Thesis Title

Anti-inflammatory activity of Kombucha Tea

Thesis submitted in partial fulfilment of the requirement
for the degree of Master of Pharmacy
Under the guidance of

Prof. (Dr.) Sanmoy KarmaKar

Submitted by

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2016

Declaration of originality and compliance of academic ethics

The author, hereby, declares that this thesis contains original research work, as part of his Master of Pharmacy program. This indicates that the work was done entirely by the author and the elements in the thesis are not a copy of or similar to any other thesis submitted/published elsewhere.

All works were performed under the supervision of Prof. (Dr.) Sanmoy Karmakar at the Department of Pharmaceutical Technology, Jadavpur University.

All information in this document have been obtained and presented in accordance with academic rules and ethical conduct.

The author also declares that he had fully referenced and acknowledged all materials and results that are not original to this work, in the thesis.

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Certification

This is to certify that Mr. Arup Saha has carried out all research studies under my supervision at the Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India, for the thesis titled "**Anti-inflammatory activity of Kombucha Tea**." The ideas put into effect were original and are not a copy of or similar to any other thesis submitted/published elsewhere.

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Arup Saha

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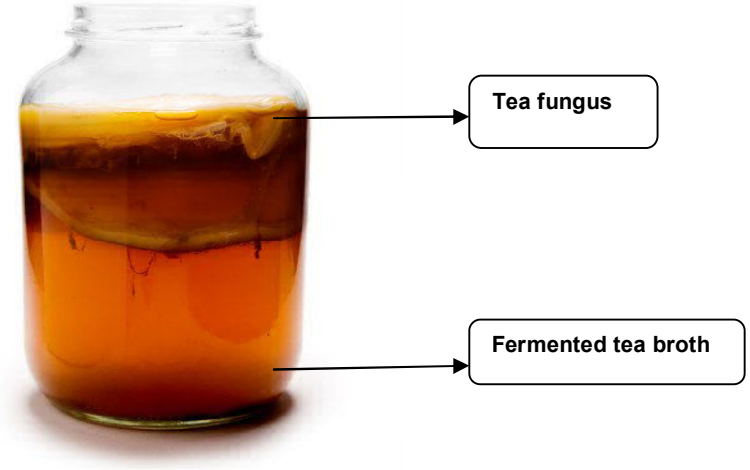
Dedicated to my beloved parents

Contents

1. INTRODUCTION

Tea is one of the most consumed beverages in the world. It is produced from the leaves of *Camellia sinensis* which is widely grown in the tropical humid climate of South East Asia. The type of tea produced depends on the length of fermentation of the leaves: green tea is not fermented, black tea is nearly completely fermented, while oolong tea is partially fermented (Sharma et al., 2007). Among these three, black tea is most popular, which accounts for 80% of the total tea consumption (Gardner et al., 2009). Several studies have shown association of tea consumption with reduced risk of chronic diseases particularly cardiovascular diseases and some types of cancer. Most of the beneficial effects of tea have been attributed to the antioxidant and free radical scavenging properties of its components like polyphenols and flavonoids (Hodgson and Croft, 2010). Kombucha tea, is another form of popular beverage. This particular type of tea is sweetened black tea which is then fermented by a consortium of yeast and acetic acid bacteria for about 14 days (Dutta and Gachhui, 2007). It is widely consumed in parts of the erstwhile Soviet Union and Central Asia, and has become popular even in Europe and the USA. The beverage has been claimed to be a prophylactic agent and is beneficial to human health (Tietze, 2000). Moreover, the polyphenols and flavonoids have been found to be higher in KT than BT. Recent studies have demonstrated that KT possesses anti-oxidant, antimicrobial, antistress, hepatoprotective, nephro-protective and hypocholesterolaemic properties, and provides relief/cure for gastric ulceration and even cancer (Bhattacharya et al., 2011). Furthermore, the US Food and Drug Administration surveying commercial producers of the starter (Kombucha mushroom or tea fungus) have found no pathogenic organisms or other hygienic violations with KT (FDA, 1995).

Fermentation of sugared tea with a symbiotic culture of acetic acid bacteria and yeast (tea fungus) yields kombucha tea which is consumed worldwide for its refreshing and beneficial properties on human health. Kombucha tea is a slightly sweet, slightly acidic refreshing beverage consumed worldwide. It is obtained from infusion of tea leaves by the fermentation of a symbiotic association of bacteria and yeasts forming “tea fungus”. A floating cellulosic pellicle layer and the sour liquid broth are the 2 portions of kombucha tea. It tastes like sparkling apple cider and can be produced in the home by fermentation using mail order or locally available tea fungus.



Chemical analysis of kombucha showed the presence of various organic acids, such as acetic, gluconic, glucuronic, citric, L-lactic, malic, tartaric, malonic, oxalic, succinic, pyruvic, usnic;

also sugars, such as sucrose, glucose, and fructose; the vitamins B1, B2, B6, B12, and C; 14 amino acids, biogenic amines, purines, pigments, lipids, proteins, some hydrolytic enzymes, ethanol, antibiotically active matter, carbon dioxide, phenol, as well as some tea polyphenols, minerals, anions, DSL, as well as insufficiently known products of yeast and bacterial metabolites. Kombucha tea has been claimed by kombucha drinkers all over the world to have many beneficial effects on human health. However, most of the benefits were studied in experimental models only and there is a lack of scientific evidence based on human models. Nonhuman studies regarding antimicrobial, antioxidant, hepatoprotective, and anticancer properties of kombucha tea have seen carried out and biological activities are reported.

The word inflammation comes from the Latin "inflammo", meaning "I set alight, I ignite". Inflammation is a protective response that involves immune cells, blood vessels, and molecular mediators. The purpose of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and to initiate tissue repair. The classical signs of acute inflammation are pain, heat, redness, swelling, and loss of function. Inflammation is a generic response, and therefore it is considered as a mechanism of innate immunity, as compared to adaptive immunity, which is specific for each pathogen. Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A series of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation, such as mononuclear cells, and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.

Cytokines are a broad and loose category of small proteins (~5–20 kDa), peptides or glycoproteins that are secreted by specific cells of immune system. Cytokines are a category of signaling molecules that mediate and regulate immunity, inflammation and hematopoiesis. Cytokines are produced throughout the body by cells of diverse embryological origin. Cytokine is a general name; other names are defined based on their presumed function, cell of secretion, or target of action. For example, cytokines made by lymphocytes can also be referred to as lymphokines. Many of the lymphokines are also known as interleukins (ILs), since they are not only secreted by leukocytes but also able to affect the cellular responses of leukocytes. Those cytokines secreted by monocytes or macrophages are termed monokines. The chemokines are cytokines with chemotactic activities. Cytokines and their receptors exhibit very high affinity for each other. Because of this high affinity, picomolar concentrations of cytokines can mediate a biological effect. There were different types of cytokines, such as- pro-inflammatory and anti-inflammatory cytokines. Proinflammatory cytokines are cytokines that are important in cell signaling and promote systemic inflammation. They are produced predominantly by activated macrophages and are involved in the upregulation of inflammatory reactions (Zhang et al., 2008). The anti-inflammatory cytokines are a series of immunoregulatory molecules that control the proinflammatory cytokine response. Cytokines act in concert with specific cytokine inhibitors and soluble cytokine receptors to regulate the human immune response. Their physiologic role in inflammation and pathologic role in systemic inflammatory states are increasingly recognized. Major anti-inflammatory cytokines include interleukin (IL)-1 receptor antagonist, IL-4, IL-6,

IL-10, IL-11, and IL-13. Specific cytokine receptors for IL-1, tumor necrosis factor-alpha, and IL-18 also function as proinflammatory cytokine inhibitors (Opal et al., 2000).

2. AIM AND OBJECTIVES

1. Activity of Kombucha, a fermented beverage from black tea against carrageenan-induced rat paws edema.

2. Effect of Kombucha against bacterial LPS-mediated inflammation in rats serum, followed by estimation of both Secreted and Inter-cellular pro-inflammatory mediators.

