**Effect of extraction temperature on antimicrobial and antioxidant properties of Assam tea**

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**Abstract**

Tea is traditionally prepared by boiling dry leaves in water. This study was taken to evaluate the effect of extraction temperature on antimicrobial and antioxidant properties of Assam tea (*Camellia sinensis var. assamica*). Dry tea leaves were extracted in water at 4 C, 37 C and by boiling at 100 C, and antimicrobial as well as antioxidant activities of the extracts were compared. Antibacterial activity was determined by agar cup diffusion assay against two important pathogens; *Staphylococcus aureus* and *Vibrio cholerae*. Antioxidant property was monitored by total reducing power (TRP) assay, ferric reducing antioxidant power (FRAP) assay and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. Our results show that maximum antioxidant activities of the extracts are obtained by boiling tea leaves. We also show that boiling of tea leaves does not lead to loss of antioxidant properties.

**Keywords:** Tea, *Staphylococcus aureus*, *Vibrio cholerae*, Antimicrobial, Antioxidant, Agar cup diffusion.

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**Introduction**

Tea is used as a beverage all over the world. It is popular because of its caffeine content which acts as mild stimulant. Various types of tea are available like green tea, black tea, red tea, oolong tea etc. It contains phytochemicals like alkaloids, saponins, tannins, catechin and other polyphenols which impart medicinal as well as pharmacological properties. Amount of these phytochemicals in the extracts often depends on the method of preparation [1]. Catechins are antioxidant and natural polyphenols present in high amount in tea leaves. Types of catechins present in tea are epicatechin (EC), epicatechin3gallate (ECG), epigallocatechin (EGC) and epigallocatechin3gallate (EGCg). ECG and EGC have antibacterial property and EC helps to prevent cancer [2-4]. Zaveri *et al* (2006) and Nagle *et al* (2006) [5,6] showed that in green tea EGCg is the major portion of total catechins which possess medicinal properties as anti-obesity, anti-inflammatory, anticarcinogen, antibacterial including antioxidant activity [7-11].

Tea extracts inhibit growth of various gastric and intestinal pathogens and used as prophylactic against typhoid [12]. It also inhibits the growth of pathogens causing dental infections like *Streptococcus mutans* [13-16]. Growth of *Staphylococcus aureus*, *S.epidermidis*, *Vibrio cholerae* 01 type, *V.cholerae* non 01 type, *V. parahaemolyticus*, *V. mimicus* were found to be inhibited by tea extracts [17]. Growth inhibition has been confirmed by observing the change in morphology of bacteria where polyphenols from green tea caused damage to the cell membranes by increasing outer and inner membrane permeability [18,19]. In addition to growth inhibition of *V.cholerae*, tea extracts are reported as potent inhibitor of cholera toxin *in-vitro* [20]. Tea extract has the ability to inhibit various types of viruses like rotavirus, enterovirus and influenza virus [21].

In addition to antibacterial activity polyphenols also exhibit antioxidant property. In green tea and black tea antioxidant property is imparted by the polyphenols and the theaflavins respectively [22,23]. According to Yang and Liu (2012) [24], green tea showed better antioxidant activity (4850.2 ± 60.7 μmol g⁻¹, P< 0.05) than black tea and oolong tea. Antioxidant activity is reported to vary in different parts of the tea plants. Different antioxidant assays like ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] assay, FRAP (ferric reducing antioxidant power) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assay showed that tea shoot has more antioxidant activity than leaves [25]. Process of tea preparation also affects the antioxidant content of tea. Baruah (2012) [26], reported that antioxidant activity is more in tea extract when prepared by microwave oven drying than when prepared by hot air oven.

Tea is prepared in different ways and variation in treatment temperature has been observed in most of the recipes. Assam tea is grown in north-eastern Himalayan region of India, and is famous all over the world for its flavour and liquor. In this study we compared the effect of temperature on antimicrobial and antioxidant activities of Assam tea.

