J. Microbiol. Biotechnol. (2009), **19**(4), 397–402
doi: 10.4014/jmb.0806.374
First published online 30 January 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Hepatoprotective and Curative Properties of Kombucha Tea Against Carbon Tetrachloride-Induced Toxicity

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Received: June 16, 2008 / Revised: November 3, 2008 / Accepted: December 8, 2008

Kombucha tea (KT) is sugared black tea fermented with a symbiotic culture of acetic acid bacteria and yeasts, which is said to be tea fungus. KT is claimed to have various beneficial effects on human health, but there is very little scientific evidence available in the literature. In the present study, KT along with black tea (BT) and black tea manufactured with tea fungus enzymes (enzyme-processed tea, ET) were evaluated for hepatoprotective and curative properties against CCl_4-induced toxicity, using male albino rats as an experimental model by analyzing aspartate transaminase, alanine transaminase, and alkaline phosphatase in plasma and malondialdehyde content in plasma and liver tissues. Histopathological analysis of liver tissue was also included. Results showed that BT, ET, and KT have the potential to revert the CCl_4-induced hepatotoxicity. Among the three types of teas tried, KT was found to be more efficient than BT and ET. Antioxidant molecules produced during the fermentation period could be the reason for the efficient hepatoprotective and curative properties of KT against CCl_4-induced hepatotoxicity.

Keywords: Kombucha tea, tea fungus, carbon tetrachloride

Carbon tetrachloride (CCl_4) is an extensively studied xenobiotic that induces lipid peroxidation and toxicity [18]. Liver cell injury induced by CCl_4 involves initially the metabolism of CCl_4 to trichloromethyl (CCl_3–) free radical by the mixed function oxidase system of the endoplasmic reticulum. The secondary mechanism could involve the generation of toxic products arising directly from CCl_4 metabolism or from peroxidative degeneration of membrane phospholipids, and causes functional and morphological changes in the cell membrane leading to accumulation of lipid-derived oxidants causing liver injury. Moreover, reactive oxygen intermediates (ROIs) generated in the hepatocytes as by-products of CCl_4 metabolism and excess of ROIs, oxidative stress, contribute to cell injury. CCl_4-induced damage also produces alteration in the antioxidant status of the tissues, which is manifested by abnormal histopathological changes. Several studies have previously demonstrated that antioxidants prevent CCl_4 toxicity, particularly hepatotoxicty, by inhibiting lipid peroxidation and increasing antioxidant enzyme activities [20].

Kombucha tea is sugared black tea fermented with tea fungus for about 14 days. Tea fungus is a symbiotic association of bacteria and yeasts. Kombucha tea is composed of two portions; a floating cellulosic pellicle layer and the under lying sour liquid broth, which tastes slightly sweet and acidic. Kombucha tea is claimed to have many beneficial effects to human health but only very few scientific evidences are available in the literature. Recent studies have suggested that kombucha tea prevents paracetamol-induced hepatotoxicity [25] and chromate (VI)-induced oxidative stress in albino rats [27]. As kombucha tea is rich in compounds known to be strong antioxidants, it is expected to ameliorate liver damage induced by CCl_4. Hence, in the present study, the prophylactic and curative effects of kombucha tea against CCl_4 induced hepatotoxicity were compared with black tea (BT) prepared by the conventional CTC process and tea fungal enzyme-processed tea (ET), using male albino rats as an experimental model.

Materials and Methods

Preparation of Black Tea

BT manufactured from Assam UPASI-3 cultivar at UPASI (The United Planters' Association of Southern India), Nirar Dam BPO, Valparai 642 127, Coimbatore District, Tamil Nadu, India was used. To 1 l of boiled distilled water, 100 g of sucrose and 10.5 g of black

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tea were added and mixed for 5 min. Then, the mixture was filtered and the filtrate was used as black tea.

Preparation of Enzyme Processed Tea

ET was manufactured by incorporating purified tea fungal cellulase and laccase (3:1 ratio) during tea leaf fermentation (Assam UPASI-3 cultivar). To 1 l of boiled distilled water, 100 g of sucrose and 10.5 g of enzyme-processed tea were added and mixed for 5 min. Then, the mixture was filtered and the filtrate was used as ET.

