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Changes in content of organic acids and tea polyphenols during kombucha tea fermentation

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Abstract

Kombucha tea is a fermented tea beverage produced by fermenting sugared black tea with tea fungus (kombucha). Tea polyphenols which includes (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG) and theaflavin (TF) have been reported to possess various biological activities. The present study focused on changes in content of organic acid and tea polyphenols in kombucha tea prepared from green tea (GTK), black tea (BTK) and tea manufacture waste (TWK) during fermentation. Concentration of acetic acid has reached maximum up to 9.5 g/l in GTK on 15th day and glucuronic acid concentration was reached maximum upto 2.3 g/l in BTK on 12th day of fermentation. Very less concentration of lactic acid was observed during the fermentation period and citric acid was detected only on 3rd day of fermentation in GTK and BTK but not in TWK. When compared to BTK and TWK very less degradation of EGCG (18%) and ECG (23%) was observed in GTK. TF and thearubigen (TR) were relatively stable when compared to epicatechin isomers. The biodegradation of tea catechins, TF and TR during kombucha fermentation might be due to some unknown enzymes excreted by yeasts and bacteria in kombucha culture.

Keywords: Kombucha; Tea fungus; Tea manufacture waste; Tea polyphenols

1. Introduction

Tea is grown in about 30 countries and is the most widely consumed beverage in the world, next to water. Tea is manufactured as green, black or oolong; black tea represents approximately 80% of tea products (Stoner & Mukhtar, 1995). Epidemiological studies suggest a protective effect of tea consumption on human cancer. Polyphenolic compounds (catechins) present in tea are capable of affording protection against cancer. (-)-Epicatechin (EC), (-)-epicatechin-3- gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG) are the four major polyphenolic derivatives present in green tea. 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 (Stoner & Mukhtar, 1995; Yang, Maliakal, & Meng, 2002; Yang, Prabhu, & Landau, 2001; Yang & Wang, 1993). Brewed tea is also found to contain significant levels of the catechins flavonoids. 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 (Bronner & Beecher, 1981).

Kombucha tea is sugared black tea fermented with a symbiotic association of acetic acid bacteria and yeasts forming "tea fungus" for about 14 days. Kombucha tea 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.

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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 is 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 hygiene 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 (SaiRam et al., 2000). Tea production waste or tea manufacture waste is dry straw and fiber of tea leaves resulting from the black tea production process. It has been reported that tea manufacture waste mixed with peat can be used as a practicable casing material for mushroom production (Gulser & Peksen, 2003).

Since the tea polyphenols are important in preventing cancer, it is therefore necessary to study the content of tea polyphenols during kombucha fermentation. Although catechins degradation in green tea, canned and bottled tea drinks have been reported (Chen, Zhu, Wong, Zhang, & Chung, 1998; Chen, Zhu, Tsang, & Huang, 2001; Su, Leung, Huang, & Chen, 2003; Zhu, Zhang, Tsang, Huang, & Chen, 1997) there is no study to date that has examined the stability of tea catechins and theaflavin during kombucha fermentation. This work will focus mainly on the changes in content of organic acids, epicatechin isomers (EGCG, EGC, ECG and EC), theaflavin and thearubigins in kombucha tea prepared from green tea (GTK), black tea (BTK) and tea manufacture waste (TWK) during tea fungus fermentation.

2. Materials and methods

2.1. Tea

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.

2.2. Chemicals

Epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals and solvents were high-analytical grade ones.

2.3. Analysis

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.

2.4. Starter culture

Starter culture or tea fungal mat was collected from local people of Coimbatore, Tamil Nadu, India and was maintained in sugared black tea.

2.5. 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. Two hundred ml of tea was poured into 500 ml glass jars that had been previously sterilized at 121 °C for 20 min. The cool 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.

2.6. Sampling

Sampling was performed periodically; each jar was sampled only once in order to avoid potential contamination. One black tea, green tea and tea manufacture waste glass jar per day were taken for the analysis of tea catechins, theaflavin, thearubigin, pH, organic acids, and protein and for total count of yeast and bacteria. The fermented tea was centrifuged at 10,000 rpm for 10 min and taken for the analysis. All analyses were carried out in triplicate.

2.7. Determination of pH

The pH of the samples was measured with an electronic pH meter (Orion model 290A).