3. Quantitative analysis for expression of pro-inflammatory cytokines specific m-RNA in rats peripheral blood mononuclear cells by real-time PCR.

3. REVIEW OF LITERATURE

Kombucha is a form of black tea and sugar that is fermented using a combination of bacterial and fungal cultures that form a "mushroom" on top of the fermentation vessel. It originated in China thousands of years ago, eventually spreading to Europe, and is today becoming increasingly popular, through celebrity use and endorsement, in the U.S. and U.K. Many home brew recipes for making kombucha may be found on the Internet but it is also manufactured and sold by companies such as Synergy Drinks (Sadjadi et al., 1998).

Most of the reports of human consumption of kombucha tea are case reports of toxicity, in some cases, life-threatening. The greatest danger from kombucha seems to arise in "home brew" versions that have become contaminated because of improper preparation and/or when kombucha interacts with alcohol or prescription drugs.

Observed adverse effects of kombucha consumption include hepatitis, xerostomia, dizziness, nausea, vomiting, headache, shortness of breath, restless legs, abdominal pain, hypotension, and tachycardia. In most cases, patients fully recovered after discontinuation of kombucha and symptomatic treatment. However there are case reports of serious and sometimes fatal cases of hepatic dysfunction and lactic acidosis (Gamundi et al., 1995).

In addition to oral ingestion, skin application of kombucha is also used as a topical analgesic. Such use has resulted in cutaneous anthrax infections from kombucha stored in unhygienic conditions; such conditions make kombucha preparations a potential medium for the growth of pathogenic microorganisms (Srinivasan et al., 1997).

Because folk medicines, herbal remedies and dietary supplements, including Kombucha tea, are not considered foods or drugs, they are not routinely evaluated by the U.S. Department of Agriculture or the U.S. Food and Drug Administration (FDA). According to the U.S. Centers for Disease Control and Prevention (CDC), drinking this tea in quantities typically consumed (approximately 4 oz daily) may not cause adverse effects in healthy persons; however, the potential health risks are unknown for those with pre-existing health problems or those who drink excessive quantities of the tea (Sabouraud et al., 2009).

Sometimes the human body struggles to release the byproducts that it creates through normal everyday use. This can result in lactic acid build up, uric acid build up, and in turn, these buildups can cause medical problems such as gout or lactic acidosis. Because Kombucha is acidic in nature, it creates a situation where the body is forced to expel the acids that you've consumed with this drink before expelling the other stored acids (Kole et al., 2009).

The nature of Kombucha is that it creates the prime working conditions for mold development, especially molds like penicillium. For those allergic to penicillin-based drugs, Kombucha could potentially create a life threatening allergic reaction if it has sat out long enough to develop mold. This is especially true for brews that have not been refrigerated. Though this is a low Kombucha health risk compared to the others, it is still a risk (Vijayaraghavan et al., 2009).

Although kombucha tea has been reported to have curative effects, there is some evidence of toxicity associated with it. Some individuals have reported dizziness and nausea after

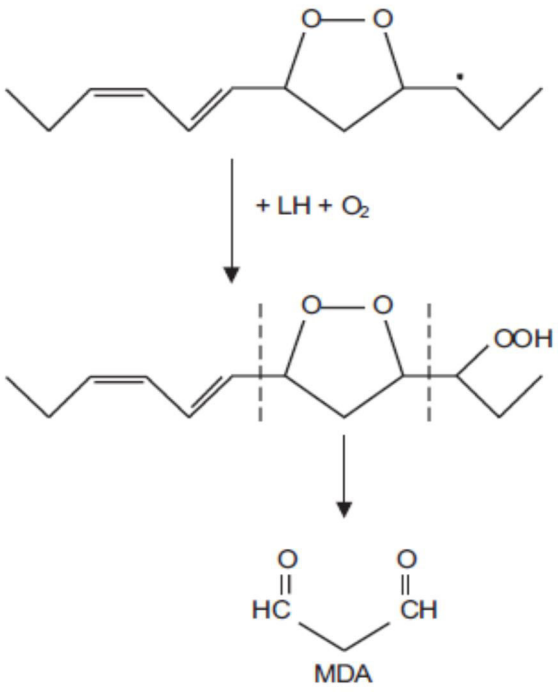
consuming certain kombucha products. Two cases of unexplained severe illness have also been reported following kombucha consumption (Centers for Disease Control and Prevention 1995). Kombucha tea is contraindicated in pregnant and lactating women. It has been found to cause lead poisoning and gastrointestinal toxicity in 2 individuals. The presence of anthrax Bacillus in kombucha tea fermented in unhygienic condition was reported by Sadjadi (1998). Further, Gamundi and Valdivia (1995) stated the risks of consuming kombucha beverage by HIV-positive patients. Side effects like allergic reactions, jaundice, nausea, vomiting, and head and neck pain related to consumption of kombucha were reported in 4 patients (Srinivasan and others 1997). A married couple who had been drinking kombucha tea for 6 mo, which was brewed in a ceramic pot, was reported to have symptomatic lead poisoning requiring chelation therapy (Phan and others 1998). It was postulated that acids in the drink eluted lead from the glaze pigment used in the ceramic pot. Sabouraud and others (2009) reported cases of lead poisoning in adults identified as anemia due to the lead-glazed earthenware jug which was used to store kombucha. A case of acute renal failure with lactic acidosis and hyperthermia within 15 h of kombucha tea ingestion by a 22-y-old HIV-positive male with a blood lactate level of 12.9 mmol/L and serum creatinine of 2.1 mg/dL was recorded (Kole and others 2009). However, all of these cases were very isolated and involved only a small number of individuals. Moreover, there is no substantial evidence to confirm the toxicity of any kombucha tea or the occurrence of illness by earlier studies (Vijayaraghavan et al., 2000).

The U.S. Food and Drug Administration and Kappa Laboratories, Miami, Florida, U.S.A. (1995), have carried out microbiological and biochemical tests and reported that kombucha tea is safe for human consumption. Vijayaraghavan and others (2000) studied the subacute (90 d) oral toxicity potency of kombucha tea using rats by recording body weight, feed intake, water intake, general behavior, and histological examinations. They concluded that kombucha feeding for 90 d to rats did not show any toxic signs. Hematological and biochemical variables of rats studied were within clinical limits. Their study indicated that rats fed kombucha tea for 90 d did not show any toxic effects. Pauline and others (2001) studied the toxicity of kombucha tea by feeding the rats orally for 15 d using 3 different doses of kombucha tea (normal dose and 5 and 10 times that dose) and by measuring various biochemical and histopathological parameters. They observed that kombucha tea displayed no significant toxicity.

Carrageenin, from the Irish word “carraigin” meaning Irish moss, refers not only to a species of red alga Chondrus crispus found along rocky areas of the Atlantic coast of the British Isles, Europe, and North America, but also refers to its mucopolysaccharide extract, discovered by the British pharmacist Stanford in 1862. The name was later changed to carrageenan so as to comply with the “−an” suffix for polysaccharides. Structurally, the carrageenans are a complex group of polysaccharides made up of repeating galactose-related monomers and are of three main types; lambda, kappa, and iota. Each has their own gel characteristics which are all thermally reversible. The lambda form does not gel strongly at room temperature and is injectable to induce an inflammatory response. Inflammation induced by carrageenan, originally described by winter, is acute, nonimmune, well-researched, and highly reproducible. Cardinal signs of inflammation—edema, hyperalgesia, and erythema—develop immediately following subcutaneous injection, resulting from action of proinflammatory agents—bradykinin, histamine, tachykinins, complement and reactive oxygen, and nitrogen