Preparation of Kombucha Tea

The tea fungus (a symbiont of two yeasts, *Pichia* sp. NRRL Y-4810 and *Zygosaccharomyces* sp. NRRL Y- 4882, and a bacterium, *Acetobacter* sp. NRRL B-2357) was obtained from the tribal people of Kolli Hills, Tamil Nadu. Tea fungus was grown and maintained in tea medium [13]. The cooled tea (BT) was poured into 500-ml glass jars that had been previously sterilized at 121°C for 20 min and inoculated with 2.5% (w/v) of freshly grown tea fungus that had been cultured in the same medium for 14 days and 20% (v/v) of previously fermented liquid tea broth aseptically. The jars were covered with clean cheesecloths and fixed with rubber bands. The fermentation was carried out under room temperature (28±2°C) for 14 days. New tea fungal mat develops over the mother culture. The tea fungal mat can either be used as starter culture or discarded.

Gas Chromatographic Analysis of Kombucha Tea

A 2-ml fraction of kombucha tea was injected into a Hitatchi G-3000 gas chromatography equipped with a flame ionization detector. A stainless steel column (2 m×2 mm) packed with Porapack Q was used for separation. The column, injector, and detector temperatures were 80, 40, and 120°C, respectively. Nitrogen gas was used as the carrier gas at a flow rate of 15 ml/min [10].

Animals and Treatments

Animal experiments were performed according to the ethical guidelines suggested by the Institutional Animal Ethics Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Culture, Government of India. Ethical care and treatment of animals were undertaken following the guidelines of the Animal Care and Ethics Committee of I.R.T. Perundurai Medical College and Research Center, Perundurai, Tamil Nadu, India (588/02/A/CPCSEA) [6]. Five-week-old male albino rats weighing about 150–180 g were used. Rats were segregated into groups of six animals each. Each group was subjected to one of the following treatments described in the next three sections.

Control Animals

Animals in group 1 were fed with normal diet and drinking water *ad libitum*. Animals in groups 2, 3, and 4 were fed with normal diet along with BT, ET, and KT (2.5 ml/kg body weight), respectively, for 30 days. Animals in group 5 were fed with normal diet and drinking water *ad libitum* for 30 days followed by a single dose (intraperitoneal injection) of CCl_4 (2 ml/kg body weight). Rats in group 1 to 5 were sacrificed on the 30th day by decapitation, and then blood and liver samples were collected.

Preventive Effect

Animals in groups 6, 7, and 8 were fed respectively with BT, ET, and KT (2.5 ml/kg body weight) and normal diet for 30 days followed by a single dose (intraperitoneal injection) of CCl_4 (2 ml/kg body weight). After 24 h of CCl_4 injection to rats in groups 6, 7, and 8, they were sacrificed by decapitation, and then blood and liver samples were collected.

Curative Effect

Animals in group 9 were fed with normal diet and were administered orally with CCl_4 (2.5 ml/kg body weight) twice a week for 30 days. Animals in groups 10, 11, and 12 were administered orally with CCl_4 (2.5 ml/kg body weight) twice a week for 30 days along with normal diet followed by feeding with BT, ET, and KT (2.5 ml/kg body weight), respectively. Blood was collected from rats in groups 9, 10, 11, and 12 on the 10th and 20th days of treatment *via* the retro-orbital plexus. They were sacrificed on the 30th day by decapitation, and then blood and liver samples were collected. This treatment was considered as curative treatment.

Enzyme Analysis and Histopathological Studies

The blood samples were collected directly from the animals by heart puncturing and mixed with the anticoagulant, heparin. The blood samples were centrifuged at 10,000 rpm for 15 min and the clear plasma were collected and stored in a refrigerator. The levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and malondialdehyde (MDA) in plasma were analyzed. Livers were excised from the rats and one portion was used to analyze the MDA content. Another portion was used for histopathological studies. Five μm sections were prepared from the liver tissue, and sections were stained with hematoxylin and eosin dye [14] and examined microscopically for cell abnormalities. ALT and AST were assayed by the method of Reitman and Frankel [26]. ALP was assayed by the method of King and Armstrong [19]. MDA was estimated by the method of Nichans and Samuelson [24].

Statistical Analysis

To assess the significant level of influence caused by tea preparations in CCl_4-injected rats, Duncan's multiple range test (DMRT) was used [7].

