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Original Article

Total phenol content, Anti-inflammatory, Anti-allergic and Antioxidant activity in differently processed *Camellia Sinensis* of Bangladesh

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ABSTRACT: The aim of present study is to investigate Total phenol content (TPC), Anti-inflammatory, Anti-allergic and Antioxidant activity (AA) in differently processed *Camellia Sinensis* (tea) of Bangladesh. TPC varied in the range of 1056.74 (branded tea) – 2348.60 (green tea) mg GAE/g respectively. Radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) was found in the range of 1269.91- 2432.76 µmol TE/ g while total Oxygen Radical Absorbance Capacity (ORAC) value in the range of 1262.81-2834.51 µmol TE/g. Both green and organic tea showed highest ORAC value when brewed for 5 min in boiling water extract while tea BT-2 and branded (mirzapur) tea exhibited highest ORAC value for 10 min brewing. Anti-inflammatory activity potential was found in the order of green tea> tea BT-2> organic tea> branded tea where both green and organic tea exhibited dose-response relationship. Anti-allergic activity ranged from (60.9-83%) with highest and lowest in green and branded tea respectively.

Key words: Anti-inflammatory activity, Anti-allergic activity, Antioxidant activity, Total phenol content, Bangladeshi tea

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INTRODUCTION

Camellia Sinensis (tea), an evergreen shrub native to Asia [1] is one of the most widely consumed beverages in the world because of its attractive and distinct color, flavor, aroma, taste. Diverse health claims made 'Tea' as an important 'health drink'. Off late several epidemiological studies have revealed variety of health benefits of tea consumption in human body including reduction in the incidence of Cardiovascular diseases (CVDs) [2, 3] and different types of cancer including lung [4], pancreatic [5], breast [6] and skin [7]. The antioxidant properties of polyphenols from tea are reported for their health beneficial roles which include, anticancer [8] antiviral, antimicrobial and anti-inflammatory [9] properties and preventing capillary fragility and platelet aggregation [10].

Depending on the manufacturing process tea can be categorized into three types: fermented (black tea), semi-fermented (oolong tea) and unfermented (green tea). Tea leaves are considered as important sources of polyphenols. They contain more than 35% of their dry weight in polyphenols. The major flavonoids in tea are catechins that include epicatechin (EC), epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG) and epicatechin-3-gallate (ECG). These four catechins comprise 30–50% of the solids in a green tea extract while in black tea catechin content goes down to 10%. Black tea is produced by fermentation of green tea and during fermentation; the polyphenols are oxidized and polymerized enzymatically to theaflavins and thearubigens. In addition to the degree of fermentation, the antioxidant activity of tea polyphenols also varies with different brewing [11, 12]. The effective time

of brewing tea leaves for attaining maximum antioxidant activity, therefore, is immensely important from health point of view.

Tea cultivation in Bangladesh is widespread over the hilly zones on the eastern part mainly in four districts (Sylhet, Moulvibazar, Habibgonj, and Chittagong) containing 163 tea estates. In 2013, Bangladesh has produced 66.2 thousand tons tea annually being the 10th largest producer in the world [14]. Despite being the well-established knowledge of protective health effect of tea very little information is available regarding the antioxidant activity and the total phenols in different varieties of teas in Bangladesh. Therefore, the present study was undertaken to investigate the (i) Total phenol content (TPC) and Antioxidant activity (AA) in Acetone :Water :Acetic acid (AWA) extract, ii) effect of different brewing time on AA and (iii) in vitro anti-inflammatory and anti-allergic activity in four differently processed tea from Bangladesh.

MATERIALS AND METHODS

Chemicals and Apparatus

2,2$^{'}$-Azobis(2-methylpropionamide) dihydrochloride (AAPH), all other solvents used for extraction (Dimethyl Sulfoxide, Hexane, Acetone and Acetic Acid) and chemicals used for assay buffer were procured from Wako Chemicals, Japan. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Aldrich, Denmark and Fluorescein isothiocyanate (FITC) from SIGMA, USA. Randomly methylated β-cyclodextrin (RMCD) was obtained from JUNSEI, Japan. Folin-Ciocalteu reagent was purchased from MP Biomedical, LLC, Ohio/France and Gallic acid from TIC, Japan. Extractions of samples were performed on an ASE 200 accelerated solvent extractor (DIONEX, Japan).

