preparation of the medium

The tea fungus samples were periodically maintained by the following procedure. Distilled water (1 l) was boiled, 10% of table sugar and 0.45% of tea powder (Tajmahal tea, India, as without any additives) were added. The sweetened black tea was filtered through a stainless steel sieve and then immediately dispensed into 250 ml glass jars (each containing 100 ml tea). Finally, the freshly prepared tea was inoculated with 2.5% (W/V) of freshly grown cellulosic pellicle or kombucha mat that had been cultured in the same medium for 18 days and 10% (V/V) of previously fermented

liquid broth aseptically [11]. The jars were covered with clean cheese cloth fixed with rubber bands. The fermentation was carried out under ambient temperature (28 ± 3^0C) for 18 days under aseptic conditions.

Kombucha fermentation and sampling

The preparation of kombucha broth was same as above including the capacity of glass jars. The jars were divided into 7 groups among fermentation carried out up to 18 days (viz., day 0, 3, 6, 9, 12, 15 and 18 of kombucha samples) with 3 jars in each group (i.e., tea fungus samples). About 2.5 % (W/V) cellulosic pellicle or kombucha mat and 10% (V/V) of broth from the above activated tea fungus sample were withdrawn and inoculated into each jar. Sampling was performed periodically each jar was sampled once only in order to avoid potential contamination and the broth was centrifuged at 10,000 g for 30 min for further analyses. All analyses were carried out in duplicate.

Analytical Procedures

Titratable Acidity

The titratable acidity was measured according to Chen and Liu method [6]. A 10 ml aliquot of tea fungus broth was taken and titrated with 0.1mol/L NaOH, the end point was determined by pH at 7. The titratable acidity was expressed as the volume consumed in millilitres of 0.1 mol/L NaOH per 100 ml sample.

Determination of Total Phenol Content

The total phenol content of kombucha was measured according to Folin-Ciocalteau method [12]. 0.1 ml of test sample was transferred to a 100 ml Erlenmeyer flask and the final volume was adjusted to 46 ml by addition of distilled water added to 1.0 ml of Folin-Ciocalteau reagent. Afterward, 1 ml of Folin-Ciocalteau reactive solution was added and incubated at room temperature for 3 min. 3 ml of 2% sodium carbonate was mixed with the above solution. The absorbance at 760 nm was then measured after 30 min. The total phenol was expressed as gallic acid equivalents (GAE, µg) from the calibration curve.

Determination of Total Antioxidant Activity by ABTS Method

The measurement of radical scavenging activity of any antioxidant is commonly associated with the using of ABTS method instead of other conventional methods because it is a quick, reliable and reproducible method to search the in vitro general antioxidant of pure compounds, as well as plant extracts [13, 14].

The method adopted for the measurement of TAA, Re et al. ABTS was dissolved in deionised water at a concentration of 7.8mM. The stock solution was mixed with 2.5mM potassium persulphate (final concentration). The mixture was kept in the dark at room temperature for 12-16 h before use in order to avoid incomplete oxidation of ABTS. A 10µL aliquot of properly diluted kombucha was added to the above solution, and then incubated for 15 min. The absorbance of mixture was detected at 734nm. The radical scavenging capacity of kombucha was calculated by the following equation: scavenging effect (%) = AC – AS / Ac X 100, where AC and AS represented absorbances measured for control and sample respectively [15].

HPLC instrumentation

Filtered sample (2ml) was purified through membrane filter (0.45µ) into HPLC vials. The filtrate obtained was subjected to analysis of acetic acid, malic acid, tartaric acid, vitamin-B_1, vitamin-B_2, vitamin-B_6, vitamin-B_{12}, vitamin-C by HPLC. A 20µl sample of filtrate was injected to a Shimadzu HPLC system equipped with phenomenex Luna C_{18}, 5µm (4.6 X 250mm) column, LC10AT VP pumps, a SCL-10AVP system controller, SIL-10AD VP auto injector and SPD-M10 AVP photodiode array detector, class VP software was used for analysis. Two kinds of mobile phases were used mixture of 0.1% v/v phosphoric acid and acetonitrile.

HPLC analytical method

We used a reverse-phase HPLC column with gradient system and isocratic system with respected mobile phases at a flow rate of 1ml/min. Detection was with a PDA detector and the column temperature was maintained at 30^0C. The detection wave lengths were set at different nm according to the detectant. The injection volume was 20µl. The methods applied to the kombucha extract and its respected reference compounds ran successfully.

Statistical Analysis

Each experiment was performed in triplicate and repeated twice. Means were compared with Duncan's multiple range tests. The results were considered statistically significant at $P<0.01$. All statistical analyses were made using SPSS 16.0 software package (SPSS, Chicago, IL, USA).