RESULTS

Gas Chromatographic Analysis

Gas chromatography analysis revealed that black tea fermented with tea fungus for 14 days contains acetic acid of 1.60 g/100 ml; succinic acid, 0.65 g/100 ml; glucuronic acid, 0.38 g/100 ml; gluconic acid, 0.20 g/100 ml; and ethanol, 0.60 g/100 ml. The results of the present study were in agreement with the results of Blanc [1] and Jayabalan *et al.* [17]. It has been reported that kombucha tea contains a wide range of organic acids (acetic acid, glucuronic acid, gluconic acid, usnic acid, citric acid, oxalic acid, malic acid, lactic acid), vitamins (B1, B2 , B6, B12, and C), and ethanol [1].

Hepatoprotective Property

Carbon tetrachloride is the best-characterized system of xenobiotic-induced hepatotoxicity and is frequently employed

Table 1. Preventive effect of kombucha tea on CCl_4-induced hepatotoxicty.

Groups	Plasma			Liver	
	AST (IU/l)	ALT (IU/l)	ALP (IU/l)	MDA (nmol/ml)	MDA (nmol/g wet liver)
1 (Control)	120	62	73	1.68	263
2 (BT)	122	62	73	1.69	263
3 (ET)	122	61	73	1.69	263
4 (KT)	121	60	73	1.69	263
5 (CCl_4)	4,735	4,220	180	5.02	542
6 (BT+CCl_4)	$1,600^{cA}$	$1,100^{c}$	90^{c}	1.50^{c}	200^{c}
7 (ET+CCl_4)	$1,580^{b}$	$1,068^{b}$	60^{b}	1.47^{b}	193^{b}
8 (KT+CCl_4)	$1,200^{a}$	720^{a}	38^{a}	1.30^{a}	150^{a}

AST: Aspartate transaminase; ALT: alanine transaminase;
ALP: alkaline phohsphatase; MDA: Malondialdehyde.
BT: Black tea; ET: Enzyme-processed tea; KT: Kombucha tea.
a, b, and c, Means with the same letter are not significantly different from each other at the 5% probability level by DMRT.

as a model to study antihepatotoxic/hepatoprotective activity of the drugs. It is metabolized in the body to a higly reactive trichloromethyl radical, which attacks membrane phospholipid-stimulating lipid peroxidation and cell lysis [2]. As a result of cell lysis, CCl_4 releases cytoplasmic hepatic enzymes into the blood circulation, resulting in the increased levels of ALT, AST, and ALP, which indicate the liver cell damage. Determination and evaluation of these parameters in the serum and tissue samples of experimental animals are used to assess the hepatotoxicity and inhibitory effects of the test drugs, as an indicator of antihepatotoxic or hepatoprotective activity.

In the present study, liver toxicity was estimated by the levels of hepatic enzymes, ALT, AST, and ALP, in plasma and malondialdehyde (MDA) levels in plasma and liver tissues. In control animals, ALT, AST, and ALP levels were 62 IU/ml, 120 IU/ml, and 73 IU/ml, respectively. The MDA levels were 1.68 and 263 nmol/ml for plasma and liver tissue, respectively. No variation was observed in the levels of marker enzymes in plasma and tissue MDA levels in test animals fed with tea preparations alone (Table 1).

Preventive Effect

In preventive treatments, CCl_4 administration (intraperitoneal) elevated the levels of these enzymes to 4220, 4735, and 180 IU/ml for ALT, AST, and ALP respectively and 5.02 nmol/ml and 542 nmol/g wet liver for NDA content in plasma and liver tissue, respectively. But in animals fed with tea preparations, these enzyme levels were found to be very low. In KT fed animals 82.94%, 74.66%, and 78.89% reduction was observed in ALT, AST and ALP levels respectively when compared to unfed and CCl_4 administered (intraperitoneal) animals. In ET fed animals, 74.69%, 66.63%, and 66.67% reduction was observed and in BT feeding the reduction was 73.93%, 62.20%, and 50.0% respectively (Table 1).

Curative Effect

In curative treatments, the marker enzymes, ALT, AST, ALP and MDA levels were analyzed on 10^{th}, 20^{th}, and 30^{th} day. The level of hepatic enzymes tends to decrease with increasing treatment periods. Animals orally fed with $CC1_4$ had high levels of ALT, AST, ALP and MDA levels on 10^{th} day. On 10^{th} day the ALT level was 4486 IU/ml,

Table 2. Curative effect of kombucha tea on CCl_4-induced hepatotoxicty.