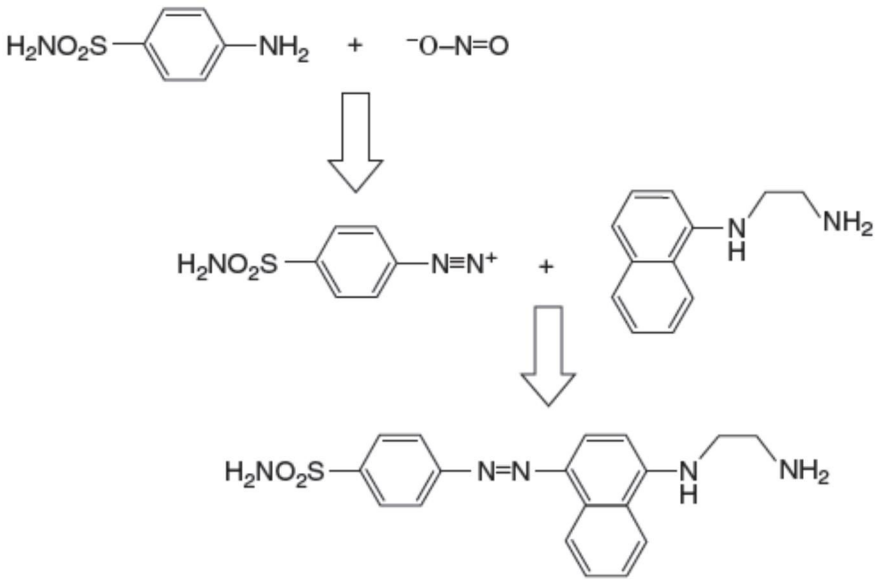
species. Such agents can be generated in situ at the site of insult or by infiltrating cells. Neutrophils readily migrate to sites of inflammation and can generate proinflammatory reactive oxygen and other species.

Malondialdehyde (MDA) is the organic compound with the formula $CH_2(CHO)_2$.This reactive species occurs naturally and is a marker for oxidative stress. Reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde. This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells and form covalent protein adducts referred to as advanced lipoxidation end-products (ALE), in analogy to advanced glycation end-products (AGE). The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism (Pryor et al., 1975).



Mechanism of lipoxigenation of polyunsaturated fatty acids

Nitrite and nitrate represent the final products of nitric oxide (NO) oxidation pathways, and their hematic concentrations are frequently assessed as an index of systemic NO production. However, their intake with food can influence their levels. Nitrite and nitrate could have a role by producing NO, because nitrite can release NO after reaction with deoxyhemoglobin and dietary nitrate can be reduced substantially to nitrite by commensal bacteria in the oral cavity (Gally et al., 1990).



Schematic diagram representing the Griess reaction principle

Superoxide anion radical (O_2^-) is generated by four-electron reduction of molecular oxygen into water. This radical also formed in aerobic cells due to electron leakage from the electron transport chain. Superoxide radicals (O_2^-) are also formed by activated phagocytes such as monocytes, macrophages, eosinophils and neutrophils and the production of O_2^- is an important factor in the killing of bacteria by phagocytes. In living organisms, O_2^- is removed by the enzymes called superoxide dismutases (SOD) (Farmer et al., 2007).

4. MATERIALS AND METHODS

CHEMICALS

Carrageenan (κ 80% and λ 20%); lipopolysaccharide (LPS from *E. coli*, serotype O127:B8) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other unlabelled chemicals and reagents were of analytical grade (SRL Mumbai, E.Merck India).

ANIMAL HUSBANDRY AND MAINTENANCE

Healthy adult male Wistar rats weighing 120-140 g procured from M/S Chakraborty Enterprise, 3/1D Girish Vidyaratna lane, Narkeldanga, Kolkata-700011, were used for the study. The animals were grouped and housed in wire cages with not more than six animals per cage in a controlled environment (12 h light and dark cycle, temperature of 25 ± 2°C and 50 ± 20% relative humidity). During the period of study, the animals had free access to standard dry pellet diet (Nutrilab Rodent; Provimi) and water ad libitum. The study was conducted in accordance with the Institutional Ethical Committee (constituted under the Guidelines Committee for the Purpose of Control and Supervision of Experiments on Animals) (Nag Chaudhuri et al., 2005).

PREPARATION OF BLACK TEA (BT) AND KOMBUCHA TEA (KT)

Eight grams black tea was added to 300 ml water and allowed to boil for 5 min. Then it was filtered through a sterile sieve and cooled to room temperature. It was then adjusted to 1600 ml with water. 10% (w/v) sucrose was added to it. The cooled tea was poured into 3 l glass beaker that has been previously sterilized at 121°C for 20 min. This was used as black tea (BT). It was then inoculated with freshly grown Kombucha mat that had been cultured in the same medium for 14 days and 10% (v/v) of previously fermented liquid tea broth to prepare Kombucha tea. The beaker was covered with clean cheese cloths and fixed with rubber bands. The fermentation was carried out under room temperature for 14 days. New Kombucha mat developed over the mother culture. The fermented tea was centrifuged at 10,000g for 15 min and the supernatant was lyophilized to dryness (LEx) which was then used for experimental analysis (Bhattacharya et al.,2011).

CARRAGEENAN INDUCED RAT PAW OEDEMA (PRE- AND POST-TREATMENT)

The control vehicle (isotonic saline 0.9%, w/v), test (2 groups) and standard drugs were administered 30min prior to the administration of carrageenan (Sigma Type I; 0.1ml of 1%, w/v solution) in the subplantar tissue of the hind paw. The paw volumes were measured using Plethysmometer (Model No 7141; UGO Basile, Italy), immediately and thereafter at hourly intervals, for 5 h, following administration of carrageenan (Chattopadhyay et al., 2004; Nag Chaudhuri et al., 2005). In the post-treatment study, the same procedure was repeated where the test drugs were administered (orally), 2 h after the administration of carrageenan (Boughton-Smith et al., 1993). The groups were divided as-

Group I- Control (Normal saline treated)
Group II- Kombucha treated group (once a day at 0.1ml/10 gm body weight)

Group III- Kombucha treated group (twice a day at 0.1 ml/ 10 gm body weight)
Group IV- Standard drug treated (Aceclofenac treated) (Dose 20 mg/kg, s.c., a stock solution containing 4 mg/ml of the drug and inject 0.5 ml/100 gm of body weight of the animal)

ANIMAL TREATMENTS AND TISSUE COLLECTION

Adult male Wistar rats of 70–80 days old weighing 150–170 g were taken. The rats were fed standard rat chow and water ad libitum and maintained at ambient temperature of 22–25°C under standard lighting regimens (12 h light: 12 h darkness, temperature of 25 ± 2^0C and 50 ± 20% relative humidity). All the protocols used in the present study were approved by the Institutional Animal Ethics Committee. For 7 days prior to the experiment, rats were handled daily, for 5 min. This was undertaken to acclimatize the rats to their surrounding environment and human contact and to decrease any hypothalamopituitary-adrenal axis responses to the handling involved in the subsequent experimental manipulations (Ma. et al., 1998). The LPS dissolved in 0.5 ml of sterile saline was injected intraperitoneally (i.p.) at the dosage of 5 mg/kg body weight of the animals and sacrificed after 3 h LPS treatment. Each group consisted of 6 animals. Saline treated animals served as the control group. The animals were maintained under continuous observation and their condition was noted. At appropriate time intervals after injection, rats were anesthetized with ketamine and xylazine mixture (12:5) ratio according to their body weight). After that the blood samples were collected through cardiac puncture.

Dose of ketamine and xylazine:

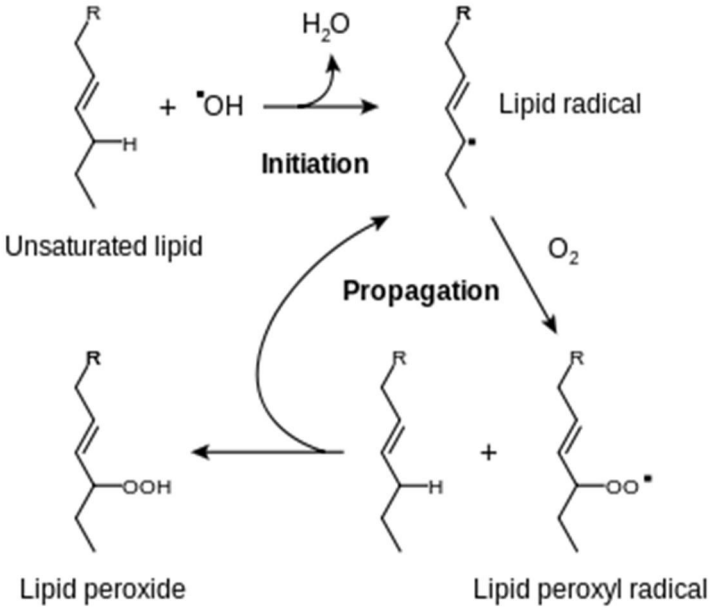
Ketamine = (Body weight in gm x 12)/10,000 ml
Xylazine = (Body weight in gm x 5)/10,000 ml

SERUM SEPERATION

A volume 2 ml of blood collected in heparinized tube was centrifuged at 1000 X g for 10 min at 4°C and the supernatant was collected and stored at -80°C.