2.8. HPLC analysis of organic acids

Filtered sample (5 ml) was passed through membrane filter (0.45 μM) into HPLC vials. The filtrate obtained was subjected to analysis of D-glucuronic acid, acetic acid, lactic acid and citric acid by HPLC. A 10 μl sample of fil-

trate was injected to a HPLC system equipped with a diode array detector. Phenomenex Luna C-18(2) column (4.6 mm ID × 25 cm, 5 μm) was used for the analysis. The mobile phase was a mixture of 20 mM potassium dihydrogen phosphate, pH 2.4 and methanol (97:3). The flow rate and column temperature was maintained as 1.0 ml/min and 28 °C, respectively. Detection was carried out at 220 nm. The resolution peaks were recorded on the HPLC chart according to the retention time of each compound. The concentrations of organic acids were quantified from standard curves.

2.9. Protein estimation

Protein content in the fermented tea broth was determined according to the method as previously described using bovine serum albumin as standard (Lowry, Rosenbrough, Farr, & Randall, 1951).

2.10. Microbiological analysis

Enumeration of yeasts and acetic acid bacteria was performed on OGYA (oxytetracycline glucose-yeast extract agar) containing oxytetracycline and nutrient agar containing 4 mg of cycloheximide per liter, respectively. Samples were plated on the respective medium and were incubated at 30 °C for acetic acid producing bacteria and at 25 °C for yeasts. (Sreeramulu et al., 2000)

2.11. HPLC analysis of tea polyphenols

Tea catechins (EC, ECG, EGC and EGCG) in kombucha tea during fermentation period were measured by the method as previously described (Anon, 1999). 5 ml of sample was extracted with 25 ml methanol. The contents were filtered through membrane filter (0.45 μM) into HPLC vials. The filtrate obtained was subjected to analysis by HPLC. A 10 μl sample of filtrate was injected to a Shimadzu (Kyoto, Japan) HPLC system equipped with a diode array detector (SPD-M10Avp). Phenomenex Luna C-18(2) column (4.6 mm ID × 25 cm, 5 μm) was used for the analysis. The mobile phase was a mixture of 0.1% orthophosphoric acid (A) and acetonitrile (B). The gradient used was: 0–12 min, 15% B; 12–22 min, 25% B; 22–30 min, 15% B. The flow rate and column temperature was maintained as 1.0 ml/min and 35 °C, respectively. Tea polyphenols were detected at 280 nm. The resolution peaks were recorded on the HPLC chart according to the retention time of each compound. The concentrations of tea polyphenols were quantified from standard curves.

2.12. Analysis of theaflavin (TF) and thearubigins (TR)

Content of theaflavin and thearubigins in fermented tea broth were estimated by the method of Takeo and Oosawa (1976) as modified by Ramaswamy (1978) and Thanaraj and Seshadri (1990). Twenty five ml of fermented tea broth was extracted with 25 ml of isobutyl methyl ketone (IBMK) at room temperature. After phase separation, 1 ml of upper IBMK phase was mixed with 9 ml of 45% ethanol and absorbance was measured at 380 nm (A) and the aqueous phase was retained for thearubigins estimation. Ten ml of IBMK phase was extracted with 10 ml of 2.5% disodium hydrogen phosphate. After extraction and phase separation, 1 ml of IBMK phase was mixed with 9 ml of 45% ethanol and absorbance was measured at 380 nm (B). 10 ml of aqueous phase from the first step was extracted with 10 ml of *n*-butanol. After phase separation, 1 ml of *n*-butanol layer was mixed with 9 ml of 45% ethanol and absorbance was measured at 380 nm (C). Concentration of theaflavin and thearubigins was calculated from the absorbance values as given below.

$$\text{TF}\ (\%) = 4.313 \times \text{B} \quad (1)$$

$$\text{TR}\ (\%) = 13.643(\text{A} + \text{C} - \text{B}) \quad (2)$$

Multiplication factors for the calculation of TF and TR were derived from molar extinction coefficients of pure compounds and dilution factors (Roberts & Smith, 1963).

3. Results and discussion

Until now there have been few reports about the influence of microbial activity on the component changes during kombucha fermentation. It has been shown that the composition of different kombucha preparations is greatly affected by the individual tea fungus culture used. This probably results from the variability of the normal microflora present in different tea fungus samples (Blanc, 1996; Chen & Liu, 2000; Reiss, 1994).

3.1. pH

The pH of BTK, GTK and TWK was decreased with fermentation time (Fig. 1). During the fermentation process, bacteria and yeasts metabolize sucrose into a number of organic acids such as acetic acid and glucuronic acid.