	AST (IU/l)			ALT (IU/l)			ALP (IU/l)			MDA plasma (nmol/ml)			MDA liver (nmol/g liver)		
Days	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30
Groups															
9 (CCl_4)	4,650	4,942	4,930	4,486	4,480	4,475	215	210	203	6.25	6.23	6.2	610	604	598
10 (BT)	$1,850^{cA}$	$1,768^{c}$	$1,700^{c}$	$1,600^{c}$	$1,575^{c}$	$1,560^{c}$	98^{c}	85^{c}	78^{c}	2.85^{c}	2.80^{c}	2.72^{c}	280^{c}	268^{c}	250^{c}
11 (ET)	$1,843^{b}$	$1,751^{b}$	$1,682^{b}$	$1,585^{b}$	$1,555^{b}$	$1,513^{b}$	82^{b}	69^{b}	57^{b}	2.71^{b}	2.65^{b}	2.50^{b}	268^{b}	252^{b}	228^{b}
12 (KT)	$1,600^{a}$	$1,525^{a}$	$1,402^{a}$	$1,502^{a}$	$1,400^{a}$	$1,425^{a}$	77^{a}	62^{a}	49^{a}	2.00^{a}	1.90^{a}	1.75^{a}	240^{a}	225^{a}	204^{a}

AST: Aspartate transaminase; ALT: alanine transaminase; ALP: alkaline phosphatase.
MDA: malondialdehyde.
BT: Black tea; ET: Enzyme-processed tea; KT: Kombucha tea.
a, b, and c, Means with the same letter are not significantly different from each other at the 5% probability level by DMRT.

AST was 4,950 IU/ml, and ALP was 215 IU/ml. After 30 days of treatment, KT reduced the levels of these enzymes by 70.15%, 71.56%, and 75.86%, respectively; ET reduced the enzyme levels by 66.19%, 65.88%, and 71.92%, and BT by 65.14%, 65.52%, and 61.58%, respectively. MDA levels in plasma were reduced by 71.77%, 59.68%, and 56.13% and in liver tissues by 65.89%, 61.87%, and 58.19% by KT, ET, and BT, respectively. Among the three tea preparations, KT was found to be more effective in reducing the levels of hepatic enzymes and MDA levels. Among the two treatments, preventive treatment was more efficient than the curative treatment (Table 2). The efficiency of the three teas against hepatic injury (necrosis) in both the treatments was in the order of KT>ET>BT.

Histopathological studies reveal that the three teas, BT, ET, and KT, had no negative impact on the liver tissues and resemble the control (Fig. 1). CCl_4 administration induced the formation of macro and micro vesicles in the hepatic tissues. Feeding with KT exhibited a more pronounced reduction for the macro and micro vesicular zonal necrosis in preventive and curative treatments than BT and ET. The regeneration/reverting rate of necrotic tissues to normal condition was more pronounced in KT-fed animals than the animals fed with BT or ET (Figs. 2 and 3).

Discussion

In the present study, BT, ET, and KT were used as hepatoprotective and curative agents against CCl_4-induced hepatotoxicity in male albino rats. The sucrose in the medium was hydrolyzed to glucose and fructose by yeast invertase [4]. Glucose and fructose are utilized by yeasts *via* glycolysis and produce ethanol and glycerol. Part of the glucose was utilized by *Acetobacter xylinum* for the production of gluconic acid *via* the pentose phosphate pathway and cellulose biosynthesis. Mostly, the *Acetobacter* sp. are known to oxidize glucose and produce gluconate. The *Acetobacter* can utilize ethanol produced by yeasts for growth and produce acetic acid. The acetic acid production in turn induces the yeasts to produce ethanol. This type of symbiotic interaction can be seen between the yeast (*Saccharomyces cerevisiae*) and *Gluconobacter oxydans* in fermentation of orange juice [3]. The presence of glucuronic acid in kombucha tea was already reported by Jayabalan *et al.* [17]. Glucuronic acid is a potent detoxifying agent found in human systems, and its presence may be one of the reasons for the efficient activity of kombucha tea observed in the present study.