Sample collection & preparation

Four differently processed tea samples (green tea, tea organic, tea BT-2, and branded tea -mirzapur) were collected from markets. The collected tea samples were processed in the Food Analysis Laboratory of Institute of Nutrition and Food sciences (INFS), University of Dhaka, Bangladesh. Tea samples were kept at -20˚C in their original dried form until analysis. All samples were ground well by miller-800DG, IWATANI, Japan, before extraction and 0.5 g ground food samples was mixed with food matrix (Varian, sample preparation products, A) to make final weight 2.5g. Samples and food matrix were transferred to a 22-mL extraction cell and initially extracted with hexane/dichloromethane (1:1, Hex/Dc) followed by acetone:water:acetic acid (70:29.5:0.5; AWA). Both the hexane/dichloromethane and AWA extracts were transferred to 25 ml measuring cylinder and adjust the volume with the extraction solvent to 25 mL of total volume. AWA extracts were used to measure the Hydrophilic ORAC (H-ORAC) and Total phenol after proper dilution. After adjustment of Hexane/Dichloromethane extracts to a final volume of 25 mL, an aliquot of the extracts was transferred to screw-capped glass tube to store at 4˚C. Before estimation of Lipophilic ORAC (L-ORAC) an aliquot of Hex/Dc extracts were dried under nitrogen flow in a 37˚C heating block, then dissolved by acetone (half of the volume of the aliquot of extracts was taken) and after mixing well, equal volume of 7% RMCD in a 50% acetone-water mixture (v/v) was added and shaken for 1h at room temperature on an orbital shaker at 400 rpm. The sample solution was ready for analysis of L-ORAC after further dilution with 7% RMCD solvent.

TPC estimation

TPC of the samples was measured by modified Folin-Ciocalteu method [14]. Gallic acid calibration solutions (100, 50, 30, 20 and 10 mg/g) were made and run on the multi detection microplate reader (Powerscan^{TR}, Dainippon Sumitomo Pharma, Japan.) to obtain a standard curve for Gallic acid and estimation of TP content of the AWA extracts of samples. The results were expressed as mg of Gallic Acid Equivalents per gram fresh weight of sample (mg of GAE/g FW).

Antioxidant activity of sample extract by DPPH Radical scavenging assay (DPPH RSA)

Antioxidant activity of AWA extracts of samples was estimated by DPPH RSA method [15]. The DPPH$^{\bullet}$ (stable radical) was dissolved in ethanol at the concentration of 800 µM and preparation prior to use and stirring 30 min to 1 h in dark container. Sample extracts (AWA) was mixed with equal volume of 30% acetone and if necessary further dilution with 50% acetone. 100 µl of calibration (160, 120, 80, 4, 0 µM of Trolox) and 3 different dilution (100, 50, 25 %) of samples were added to 50 µl of 200 mM MES buffer. 50 µl DPPH solutions were added except well for blank and100 µl of 100% samples were used for the subtraction of original color of the sample extracts. After 20 min at ambient temperature, absorbance was measured at 520 nm in UV spectrophotometer. A

standard curve was constructed by plotting varying Trolox concentrations (0, 20, 40, 60 and 80 nmol/µL) on abscissa and absorbance on ordinates. The antioxidant activity of the assayed samples was calculated by kinetics using following formula: AC=m2/m1, where m1 is the slope of the standard curve, and m2 is the slope of the triplicate sample. The results were expressed in terms of Trolox equivalent per gram of fresh weight of sample (µmol TE/g FW).

Antioxidant activity of the sample extracts by ORAC assay

The ORAC assay was performed by the procedure described by [16]. A Stock solution of fluorescein sodium salt (Sigma, St. Louis, MO) (7.17 µM) was prepared in phosphate buffer (pH 7.0). The working solution (71.7 nM) was obtained by subsequent dilution of stock solution with PBS. AAPH, (Wako Pure Chemicals Industries, Japan) was prepared freshly at a concentration of 200 mM and used after 10 min. heating at 37˚C and zero-time absorbance. Calibration curve (50, 25, 12.5, 6.25 µM) of Trolox (Sigma, St. Louis, MO) were prepared during every estimation by diluting the stock solution (2mM) with PBS. Diluted samples (25µL) solution was mixed with Fluorescein (150 µL) and incubated at 37˚C for 10 min. in the microplate reader. The analyzer was programmed to record the fluorescence of FL every minute after addition of AAPH (25 µL) solution and the microplate were shaken. The fluorescence was measured at 490 nm for excitation and 535 nm for emission in a multilabel counter (Wallac, $ARVO_{TM}SX$, Perkin Elmer, Life Science) and previously before addition of AAPH zero fluorescence was measured. All measurements were expressed relative to the initial reading. Final results were calculated using the differences of areas under the FL decay curves between the blank and a sample. These results were expressed as µmoles Trolox equivalent (TE) per gram fresh weight of sample.