MALONDIALDEHYDE (MDA) MEASUREMENT

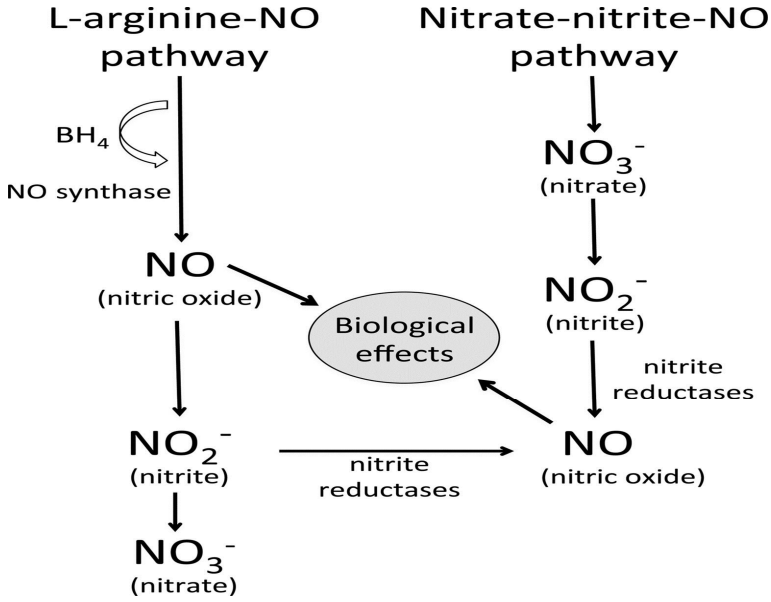
Plasma malondialdehyde (MDA) levels were determined as an indicator of lipid peroxidation (Ohkawa et al., 1979). An aliquot (100 µl) of the plasma collected at the specified time was added to a reaction mixture containing 200 µl of 8.1% SDS, 1500 µl of 20% acetic acid (pH 3.5), 1500 µl of 0.8% thiobarbituric acid and 700 µl distilled water. Samples were then heated for 1 h at 95°C and centrifuged at 3000g for 10 min. The absorbance of the supernatant was measured at 650 nm by Jasco UV/Vis Spectrometer.



Lipid peroxidation process

MEASUREMENT OF NITRITE/NITRATE

Nitrite+nitrate production, an indicator of NO synthesis, was measured in the plasma samples as previously described (Cuzzocrea et al., 1999) with slight modification. The nitrite concentration in the samples was then measured by the Griess reaction, by adding 100 µl of Griess reagent (0.1% naphthylethylendiamide dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_3PO_4; vol. 1: 1) to 100 µl samples. The optical density at 550 nm (OD_{550}) was measured using ELISA microplate reader (Epoch, Biotech, USA). Nitrite concentrations were calculated from standard curves of sodium nitrite at different concentrations in distilled water.



SUPEROXIDE ANION SCAVENGING ACTIVITY

The reaction mixture containing nitroblue tetrazolium solution (156 µM NBT in 100 mM phosphate buffer pH 7.4), NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4) were mixed with the serum samples and the reaction was started by the addition of phenazine methosulphate solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4). The final reaction mixture was incubated at 25 °C for 5 min, and then absorbance was measured at 560 nm in a Jasco UV/Vis spectrophotometer. A decrease in absorbance of the reaction mixture is indicative of an increasing superoxide anion scavenging activity (Nishimaki et al., 1972).

EXTRACTION OF TOTAL RNA FROM LEUKOCYTES AFTER LYSIS OF ERYTHROCYTES (LY)

The isolation of leukocytes or white blood cells (WBC) from 2 ml whole blood collected in heparinized tubes was done by alkaline lysis of erythrocytes. The whole blood was diluted 1:1 (v/v) with RBC lysis buffer [8.26 gm of NH_4Cl, 1.19 gm of $NAHCO_3$, 200 µl of EDTA (0.5 M, pH 8) in 100 ml water and adjust pH to 7.4] and incubated for 15 min at room temperature. The solution was then centrifuged for 10 min at 2,000 rpm at 25^oC. Supernatants were discarded, the cell pellet was resuspended in lysis buffer and centrifugation was repeated. This step was repeated thrice until the color of the pellet turns white. Total RNA from the WBC pellet was extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol. The RNA was treated with DNAse I and extracted again with Trizol (Chakraborty et al., 2016).

CYTOKINE ASSAYS BY RT-PCR

Total RNA from the WBC pellet was extracted with Trizol reagent (Invitrogen) according to the manufacturer's protocol. The RNA was treated with DNase I and extracted again with Trizol. Complementary DNA (cDNA) was prepared from 2µg of total RNA using iScript reverse transcription supermix for Quantitative Real Time-PCR (Bio rad, USA) according to the manufacturer's protocol. Expression of the genes encoding IL-10, NF-κB, TNF-α was measured with 1 µl of cDNA in StepOne^{TM} Real-Time PCR System (Applied Biosystems, USA) with kapa SYBR fast master mix universal. PCR conditions were 95°C for 15 min and then 40 cycles of 95°C for 30 s, 51°C for 30 s, 72°C for 30 s. The primers used were:

TNF α Forward primer for rat	5′-AGATGTGGAACTGGCAGAGG-3′
TNF α Reverse primer for rat	5′-CCCATTTGGGAACTTCTCCT-3′
NF κB Forward primer for rat	5′-TAC CAT GCT GTT TTG GTT AC-3′
NFκB Reverse primer for rat	5′-TCA AGC TAC CAA TGA CTT TC-3′
GAPDH Forward primer for rat	5′- TGATTCTACCCACGGCAAGT - 3′
GAPDH Reverse primer for rat	5′- AGCATCACCCCATTTGATGT- 3′
β actin Forward primer for rat	5′- CTATGAGCTGCCTGACGGTC-3′
β actin Reverse primer for rat	5′-AGTTTCATGGATGCCACAGG-3′

The internal control genes β-actin and GAPDH were amplified simultaneously in separate reaction tubes to normalize the data. Threshold cycle number (CT) of six reactions was determined using the ABI-SDS software and the mean CT of six reactions was determined.

The levels of expression of the genes of interest were normalized to both β-actin and GAPDH using the formula $2^{-\Delta\Delta CT}$, where $-\Delta\Delta CT = \Delta CT$ (sample) $-\Delta CT$ (calibrator) and ΔCT is the CT of the target gene subtracted from the CT of the housekeeping gene (GAPDH). The calibrator used in our experiments was the untreated normal rats (Chakraborty et al., 2016).

STATISTICAL ANALYSIS

Results were expressed as mean ± standard deviations. (n = 6). Statistical analyses were performed using Origin software: Version Pro8 and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. "P" value less than 0.05 were considered to be statistically significant.

5. RESULTS

CARRAGEENAN-INDUCED RAT PAW OEDEMA

Fermented black tea kombucha (once daily and twice daily) significantly inhibited both the early and late phases of carrageenan-induced paw oedema (Fig. 1). In the post-treatment model (drug administration after 2 hr), the BTK significantly decreased the carrageenan induced rat paw oedema, however, aceclofenac was found to produce no significant inhibitory activity (Fig. 1).