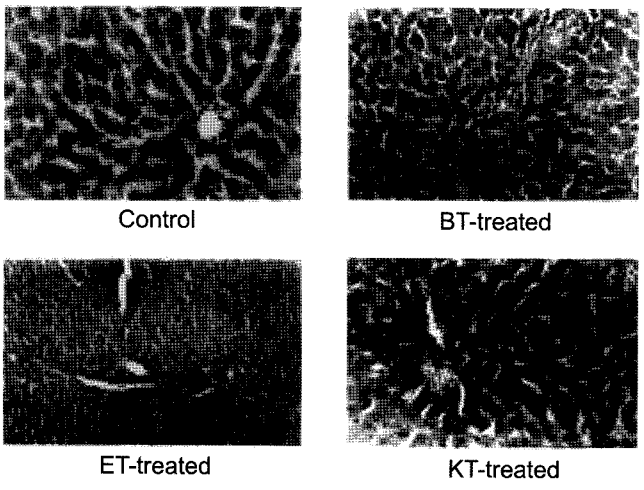

Fig. 1. Liver sections of control animals and rats treated with BT, ET, and KT alone.

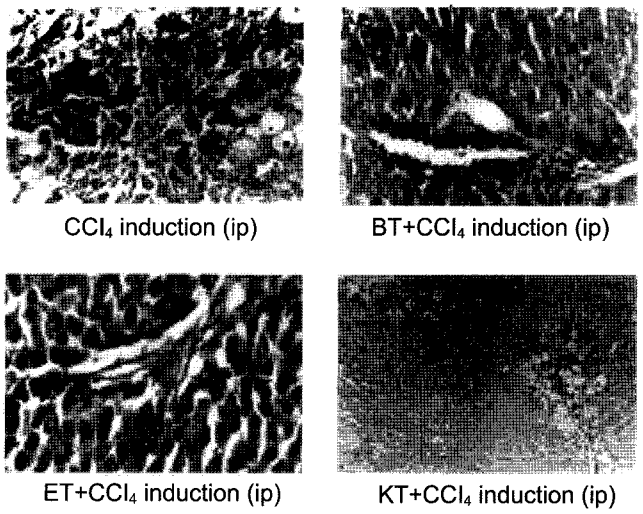

Fig. 2. Liver sections of rats treated with CCl_4 alone and rats fed with BT, ET, or KT followed by CCl_4 injection (preventive treatment).

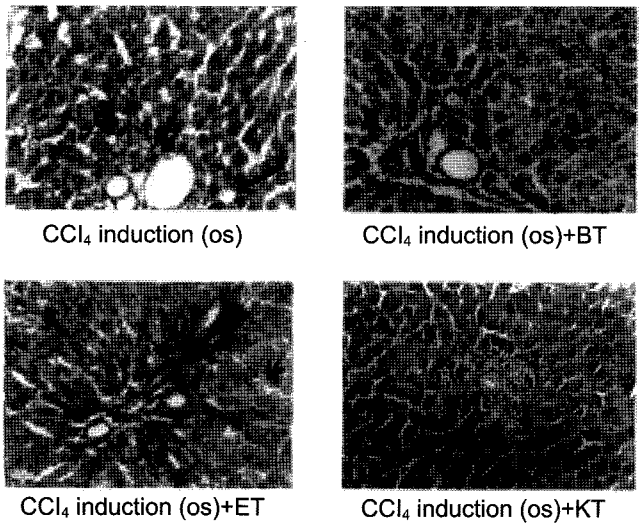

Fig. 3. Liver sections of rats treated with CCl_4 alone and rats injected with CCl_4 in a 10-day interval along with BT, ET, or KT feeding (curative treatment).

Fructose is converted into acetic acid and none to gluconic acid. However, *Acetobacter* is also capable of hydrolyzing sucrose, by levansucrase, into glucose and a polysaccharide of fructose, levan. The composition of the liquid broth determines the flavour and taste of tea fungus products. Volatile acetic acid produces an astringent and acidic flavour and the gluconic acid was mild. Kombucha beverage produced within 6–10 days of incubation has a refreshing fruit-like taste, but prolonged fermentation results in a vinegar-like flavour [4].