Anti-inflammatory activity on J774.1A cell assay

To estimate in vitro anti-inflammatory activity, 0.2 g samples were mixed with 5 ml of Dimethyl sulphonic-oxide (DMSO). The mixture of sample and DMSO was stirred in a shaker at 130 rpm over night at room temperature and centrifuged at 3000 rpm for 5 minutes. After centrifugation, the supernatant collected as DMSO extraction was transferred by pipetting and 500µl of aliquots were stored at 25 °C for in vitro cell analysis. In vitro anti-inflammatory activity was assessed Herath's (2003) method [17].

The mouse macrophage (J774A cell) suspension with a concentration of 5.0 X 105 cells/well was seeded into each well of 96-wells plates and incubated overnight at 37° C in an atmosphere of 5% CO2 and 95% air which was followed by removal of cultured medium by washing with Hank's solution. After removal of cultured medium, 160 µl DMEM with 20 µl of LPS (1.0 µg/ml) (LPS plus) were added to 48 wells (LPS minus) and 180 µl DMEM with 20 µl of diluted sample extracts (40 µg/ml) to rest of the 96 well which were treated as control. The culture plate was incubated for 4 hours, and the supernatant was then collected and assayed for TNF-α content using the mouse TNF-α enzymelinked immunosorbant assay kit (Ready-Set-Go, eBioscience, USA). After this preliminary screening, samples extracts with at least 75% inhibition activity were assessed for dose response.

Dose response of the samples was carried out using different concentration of samples extracts; stored sample extracts were diluted by phosphate buffer saline (PBS) to get final concentration of 1, 3, 10, and 40 µg/ml.

Anti-allergic activity on RBL-2H3 cell line

RBL-2H3 cells were grown in 10% FCS-containing DMEM (Sigma, USA) and for the evaluation of the anti-allergic effect of the selected food samples β-hexaminidase enzyme assay was done. The RBL-2H3 cells were grown in 24 well plate at the concentration of $5x10^5$ cells/ml cell and were incubated overnight at 37˚C in an atmosphere of 5% CO_2, 95% air.

Food extracts were diluted to 2 µg/ml concentrations with Mt Modified Tyrode (MT) buffer and were added to in each experimental well (490 ul). On the other hand, 490 µl of MT and 200 times diluted wortmannin solution (wortmannin stock -20 mM) were added to Blank, negative control, and positive control well respectively. After 10 min incubation, 10 µl of DNP-labeled Human Serum Albumin (final concentration 50 ng/ml) was added to each well and the mixture with cell culture incubated for 30 min. The supernatant was collected and transferred into the lower wells and the cells were lysed with 500 µl of Triton X-100. The supernatant and the lysate (50 µl) was transferred to 96 well multiwell plate and mixed with 100 µl of 0.1 M citrate buffer (pH-4.5) containing 3.3 mM p-nitrophenyl-2-

acetamide-β-D-glucopyranoside. The mixture was incubated for 25 min. The reaction was stopped by adding 100µl of 2M glycine buffer (pH 10) and the absorbance was measured in a micro plate reader (model 550: Bio Rad, CA, USA). Lower four-lane used as control by adding stop solution first and then addition of substrate at the same time as experimental wells. Finally, the % degranulation was calculated according to [18].

Statistical analysis

All the analyses were carried out in triplicate, and the results are shown as mean value and standard deviation. Pearson correlation was used for Correlational analysis between TPC, antioxidant activity, and ORAC value of sample extracts. P value <5% level being used to determine the significance.

RESULTS AND DISCUSSION

TPC and AA of analyzed tea samples

TPC and AA of tea samples are presented in table 1. TPC varied in the range of 1056.74 - 2348.60 mg GAE/ g FW with the highest amount in green tea and lowest in branded (mirzapur) tea. In present study TPC of black tea varied in the range of 1056.74 (branded tea) -1501.70 (tea BT-2) mg GAE/ g FW. The higher content of total phenols in green tea than in black tea reported in previous studies [19, 20] is in accordance with present study findings.