RESULTS

All groups of kombucha samples (except 0^{th} day samples) showed increased antioxidant activity with increase of fermentation time (Table 1). Total phenol content of kombucha among all samples increased linearly with the fermentation time, and 18^{th} day samples had higher content. The titratable acidity of kombucha increased steadily with fermentation time. The final mean titratable acidity was 14.10 ml of 0.1 mol L^{-1} NaOH 100 ml^{-1}.

The specificity of the method was ascertained by analyzing the standards and the sample. The peaks for acetic acid, malic acid, tartaric acid, vitamins B_1, B_2, B_6 and vitamin C in the sample were confirmed by comparing the retention times of the peaks with that of standards (Figure 1a, 1b, 1c, 1d, 1e, 1f). The results proved that the kombucha extract contained acetic acid, malic acid, tartaric acid, vitamins B_1, B_2, B_6 and vitamin C as the main constituents apart from many other components.

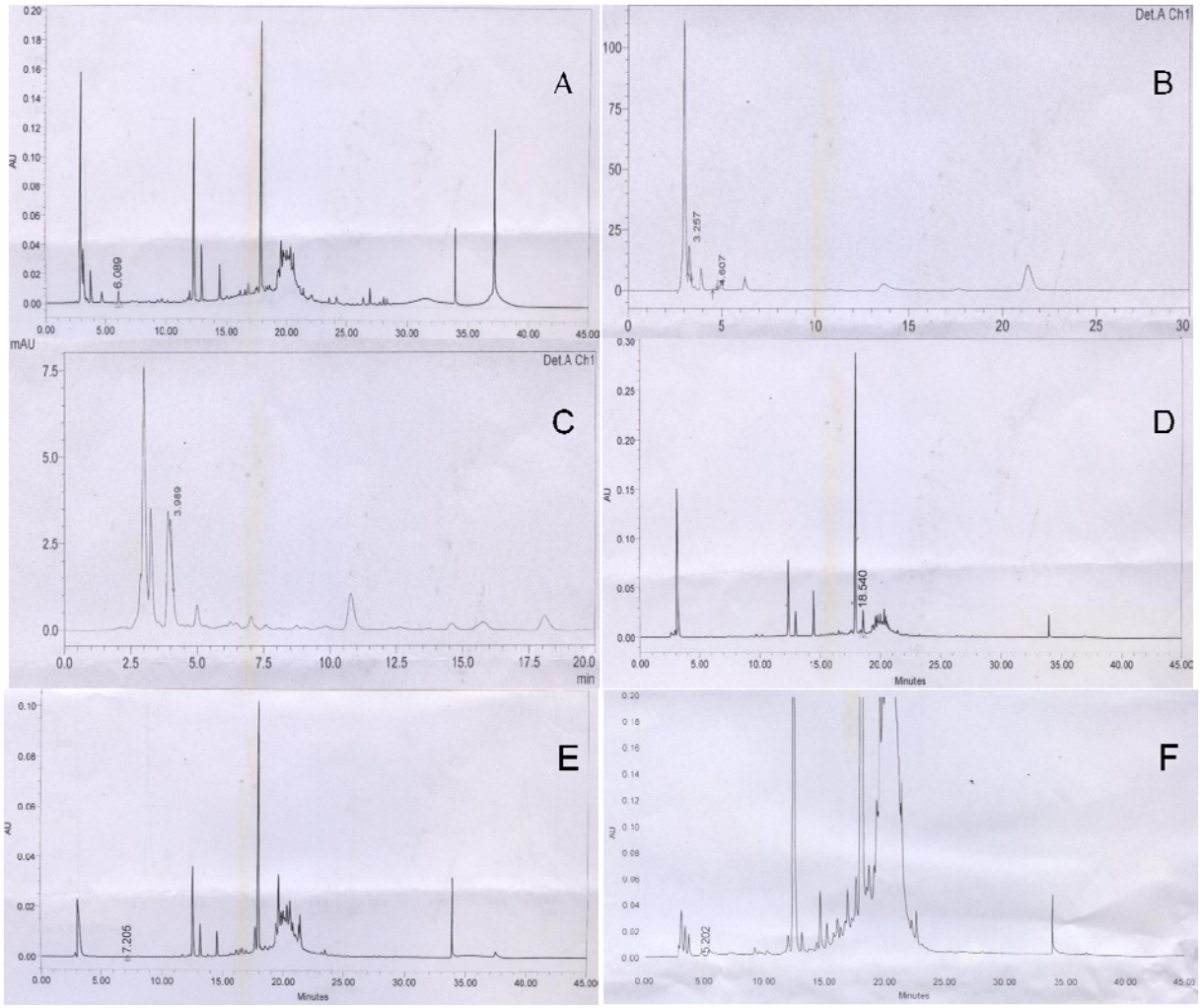
DISCUSSION

Tea decoction, prepared with black tea leaf exhibited reasonably good antioxidant activity. The ABTS scavenging ability increased gradually up to 18th day. Recent studies demonstrated that kombucha had *in vivo* antioxidant activities, but the cause of this remained unclear. The increased potential against DPPH (another free-radical generating system) radical might explain the phenomena that