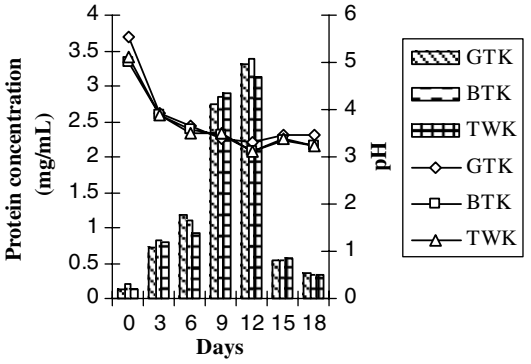

Fig. 1. Changes in protein content and pH during kombucha fermentation: GTK = green tea kombucha; BTK = black tea kombucha; TWK = tea waste kombucha. Data are expressed as mean ± SD of $n = 3$ samples.

Due to an increased concentration of these organic aids, the pH decreased from 5 to 3 within 12 days of fermentation. A slight increase in pH was observed after 12th day. These observations are in agreement with the findings of other studies (Chen & Liu, 2000; Reiss, 1994; Sreeramulu et al., 2000).

3.2. Organic acids

The production of organic acids, varying with time is shown in Tables 1–3. The concentration of acetic acid increased slowly with time until a maximum of 9.5 g/l in GTK on 15th day of fermentation followed by a slow decrease. L-glucuronic acid was the other major organic acid found in the metabolites of tea fungus and it reached a maximum concentration of 2.3 g/l in BTK on 12th day of fermentation. Glucuronic acid is considered to be one of the important key components found in kombucha tea due to its detoxifying action through conjugation (Loncar, Petrovic, Malbasa, & Verac, 2000). Concentration of lactic acid and citric acid was very less in all three types of kombucha tea studied. Maximum concentration of lactic acid was detected on 3rd day of fermentation in GTK followed by BTK and TWK. Citric acid was detected only on 3rd day of fermentation in GTK and BTK, and it was not detected in TWK throughout the fermentation period. Yeast cells hydrolyse sucrose into glucose and fructose by yeast invertase and produce ethanol via glycolysis with preference for fructose as substrate. Acetic acid bacteria utilize glucose to produce gluconic acid and ethanol to produce acetic acid. One of the possible ways of glucose transformation is also its oxidation at C-6 position into glucuronic acid. Lactic acid is formed by the acetic acid bacteria from ethanol and acetic acid (Dufresne & Farnworth, 2000). Green tea and black tea was found to be the best substrate for acetic acid and glucuronic acid production respectively by kombucha culture.

Table 1
Changes in content of organic acids in green tea during kombucha fermentation

Organic acids (g/l)	Fermentation days						
	0[a]	3	6	9	12	15	18
Acetic acid	ND	0.22 ± 0.1[b]	1.64 ± 0.06	3 ± 0.15	3.37 ± 0.39	9.51 ± 0.35	8.36 ± 0.87
D-Glucuronic acid	1.05 ± 0.19	1.63 ± 0.29	1.86 ± 0.31	1.39 ± 0.2	1.73 ± 0.14	1.57 ± 0.14	1.73 ± 0.09
Lactic acid	ND	0.54 ± 0.06	0.15 ± 0.02	0.13 ± 0.025	0.12 ± 0.01	0.15 ± 0.02	0.12 ± 0.02
Citric acid	ND	0.03 ± 0.01	ND	ND	ND	ND	ND

ND = Not detected.
[a] Number indicates the day when sampling was performed.
[b] Values are mean ± standard deviation; $n = 3$ samples.

Table 2
Changes in content of organic acids in black tea during kombucha fermentation

Organic acids (g/l)	Fermentation days						
	0[a]	3	6	9	12	15	18
Acetic acid	ND	0.33 ± 0.07[b]	1.5 ± 0.16	2.44 ± 0.3	4.72 ± 0.84	6.17 ± 0.3	4.69 ± 0.54
D-Glucuronic acid	0.94 ± 0.09	1.08 ± 0.03	1.38 ± 0.16	1.69 ± 0.21	2.33 ± 0.24	1.5 ± 0.17	1.71 ± 0.1
Lactic acid	ND	0.44 ± 0.08	0.32 ± 0.08	0.25 ± 0.07	0.24 ± 0.1	0.33 ± 0.07	0.18 ± 0.08
Citric acid	ND	0.11 ± 0.06	ND	ND	ND	ND	ND

ND = Not detected.
[a] Number indicates the day when sampling was performed.
[b] Values are mean ± standard deviation; $n = 3$ samples.