Many plant products have been reported to protect against hepatic injury. Zhen *et al.* [29] demonstrated that administration of the green tea polyphenol epigallocatechin-3-gallate was useful in the treatment and prevention of hepatic fibrosis. Nevin and Vijayammal [23] reported that a partially purified petroleum ether extractable fraction of the whole plant *Aerva lanata* contained antioxidant alkaloids capable of ameliorating the CCl_4-induced hepatic injury by virtue of its antioxidant activity. The results of Jain *et al.* [16] strongly indicated that *Momordica dioica* Roxb. leaves have potent hepatoprotective action against CCl_4-induced hepatic damage in rats. Yang *et al.* [28] demonstrated that pycogenol, a standardized extract from the bark of the French maritime pine (*Pinus maritima*), has a protective effect against acute hepatotoxicity induced by the administration of CCl_4 in rats, and that the hepatoprotective effects of pycogenol may be due to both the inhibition of lipid peroxidation and the increase of antioxidant activity. Lee *et al.* [21] demonstrated that the appropriate mixture of *Artemisiae capillaris* herba and *Picorrhiza* rhizome had favorable synergistic effects on CCl_4-induced subacute hepatic damage. The results of Lin *et al.* [22] suggest that a water extract of *Solanum nigrum* Linn could protect liver against the CCl_4-induced oxidative damage in rats, and this hepatoprotective effect might be attributed to its modulation on detoxification enzymes and its antioxidant and free-radical scavenger effects.

Different tea preparations have also been used as liver protectants. Gong *et al.* [11] reported that oral feeding of tea polyphenols and tea pigments (theaflavins and thearubigins) exhibited protective action against *N*-nitrosodiethylamine (NDEA) injections, followed by intraperitoneal CCl_4 injection. He *et al.* [12] reported that oral feeding of green tea (0.4–1.2 g/kg body wt) for two weeks, suppressed the lipopolysaccharide-induced liver injury in D-galactosamine sensitized rats by decreasing the plasma ALT and AST levels. Jadon *et al.* [15] revealed that 200 mg/kg dose of gallic acid could act as an effective drug against CCl_4-induced liver and kidney damage. Elhalwagy *et al.* [8] reported that supplementation of green tea extracts to rats partially ameliorates the toxic effect of fenitrothion pesticide on the liver and kidney tissues and their functions.

Tea contains polyphenols, flavanols (theaflavins and thearubigins), catechins, caffeine, adenine, theobromine, theophylline, xanthine, gallotannin, and small amounts of aminophylline. Theaflavin and thearubigins are products of the enzymatic oxidation of polyphenols. Most of the beneficial effects of tea have been attributed to the antioxidant and free-radical scavenging properties of tea components such as polyphenols and flavanols. Fadhel and Amran [9] reported that black tea components (0.7%) exhibit antioxidative effects against CCl_4-induced toxicity in liver of male and female rats. Black tea might act to prevent tissue damage by shielding cells from toxic agents and protecting them against cytotoxic agents, CCl_4, and its free radicals. Chu and Chen [5] reported that the average antioxidant potentials of kombucha tea after fermenting for 15 days were raised to about 17%, 40%, and 49%, respectively, as determined by the assays of DPPH, ABTS radical scavenging, and inhibition of linoleic acid peroxidation. They have also reported that the phenolic content was decreased, which implied that thearubigin might be subjected to biodegradation during fermentation, resulting in the release of smaller molecules with higher antioxidant activities. Jayabalan *et al.* [17] observed that 5% of theaflavin and 11% of thearubigin were lost during kombucha fermentation. The brown color of the black tea was mainly from the chromophoric group of thearubigin. Since the color intensity of the kombucha broth is decreasing during fermentation period, it is suggested that thearubigin undergoes microbial transformation. It has been reported that kombucha starters secreted some unknown enzymes, which are capable of catalyzing the biodegradation of thearubigin, and the hydrolysates were potent antioxidant molecules [5, 17].

When compared with these findings, the data obtained in the present study revealed that oral feeding of Kombucha tea expressed higher inhibitory activity towards CCl_4-induced hepatic injury as a preventive and curative agent in rats. Kombucha tea prepared by fermentation of black tea with tea fungus could be used as a preventive and curative agent against CCl_4-induced hepatotoxicity.

Acknowledgments

The research was partly supported by the Research Center for Industrial Development of Biofood Materials in the Chonbuk National University, Jeonju, Korea. The center is designated as a Regional Research Center appointed by the Ministry of Commerce, Industry and Energy (MOCIE), Jeollabuk-do Provincial Government and Chonbuk National University.

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