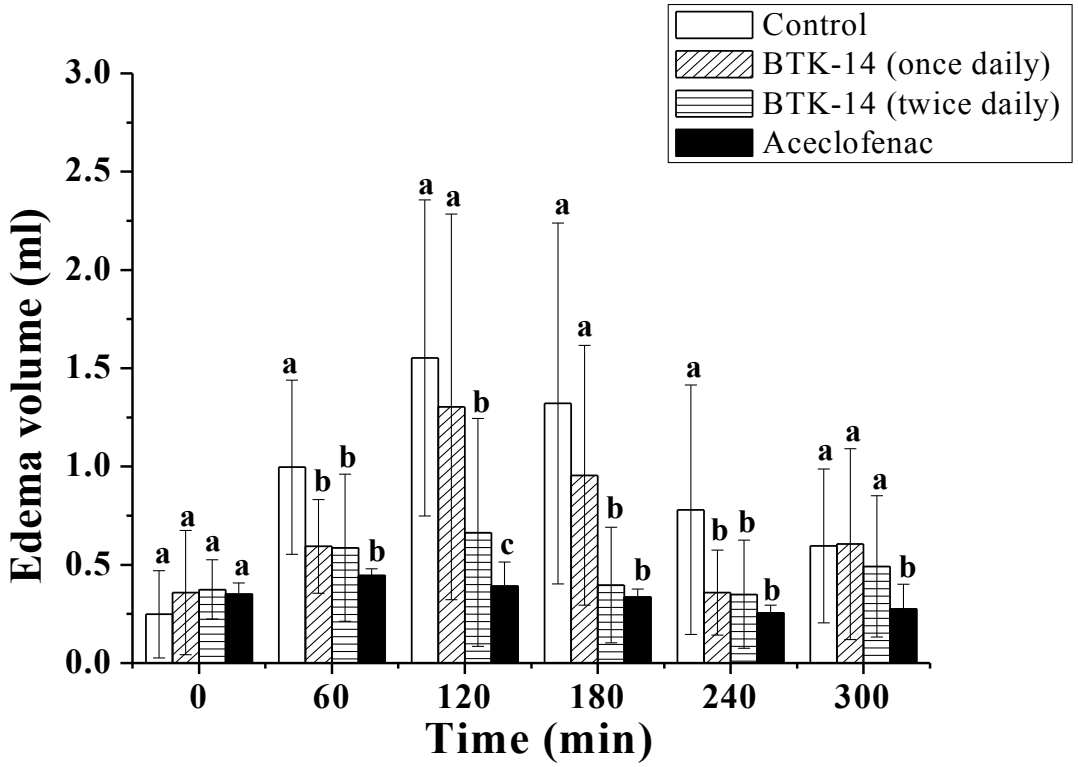


Figure 1.Effect of Kombucha tea (pre-treatment) on carrageenan-induced rat paw edema. The data are expressed as mean ± standard deviations (n = 6). The results at each time interval not sharing a common value (a-c) differ significantly at $P<0.05$.

EFFECT OF BLACK TEA KOMBUCHA (BTK) ON MALONALDEHYDE (MDA; nM) LEVELS IN PLASMA OF RATS AFTER LPS INJECTION

LPS treated animals exhibited a substantial increase in the plasma MDA levels. Treatment of rats with fermented black tea kombucha significantly attenuated the increase in MDA caused by LPS. No increases in plasma MDA levels were observed with normal rats (Figure 2).

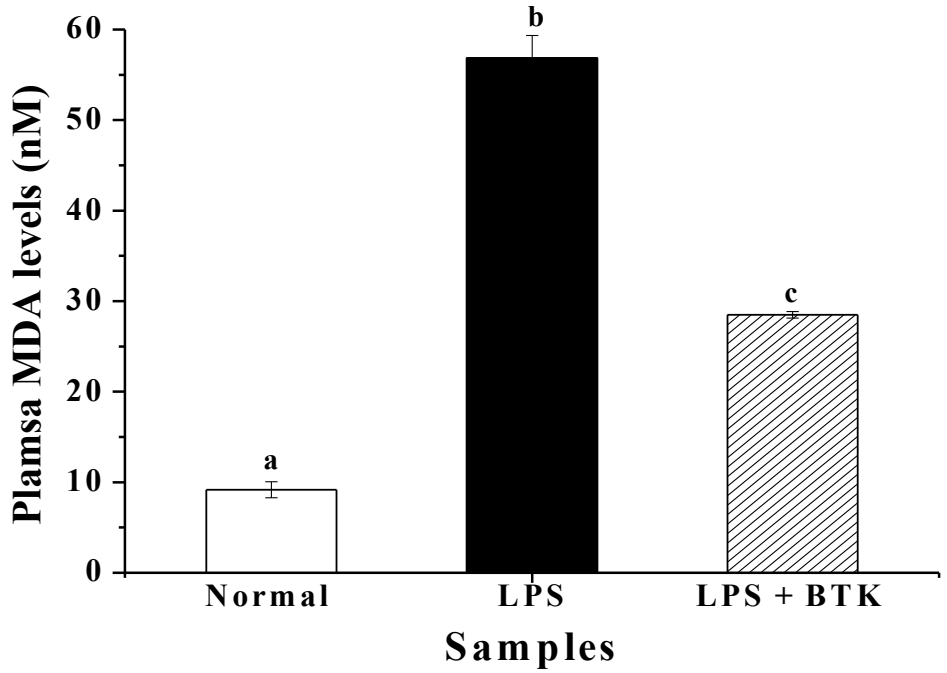


Figure 2. Effect of Black tea Kombucha (BTK) on malonaldehyde levels in plasma of rats after LPS injection. The results are expressed as Mean ± standard deviations of 6 animals for each group. The results not sharing a common value (a-c) differ significantly at $P<0.05$.

EFFECT OF BLACK TEA KOMBUCHA (BTK) ON NITRITE LEVELS (mM) IN PLASMA OF RATS AFTER LPS INJECTION

The levels of NO_x were significantly increased in the plasma from LPS treated rats. In contrast, the levels of NO_x were significantly lower in the plasma of kombucha treated rats. No increases in plasma NO_x levels were observed with normal rats (Figure 3).

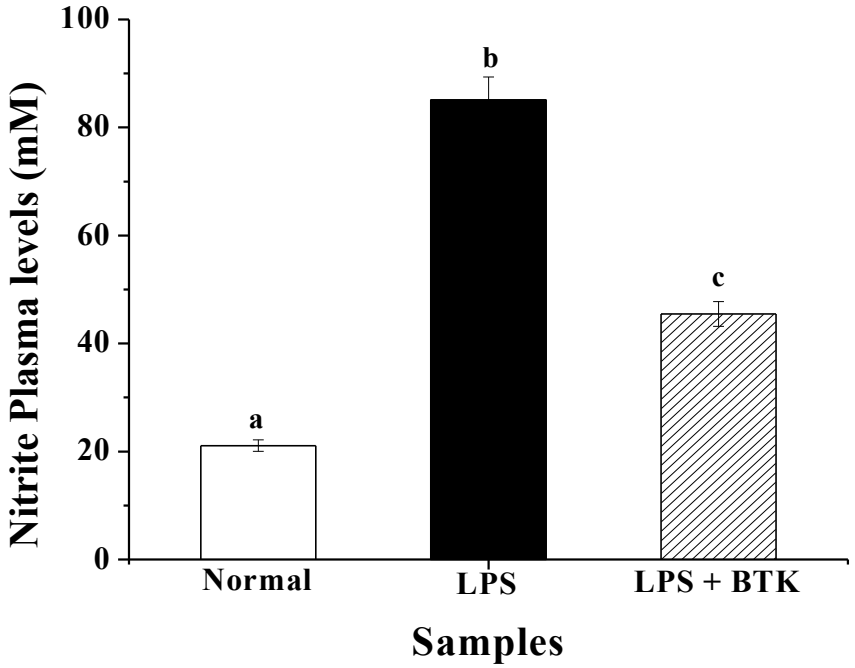


Figure 3. Effect of Black tea Kombucha (BTK) on nitrite levels in plasma of rats after LPS injection. The results are expressed as Mean ± standard deviations of 6 animals for each group. The results not sharing a common value (a-c) differ significantly at $P<0.05$.