Table 3
Changes in content of organic acids in tea manufacture waste tea during kombucha fermentation

Organic acids (g/l)	Fermentation days						
	0[a]	3	6	9	12	15	18
Acetic acid	ND	0.32 ± 0.12[b]	1.32 ± 0.11	2.52 ± 0.53	3.75 ± 0.48	5.72 ± 0.41	5.37 ± 0.66
D-Glucuronic acid	0.88 ± 0.28	1.08 ± 0.45	1.26 ± 0.25	1.41 ± 0.32	1.63 ± 0.32	1.16 ± 0.21	1.22 ± 0.22
Lactic acid	ND	0.173 ± 0.07	0.223 ± 0.066	0.203 ± 0.061	0.196 ± 0.04	0.173 ± 0.02	0.18 ± 0.01
Citric acid	ND	ND	ND	ND	ND	ND	ND

ND = Not detected.
[a] Number indicates the day when sampling was performed.
[b] Values are mean ± standard deviation; $n = 3$ samples.

3.3. Protein content

Although no other nitrogen source was added to the tea before fermentation, the protein level increased rapidly with fermentation time. These proteins likely represent extra cellular proteins secreted by bacteria and yeasts during the fermentation time (Sreeramulu et al., 2000). The protein content was rapidly increased from 0.1 to 3.0 mg/ml through 12 days of fermentation and thereafter continued to decrease due to decrease in extracellular proteins secreted by the bacteria and yeast (Fig. 1).

3.4. Microbiological analysis

The total count of bacteria and yeast were increased rapidly until 9 days of fermentation and thereafter continued to decrease (Fig. 2). The decreased number of bacteria and yeast after 9 days of fermentation was likely caused by acid shock (low pH), which influenced the multiplication of bacteria and yeast. Chen and Liu (2000) reported that anaerobic and starved environment created could also be the reason for the decrease in microbial content during the fermentation period. Carbon dioxide generated as a result of alcohol fermentation by yeasts accumulated in the interface between the pellicle and broth. This separates the pellicle from the broth and creates an anaerobic and starved environment due to block of transfer of nutrients from broth to pellicle and transfer of oxygen from the surface of the pellicle to broth.

3.5. Tea polyphenols

Four epicatechin isomers (EGCG, EGC, ECG and EC) examined demonstrated varying stability during kombucha fermentation (Fig. 3). It was found that catechins in tea during kombucha fermentation were degraded. All catechins studied were degraded upto 9th day of kombucha fermentation and they showed marked increase on 12th day. Release of catechins from the acid-sensitive microbial cells might be the reason for the increased concentration of epicatechin isomers observed on 12th day. Degradation of

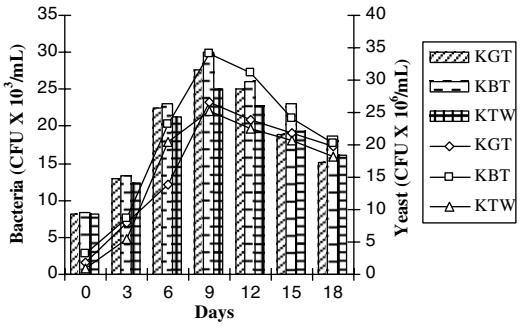

Fig. 2. Changes in total bacterial count and total yeast count during kombucha fermentation: GTK = green tea kombucha; BTK = black tea kombucha; TWK = tea waste kombucha. Data are expressed as mean ± SD of $n = 3$ samples.