**Materials and Methods**

Chemicals

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experiment was done in triplicate. Inhibition of the samples were measured. Each reading indicates

C

petriplate was incubated overnight at 37°C. Before analyzing the samples were dehydrated

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and kept in 4°C for twenty minutes along with 2.5ml of 0.2M phosphate buffer adjusted to pH 6.6 and 2.5ml of 1% potassium ferricyanide. After that 2.5ml of 10% trichloroacetic acid was added to it and centrifuged at 3000 rpm for ten minutes. 2.5ml of distilled water and 0.5ml of 0.1% ferric chloride was added to 2.5ml aliquot of upper layer. The absorbance was measured at 700nm and ascorbic acid was used as standard.

FRAP assay

The tea leaf extracts were checked for the presence of ferric reducing antioxidant power content (FRAP Assay). The stock solutions included 20mM FeCl₃.6H₂O solution, 10mM TPTZ (2, 4, 6- tripyridyl-s-triazine), 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), trolox and ascorbic acid were obtained from Sigma.

Antioxidant activity of tea extracts

TRP assay

Total reducing power of tea leaf, extracted in different temperature was measured to detect the antioxidant activity. 1ml of extract was taken and incubated at 50°C for twenty minutes along with 2.5ml of 0.2M phosphate buffer adjusted to pH 6.6 and 2.5ml of 1% potassium ferricyanide. After that 2.5ml of 10% trichloroacetic acid was added to it and centrifuged at 3000 rpm for ten minutes. 2.5ml of distilled water and 0.5ml of 0.1% ferric chloride was added to 2.5ml aliquot of upper layer. The absorbance was measured at 700nm and ascorbic acid was used as standard.

Plant materials

The tea leaves were provided by the Senior Manager, Chabua Tea Estate, Dibrugarh, Assam who is maintaining the plants in nursery through vegetative propagation. Further the leaves were identified through a Horticulturist, Archaeological Survey of India, Government of India, Agra, as described earlier (Mehrotra et al 2010)

Preparation of extracts

Dried tea leaves were taken and crushed in mortar and pestle. 10gm of powdered tea was taken in three different conical flasks and 40 ml of autoclaved distilled water was added in each flask and kept in 4°C, 37°C and 100°C for six hours. After temperature treatment these samples were filtered using Whatman filter paper no 1 and filtrates were lyophilized and stored for future use.

Inoculation of bacterial culture

Luria-Bertani broth 2 ml was taken in autoclaved test tubes, and isolated bacterial colonies were inoculated in the broth separately and kept overnight in the shaker at 37°C. S.aureus clinical isolates provided by NICED, Kolkata and V.cholerae clinical strains were isolated by authors of Assam medical college) were inoculated in the broth separately and kept overnight in the shaker at 37°C.

Antibacterial activity of tea extracts

Antibacterial activity was checked by agar cup diffusion assay. The Luria-Bertani agar media was spread separately with 100µl of overnight grown bacterial cultures (S.aureus and V.cholerae) and wells were made in the plate using sterile cork borer. 100µl of 4°C, 37°C, 100°C treated tea leaf extracts were loaded in wells and autoclaved distilled water was included as negative control. The petriplate was incubated overnight at 37°C. Next day the zone of inhibition of the samples were measured. Each reading indicates diameter of zone of inhibition excluding the well diameter and experiment was done in triplicate.

Scanning electron microscopy

Bacterial strains were grown to optical density 0.5 at 600nm and treated overnight with sub-lethal doses of tea leaf extract. Next day bacterial cells were harvested and fixed in 2.5% glutaraldehyde and stored at 4°C. Before analyzing the samples were dehydrated with graded ethanol.
showed higher antibacterial activity against *S. aureus* than to *V. cholerae*. Antimicrobial activity against both the strains was highest in 100 °C followed by 4 °C and 37 °C (Fig 1). It was reported that, with the increase of extraction temperature, solubility of the phenolic compound, which is responsible for antibacterial activity, increases [29]. Thus tea leaf extract prepared at 100 °C showed maximum growth inhibition activity as it contains highest amount of phenolic compound. On the contrary, extract prepared at 37 °C showed least bioactivity amongst the extraction methods used. The possible reason of the loss of the antibiotic activity could be due to oxidation of phenolic compounds. Polyphenol oxidase enzyme causes oxidation of phenolic compound and remains active within 20 °C to 70 °C and it is inactive when temperature rises beyond 70 °C or falls below 20 °C [30,31]. This is also interesting to observe that in contrast to most synthetic antibiotics that get inactivated by boiling, antimicrobial activities of tea-extracts are quite heat-resistant. This observation is similar to earlier study made on plant-based antimicrobial activities [27] in which extracts were treated to 100 °C for one hour and observed to retain its activity. The results suggest that boiling extract method is best for antimicrobial activities of the tea extracts. Scanning electron microscopy with tea extract (100 °C) showed morphological changes in both *S. aureus* and *V. cholerae*. Deformation and surface ruffling of the bacterial cell were observed after treatment with tea extract prepared at 100 °C (Fig 2).