EFFECT OF BLACK TEA KOMBUCHA (BTK) ON SUPEROXIDE DISMUTASE (SOD) ACTIVITY at 560nm IN PLASMA OF RATS AFTER LPS INJECTION

The fermented black tea kombucha significantly scavenged superoxide anion produced in LPS treated rats. The levels of superoxide anion were significantly increased in the plasma from LPS treated rats. No increases in plasma SOD levels were observed with normal rats (Figure 4).

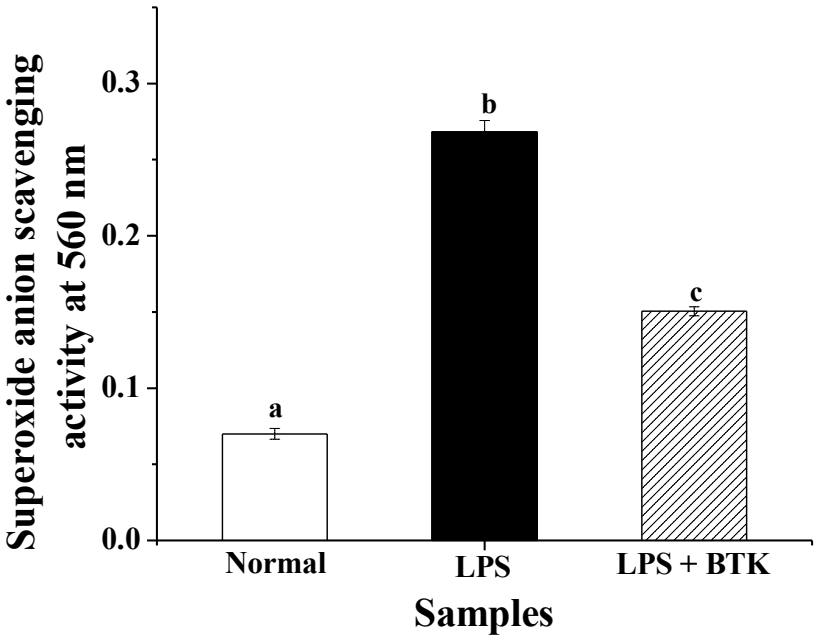


Figure 4. Effect of Black tea Kombucha (BTK) on superoxide dismutase activity in plasma of rats after LPS injection. The results are expressed as Mean ± standard deviations of 6 animals for each group. The results not sharing a common value (a-c) differ significantly at $P<0.05$.

REAL TIME PCR DETERMINATION OF FOLD CHANGES OF IL-10 mRNA EXPRESSION IN RATS AFTER LPS INJECTION COMPARED WITH THAT OF NORMAL UNTREATED RATS, TAKING β-ACTIN AS THE INTERNAL CONTROL GENE

The mRNA expression of the anti-inflammatory cytokine IL-10 was significantly up-regulated in BTK-treated rats after injection with LPS when compared with the normal untreated animals. However no significant expression of IL-10 was observed in LPS-treated rats when compared with the normal untreated rats.

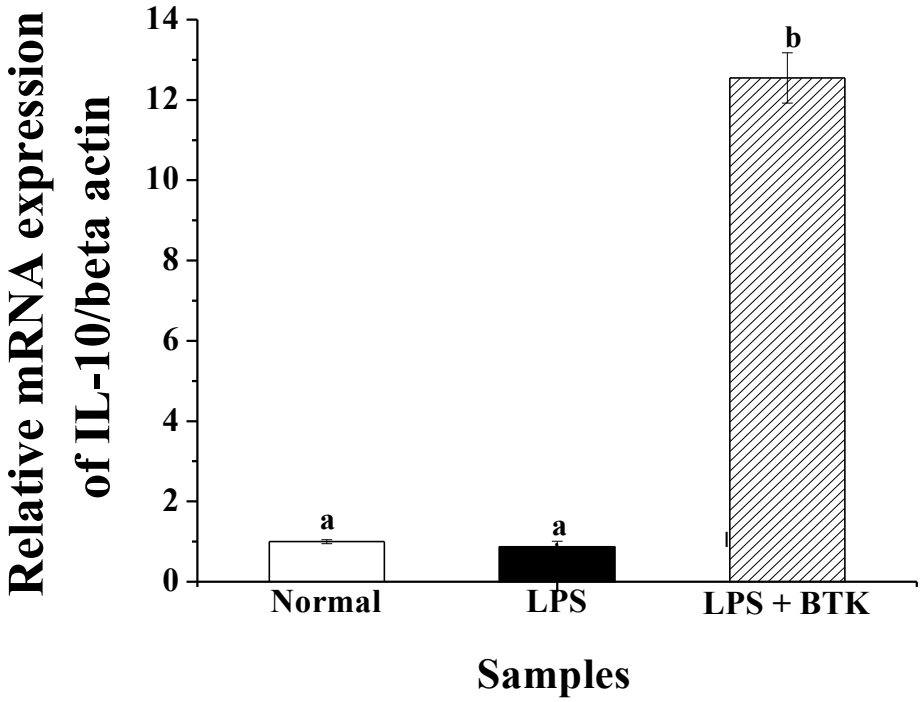


Figure 5. Real time PCR determination of fold changes of IL-10 mRNA expression in rats after LPS injection compared with that of normal untreated rats, taking β-actin as the internal control gene. The results are expressed as Mean ± standard deviations of 6 animals for each group. The results not sharing a common value (a-b) differ significantly at $P<0.05$.

RT- PCR DETERMINATION OF FOLD CHANGES OF IL-10 mRNA EXPRESSION IN RATS AFTER LPS INJECTION COMPARED WITH THAT OF NORMAL UNTREATED RATS, TAKING GAPDH AS THE INTERNAL CONTROL GENE

Similar results were obtained when gene expression was determined taking GAPDH as the housekeeping gene. The mRNA expression of the anti-inflammatory cytokine IL-10 was also significantly up-regulated in BTK-treated rats after injection with LPS when compared with the normal untreated animals. Moreover the expression of IL-10 mRNA was significantly down-regulated in LPS-treated rats when compared with the normal untreated rats.

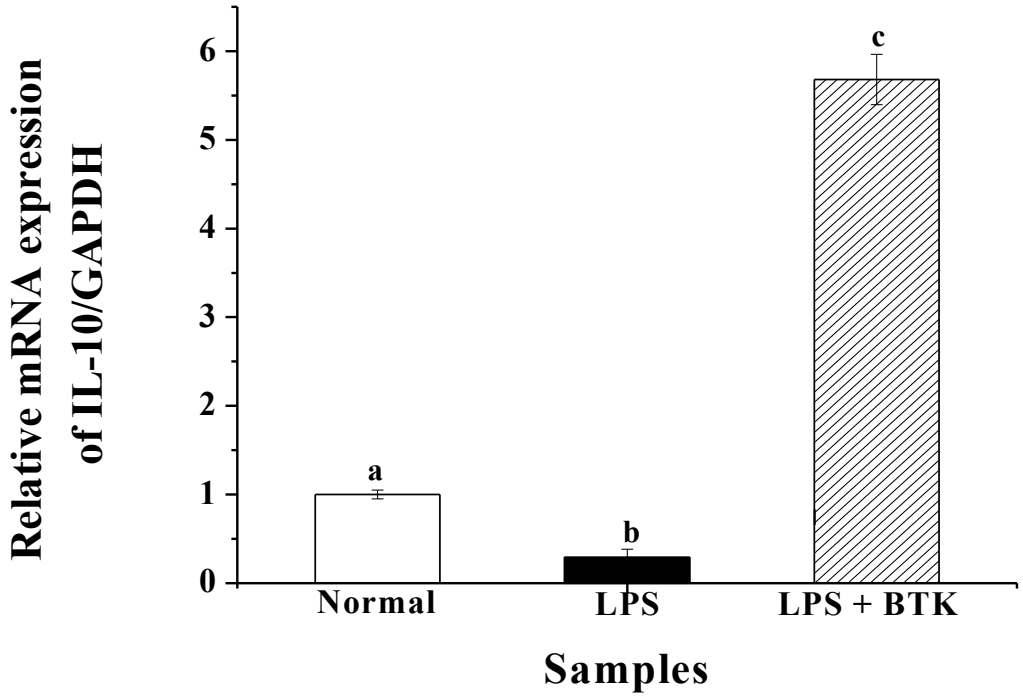


Figure 6. Real time PCR determination of fold changes of IL-10 mRNA expression in rats after LPS injection compared with that of normal untreated rats, taking GAPDH as the internal control gene. The results are expressed as Mean ± standard deviations of 6 animals for each group. The results not sharing a common value (a-c) differ significantly at $P<0.05$.