Antioxidant activity of tea samples were evaluated by DPPH radical scavenging assay (DPPH RSA) and ORAC assay. DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant compounds because of its stability (in radical form) and simplicity of the assay. The ORAC assay is instead a direct method that measures antioxidant inhibition of peroxyl radical-induced oxidations and reflects classical, radical chain-breaking antioxidant activity by hydrogen atom transfer. ORAC assay is widely used as it can measure both duration of anti-oxidative action and reactivity [21].

In terms of DPPH radical scavenging activity green tea showed highest AA (2432.76 ± 110.3 µmol TE/ g FW) while branded tea (mirzapur) showed lowest (1269.91 ± 38.65 µmol TE/ g FW) (Table 1). Similarly, highest total ORAC value was observed in green tea (2834.51 µmol of TE/g FW) and lowest in branded tea (1262.81 µmol of TE/g FW) (Table 1). L-ORAC value was negligible in all four tea samples. All four types of tea showed high H-ORAC values (1242.10 - 2819.81 µmol TE/g FW) and green tea (2819.81 µmol TE/g FW) showed highest antioxidant activity as compared to other teas. The difference in ORAC values in tea samples is due to their variation in catechin content which changes during the fermentation process. During the fermentation process polyphenol oxidase enzyme of the leaves comes to the contact of catechins and leads to the conversion of catechins to their dimmer and polymers known as theaflavins and thearubigins, which affects the ORAC values [22].

Table 1: TPC, DPPH scavenging activity, H-ORAC, L-ORAC, and total ORAC of different types of tea

Sample name	TPC (mg GAE/ g FW)	DPPH scavenging activity (µmol TE/ g FW)	H-ORAC (µmol of TE/g FW)	L-ORAC (µmol of TE/g FW)	Total ORAC (µmol of TE/g FW)
Green tea	2348.60	2432.76±110.3	2819.81± 276.57	14.7 ±2.0	2834.51
Organic tea	1271.10	1532.60±82.86	1528.54± 49.5	12.66±2.8	1578.05
Tea BT-2	1501.70	1376.67±66.91	1573.73± 162.5	9.62±0.9	1583.33
Branded (mirzapur) tea	1056.74	1269.91±38.65	1242.1± 105.92	20.74±3.47	1262.81

Values are mean ± SD of triplicate analysis. *GAE- Gallic acid equivalent, * TE- Trolox equivalent

In present study green tea showed higher antioxidant activity as compared to black teas which is in well agreement with some other previous studies [23, 24] while insignificant studies reported that black teas are better than green ones [25, 26].

Effect of brewing time on Antioxidant activity in different types of tea in Bangladesh

Antioxidant activity of tea is influenced by different brewing conditions including extraction solvent, extraction time, temperature, pH, tea particle size, tea to water ratio, type of water used for brewing [27]. From a nutritional point of view, it is immensely important to find out the optimal

brewing time of particular tea at which it exhibits highest antioxidant activity. Again, this optimal time of brewing for highest extraction of polyphenol and antioxidant activity may vary for different tea samples. One of the objectives of this study was to determine the effect of brewing time and temperature effect on antioxidant activity in different tea samples of Bangladesh which are presented in table 2. For green tea and organic tea, 5 min brewing in boiling water extract showed highest ORAC value on the contrary 10 min brewing exhibited highest ORAC value for tea BT-2 and branded tea. This information regarding optimum brewing time for a particular tea is extremely useful to attain maximum extraction of polyphenols and their corresponding antioxidant activity through tea consumption.

Table 2: Comparison of ORAC value (µmol of TE/g FW) of analyzed teas in hot water extraction in different brewing time

Type of Tea samples	Hot water Extract (2min)	Hot water Extract (5min)	Hot water Extract (10min)
Green tea	907.64±45.00	1640.73±110.30	1521.12±106.22
Organic tea	1014.23±43.76	1584.12±82.86	1161.82±94.54
Tea (BT-2)	733.00±66.23	817.65±66.91	1005.41±72.65
Tea (Mirzapur)	769.07±32.88	973.44±38.65	1070.45±40.55

Values are mean ± SD of triplicate analysis

In literature, the optimal brewing time was reported to be 3-5 min [28] for green tea to achieve maximum antioxidant activity which corroborated with our findings. A higher optimal brewing time (10 min) for green tea was reported by Molan et al. [11] while Vuong et al. [27] reported 30 min hot water extraction as the most efficient brewing time for maximal extraction of catechins per gram of green tea. Other than green and organic tea we found a gradual increase in ORAC value in other two tea samples with an increase in brewing time. Similarly, [12] reported that longer the time of brewing, higher the antioxidant activity as well as total phenols and catechin contents for black tea.