![Graph showing antimicrobial activity of tea leaf extract](image)

**Fig 1:** Antimicrobial activity of tea leaf extract (A) Zone of inhibition obtained by agar-cup assay. Each bar represents mean ± standard deviation from triplicate readings. (B) and (C) shows representative inhibition assay with *V. cholerae* and *S. aureus* respectively.
Boiling of leaves retains antioxidant activity of tea extracts

Antioxidant activities are usually monitored by several assays [32]. Three different antioxidant assays were done to include different kinds of antioxidant moieties present in tea extract, whose solubility varies with different reagents. One of the assay systems measures total reducing power (TRP) by monitoring the reduction of ferric ion (Fe3+) to ferrous ion with the help of ferric chloride [33]. Commercially available ascorbic acid served as a standard of our assay (Fig 3B). It was observed that tea extract prepared at 4°C, 37°C and 100°C had the ascorbic acid equivalent antioxidant capacity of 56.53 mg/gm, 65.55 mg/gm and 80 mg/gm of dry weight of extract respectively by TRP assay with reference to standard curve (y = 0.002 x, R² = 0.996). As shown in the Fig 3A, 100°C treated tea extract showed maximum reductive ability to transform Fe3+ to Fe2+ followed by the extracts that were prepared at 4°C and 37°C. Thus, by boiling the antioxidant properties of tea leaves are not compromised. Another assay system measures ferric ion reducing antioxidant power (FRAP) by monitoring the maximum ferric reducing ability with the help of ferric chloride [33] and trolox was used as a standard (Fig 4B). It was observed that tea extract prepared at 4°C, 37°C and 100°C had the trolox equivalent antioxidant capacity of 139.42 mg/gm, 220.83 mg/gm and 205.83 mg/gm of dry weight of extract respectively by FRAP assay with reference to standard curve (y = 0.0008 x, R² = 0.994) (Fig 4A). In ABTS assay maximum inhibition of oxidation of ABTS was monitored by electron transfer radical scavenging [33]. It was observed that tea extract prepared at 4°C, 37°C and 100°C had the trolox equivalent antioxidant capacity of 636.26 mg/gm, 691.66 mg/gm and 518.09 mg/gm of dry weight of extract respectively with reference to standard trolox (y = 0.0007 x, R² = 0.996) (Fig 5A, 5B). Thus, the antioxidant properties of the leaf-extracts are mostly retained in the boiling extract.
Fig 3: Antioxidant activity of tea leaf extracted at different temperature. (A) TRP assay: Ascorbic acid equivalents in mg per gram of dry weight of tea extract; (B) Standard curve for TRP assay.

Fig 4: Antioxidant activity of tea leaf extracted at different temperature. (A) FRAP assay: Trolox equivalents in mg per gram of dry weight of tea extract; (B) Standard curve for FRAP assay.
Conclusion

We conclude from the study that boiling extraction of tea leaves contains maximum antibiotic activity. Antimicrobial as well as antioxidant properties of tea extracts are retained even after boiling at 100°C for six hours. Our experiments validate the traditional method of tea preparation, which is extraction of dry tea leaves in boiling water.

Authors’ Contributions

Angana Payne Mandal and Mohd Hamza Hanfi have performed all above mentioned experiments and prepared the manuscript. Dr. Lahari Saikia and Dr. Subhajyoti Deka have helped in analysis and interpretation of data. The whole conception of the manuscript have been given by Dr. Shoma Paul Nandi and she had designed all the experiments.

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