RT- PCR DETERMINATION OF FOLD CHANGES OF TNF-α EXPRESSION IN RATS AFTER LPS INJECTION COMPARED WITH THAT OF NORMAL UNTREATED RATS, TAKING β-ACTIN AS THE INTERNAL CONTROL GENE

The mRNA expression of the anti-inflammatory cytokine TNF-α was significantly down-regulated in BTK-treated rats after injection with LPS than the LPS-treated animals. Similar expression of TNF-α was observed in both BTK-treated rats as well as in untreated normal rats. The expression of TNF-α mRNA was significantly up-regulated in LPS-treated rats when compared with the normal untreated rats.

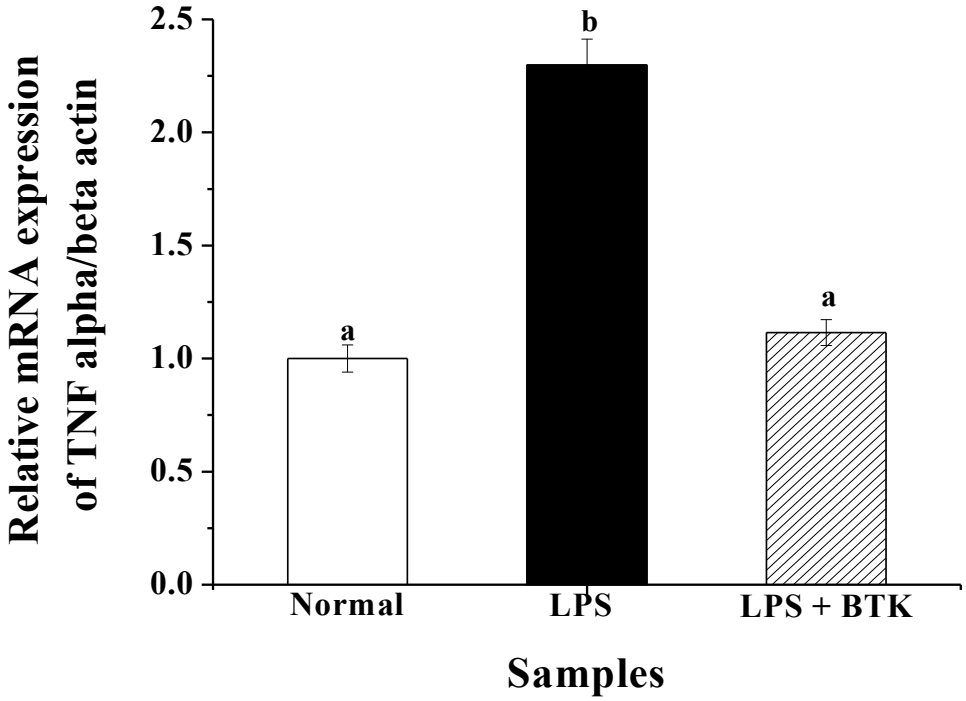


Figure 7. Real time PCR determination of fold changes of TNF-α expression in rats after LPS injection compared with that of normal untreated rats, taking β-actin as the internal control gene. The results are expressed as Mean ± standard deviations of 6 animals for each group. The results not sharing a common value (a-b) differ significantly at $P<0.05$.

RT- PCR DETERMINATION OF FOLD CHANGES OF TNF-α EXPRESSION IN RATS AFTER LPS INJECTION COMPARED WITH THAT OF NORMAL UNTREATED RATS, TAKING GAPDH AS THE INTERNAL CONTROL GENE

Similar results were obtained when gene expression was determined taking GAPDH as the housekeeping gene. The mRNA expression of the anti-inflammatory cytokine TNF-α was significantly down-regulated in BTK-treated rats after injection with LPS than the LPS-treated animals. Moreover, slight more expression of TNF-α was observed in BTK-treated rats when compared with the untreated normal rats. The expression of TNF-α mRNA was significantly up-regulated in LPS-treated rats when compared with the normal untreated rats.

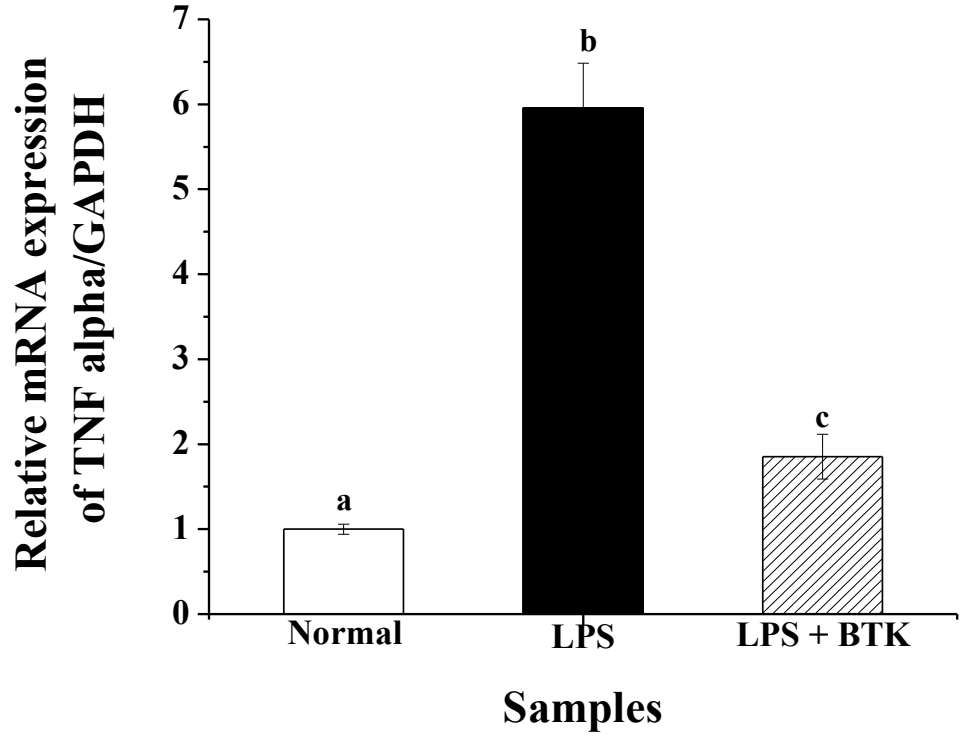


Figure 8. Real time PCR determination of fold changes of TNF-α expression in rats after LPS injection compared with that of normal untreated rats, taking GAPDH as the internal control gene. The results are expressed as Mean ± standard deviations of 6 animals for each group. The results not sharing a common value (a-c) differ significantly at $P<0.05$.

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RT- PCR DETERMINATION OF FOLD CHANGES OF NF-κB EXPRESSION IN RATS AFTER LPS INJECTION COMPARED WITH THAT OF NORMAL UNTREATED RATS, TAKING β-ACTIN AS THE INTERNAL CONTROL GENE

The mRNA expression of NF-κB was significantly down-regulated in BTK-treated rats after injection with LPS than the LPS-treated animals. Similar expression of NF-κB also was observed in both BTK-treated rats as well as in untreated normal rats. The expression of NF-κB mRNA was significantly up-regulated in LPS-treated rats when compared with the normal untreated rats.