Table 1: Effects of kombucha on ABTS, TPA, TTA during 18 days of fermentation time.

Sampling day	*Parameters*		
	ABTS	***TPA***	***Titratable Acidity***
Tea	43.30±0.4736	39.83±0.5492	0.2833±0.020
0^{th} day of kombucha	46.06±0.6297	40.69±0.6589	0.5966±0.017
3	51.46±0.4652	44.71±0.5237	1.410±0.204
6	55.18±0.3583	51.51±0.8320	2.608±0.154
9	57.02±0.5985	60.01±0.4817	5.479±0.234
12	59.89±0.6268	69.67±0.5208	10.463±0.211
15	67.17±0.6543	76.10±0.4229	12.533±0.248
18	70.11±0.7754	82.79±0.9377	14.413±0.189

ABTS-radical scavenging assay; TPA-Total phenolic assay; TTA-Titratable acidity.
Data showed were the average of three samples of duplicate with respected standard deviations.



Peaks are (a) acetic acid (b) malic acid and tartaric acid (c) vitamin-B_1 (d) vitamin-B_2 (e) Vitamin-B_6 (f) Vitamin-C,
For conditions see materials and methods section.

Figure 1: HPLC Chromatogram of kombucha

kombucha feeding significantly reversed that chromate-(IV) or lead induced oxidative injury in rats [16, 17]. 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 [18].

Phenolic compounds are regarded as high level antioxidants because of their ability to scavenge free radical and active oxygen species. Complex phenolic compounds in kombucha might be subjected to degradation in acidic environment of kombucha and by the enzymes liberated by bacteria and yeast in tea fungus consortium. It was reported that the degradation of epicatechin isomers occurs during kombucha fermentation [19]. Duenas et al., demonstrated that bioactive polyphenolic compounds of lentils were modified due to exogenous application enzymes like phytase, α-galactosidase and tannase [20]. They have also found that 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.

The results indicated that the pattern of change in the determination of titratable acidity during fermentation. Chemical analysis of kombucha beverage revealed the presence of sugars, acetic acid, gluconic acid, succinic acid, ethanol [6], and glucuronic acid, gluconic acid, lactic acid, catechins [19]. In the course of metabolic activities, yeast and bacteria in the tea fungus make use of substrates by different and complementary ways. Yeast cells hydrolyse sucrose into glucose and fructose by yeast invertase and produce ethanol via glycolysis, with a preference for fructose as a substrate. Acetic acid bacteria utilize glucose to produce gluconic acid and ethanol to produce acetic acid.

Meanwhile, we carried out the detection of organic acids and vitamins present in the kombucha based on reverse-phase HPLC separation combined with UV-detection. The best results were obtained with a Luna C_{18} column 5-µm particle size, 250 mm length and 4.6mm i.d (phenomenex), using phosphoric acid (0.1%) and acetonitrile as the mobile phase for acetic acid, vitamin B_2, vitamin B_6, vitamin C and sodium sulphite (Na_2SO_4) as the mobile phase for malic acid, tartaric acid, vitamin B_1 with a 1ml/min flow rate, enabling the baseline separation of these constituents as per their respected time profiles. Absorption measurements at different wave lengths were selected, organic acids and vitamins were efficiently detected in the sample were comparable with retention times of standard compounds with ±0.2 to ±0.3 min variation.

The present study demonstrated that kombucha prepared from black tea has good antioxidant activities, and HPLC analysis of kombucha possesses acetic acid, malic acid, tartaric acid, vitamins B_1 B_2 B_6 and C as main constituents, among many other constituents as per previous reports. The precise mechanisms in the metabolic production of these organic acids and water soluble vitamins involved are still to be determined.

CONCLUSION

The present study demonstrated that kombucha 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. Although free-redical 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.

ACKNOWLEDGEMENTS

We would like to thank the Dr. NTR University of Health Sciences, Vijayawada, Andhra Pradesh, India for the Research Fellowship to T. Srihari.

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