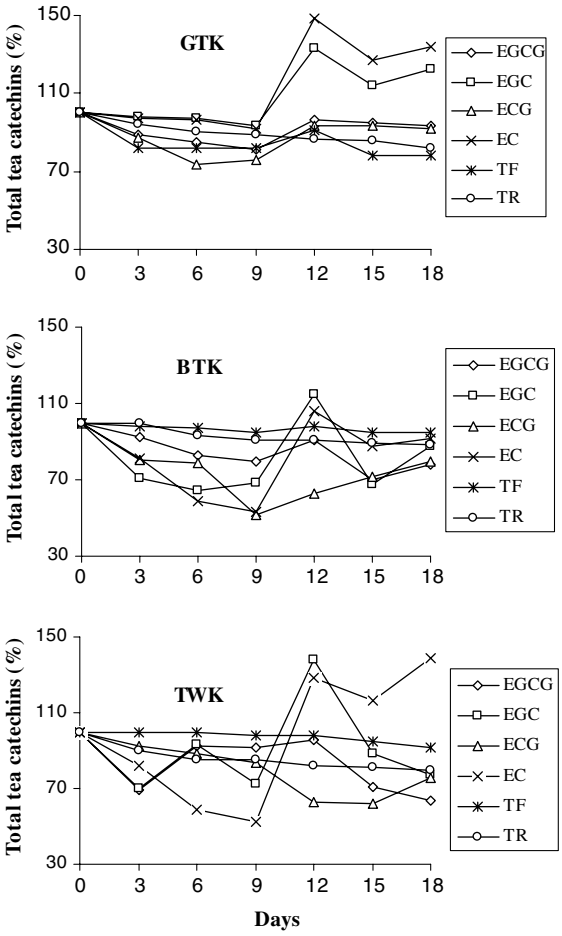

Fig. 3. Stability of tea catechins during kombucha fermentation: GTK = green tea kombucha; BTK = glack tea kombucha; TWK = tea waste kombucha. Data are expressed as mean ± SD of $n = 3$ samples.

EGCG was very less in GTK (18%) than in BTK (30%) and TWK (30%). Likewise, degradation of ECG was also very less in GTK (23%) when compared to BTK (48%) and TWK (37%). It is interesting to note that concentration of EGC and EC exceeds the initial concentration on 12th day of kombucha fermentation while it was not observed for EGCG and ECG. It is assumed that EGCG and ECG were converted to their corresponding catechin EGC and EC. Zhu et al. (1997) reported that conversion of EGCG to EGC was undetected when pure EGCG was incubated alone in both acidic and alkaline pH. Biotransformation of EGCG to EGC and ECG to EC by enzymes excreted by microorganisms in kombucha culture could be the reason for the increased concentration of EGC and EC observed on 12th day. Several reports on stability of tea catechins in alkaline solutions and acidic solutions are available in the literature (Chen et al., 2001; Su et al., 2003; Zhu et al., 1997). The stability of catechins from green tea is pH-dependent; they are very unstable in alkaline solution but stable in acidic solution (Zhu et al., 1997). Uniform degradation was observed for TF and TR during kombucha fermentation (Fig. 3). 5% of TF and 11% of TR were lost when the kombucha fermentation

was extended upto 18 days in black tea. Results revealed that TF and TR were very stable when compared to epicatechin isomers during kombucha fermentation. Su et al. (2003) reported that green tea catechins and theaflavin have short term stability in the tea drink with pH 5 or less. They have also reported the pH-dependent stability of TF and green tea catechins. The yeast *Candida tropicalis* was employed as polyphenol degrader in which the induced expression of peroxisomal enzyme such as catalase was the predominant factor (Ettayebi et al., 2003). Moreover, degradation of dietary polyphenol in the colon was regarded as the major pathway of metabolism with gut *Clostridium*, *Bacteroides* and *Eubacterium*. These gut bacteria were capable of cleaving the C ring of flavonoids and released phenolic acid as 3-(4-hydroxy phenyl)-propionic acid and 3-hydroxy phenyl-acetic acid, etc. (Rechner et al., 2004). It was therefore possible that yeasts and bacteria in kombucha secreted some unknown enzymes that were capable of catalyzing the biodegradation of tea catechins, TF and TR.

4. Conclusion

Tea polyphenols (EGCG, EGC, ECG, EC, TF and TR) and organic acids are believed to be the active ingredients in kombucha tea that possess a range of beneficial effects. The present study examined the changes in content of tea polyphenols and organic acids along with pH, protein and microbial content in green tea, black tea and tea manufacture waste tea during kombucha fermentation. Acetic acid and glucuronic acid were reached maximum on 15th (in GTK) and 12th (in BTK) day of fermentation respectively. The present study is the first to report the stability of tea polyphenols during kombucha fermentation. Results showed that TF and TR were relatively stable than epicatechin isomers during kombucha fermentation. Epicatechin isomers were degraded upto 9th day of fermentation and a marked increase on 12th day was observed. Interestingly, content of EGC and EC on 12th day was higher than the initial concentration. Biotransformation of EGCG to EGC and ECG to EC by enzymes excreted by microorganisms in kombucha culture and release of catechins from acid-sensitive cells could be the reason for the increased concentration of tea polyphenols on 12th day of fermentation. Identification and characterization of extra cellular key enzymes responsible for the degradation of epicatechin isomers, TF and TR is in progress. The present study also revealed the possibility of using tea waste material for manufacturing the kombucha tea beverage.

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