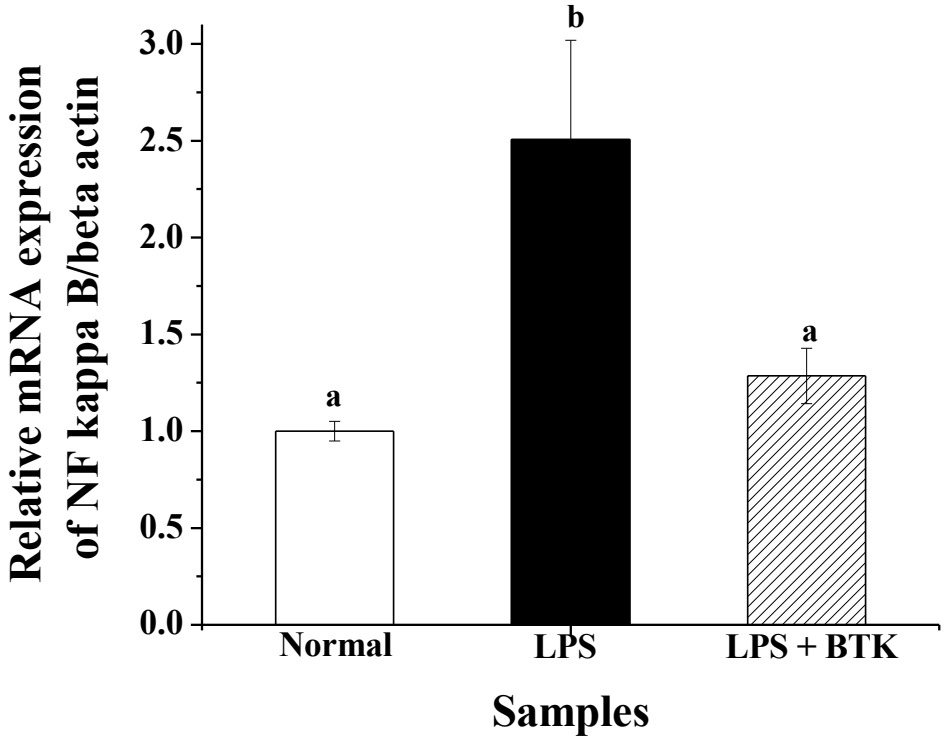

Figure 9. Real time PCR determination of fold changes of NF-κB expression in rats after LPS injection compared with that of normal untreated rats, taking β-actin as the internal control gene. The results are expressed as Mean ± standard deviations of 6 animals for each group. The results not sharing a common value (a-b) differ significantly at $P<0.05$.

RT-PCR DETERMINATION OF FOLD CHANGES OF NF-κB EXPRESSION IN RATS AFTER LPS INJECTION COMPARED WITH THAT OF NORMAL UNTREATED RATS, TAKING GAPDH AS THE INTERNAL CONTROL GENE

Similar results were obtained when gene expression was determined taking GAPDH as the housekeeping gene. The mRNA expression of NF-κB was significantly down-regulated in BTK-treated rats after injection with LPS than the LPS-treated animals. Moreover, slight more expression of NF-κB was observed in BTK-treated rats when compared with the untreated normal rats. The expression of NF-κB mRNA was significantly up-regulated in LPS-treated rats when compared with the normal untreated rats.

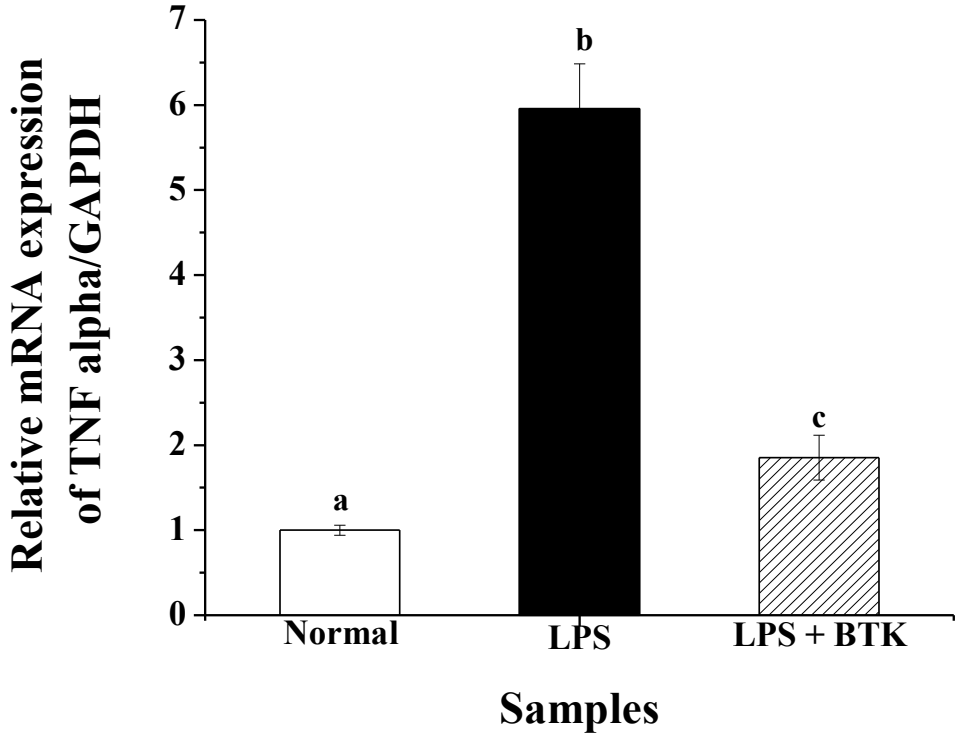

Figure 10. Real time PCR determination of fold changes of NF-κB expression in rats after LPS injection compared with that of normal untreated rats, taking GAPDH as the internal control gene. The results are expressed as Mean ± standard deviations of 6 animals for each group. The results not sharing a common value (a-c) differ significantly at $P<0.05$.

6. DISCUSSION

The present study was based on the experimental models of inflammation, closely resembling an inflammatory disorder. In this context, the studies revealed promising activity of the black tea kombucha (BTK) extract, as inferred from the results obtained from a series of experiments (Sen et al., 1993).

Earlier studies have shown that carrageenan-induced rat paw oedema is usually biphasic in nature. The initial phase is mediated by histamine and serotonin. The second phase is known to be influenced by the lipid derived eicosanoids (prostaglandins, leukotrienes, HPETEs, etc.). Our studies with the time course of carrageenan oedema formation revealed that on administration of tea extract, there was significant reduction of the paw oedema, and the effect was found to be significant up to a period of (minimum) 5 h (Sen et al., 2002).

In our present study, the fermented black tea kombucha significantly scavenged superoxide anion produced in LPS treated rats. The levels of superoxide anion were significantly increased in the plasma from LPS treated rats. No increases in plasma SOD levels were observed with normal rats.

Inflammatory processes are often accompanied by sustained dilatation of the capillaries (increased permeability), leading to excessive exudation of proteins. It has been observed that common non-steroidal anti-inflammatory drugs counteract such permeability change, thereby inhibiting exudation and subsequently oedema formation. In the present study, the tea extract showed significant anti-exudative properties, similar to that of aceclofenac (Cuzzocrea et al., 1986).

The mRNA expression of the anti-inflammatory cytokine IL-10 was significantly up-regulated in BTK-treated rats after injection with LPS when compared with the normal untreated animals, taking β-actin and GAPDH as the internal control gene. Whereas, the mRNA expression of the anti-inflammatory cytokine TNF-α and NF-κB was significantly down-regulated in BTK-treated rats after injection with LPS, taking β-actin and GAPDH as the internal control gene.

Accordingly, on the basis of the results obtained, we can suggest that the black tea kombucha extract probably interfere with: (i) the expression of COX and/or LO; (ii) enzyme (COX/LO) - catalysed reactions; (iii) functioning of the mediators (probable occupation of the receptors); and (iv) generation of free radicals (Sen et al., 1996).

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