Correlation between TPC and AA of analyzed tea samples

In this study TPC was measured by Folin-Ciocalteu assay. In most cases, total phenols determined by this method are correlated with the AA confirming the value of the Folin-Ciocalteu test [29] but it is not obvious that the higher levels of TPC will correspond to the higher antioxidant responses. The effectiveness of phenolics as antioxidants is not only attributed to their composition or relative amount of polyphenols but also by the degree of polymerization, concentration, position of OH group in the ring and interaction of their diverse chemical structures to the colorimetric assays.

The visual relationship between TPC and AA of the four analyzed tea samples is presented in figure 1. AA of all the tea samples can't be solely explained by the TPC as shown in the cases of organic tea and branded tea. Figure 1 also explains that not all poly-phenolic compounds have antioxidant potential as represented by BT-2 tea sample. The antioxidant activity demonstrated by green tea was well associated with its

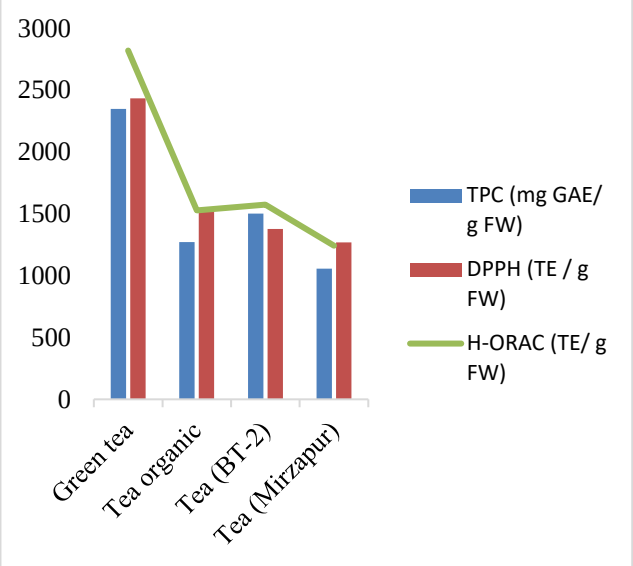

Figure 1: Relationship between TPC, DPPH and H-ORAC value of analyzed tea samples.

corresponding phenolic content. In both organic and branded tea sample similar amount of antioxidant activity was found in both DPPH and H-ORAC assay while in green and BT-2 tea antioxidant activity measured in terms of H-ORAC showed higher value than DPPH RSA.

Correlation analysis shown that both measures of AA (DPPH RSA and H-ORAC assay) has exhibited strong positive linear correlation (0.952

and 0.987) with TPC in all analyzed tea samples (Table 3). Previous studies also reported strong positive linear correlation between TPC and AA in

Table 3: Correlation coefficients (r), for the association between TPC (mg GAE/g), DPPH RSA (µmol TE/ g) and H-ORAC (µmol of TE/g) of analyzed tea samples

Relationship	R	P value
TPC * DPPH	0.952	0.048
TPC * H-ORAC	0.987	0.013
DPPH * H-ORAC	0.988	0.012

green and black tea samples [19, 30]. These results indicate that polyphenols are a major contributor to the antioxidant activity both in green and black teas.

In vitro Anti-inflammatory activity of different types of tea in Bangladesh

tea samples inhibited TNF-α production at the concentration of 40µg/mL and for anti-inflammatory activity potential the analyzed tea samples can be arranged in the following order: green tea> tea BT-2> tea Organic> branded tea (Figure 2). Further, dose response study revealed that green tea and organic tea exhibited dose-response on the inhibition of TNF-α production (40, 10, 3 & 1µg/mL) (Figure 3).

In vitro Anti-allergic activity of different types of teas in Bangladesh

Allergic reactions can be classified into four types, and the immediate hypersensitive reaction is generally termed as a type I allergy. The allergic reactions Immunoglobulin (IgE) plays a central role for the allergic response in Mast cells or basophils through the multivalent binding of allergens to the IgE receptors on the surface of

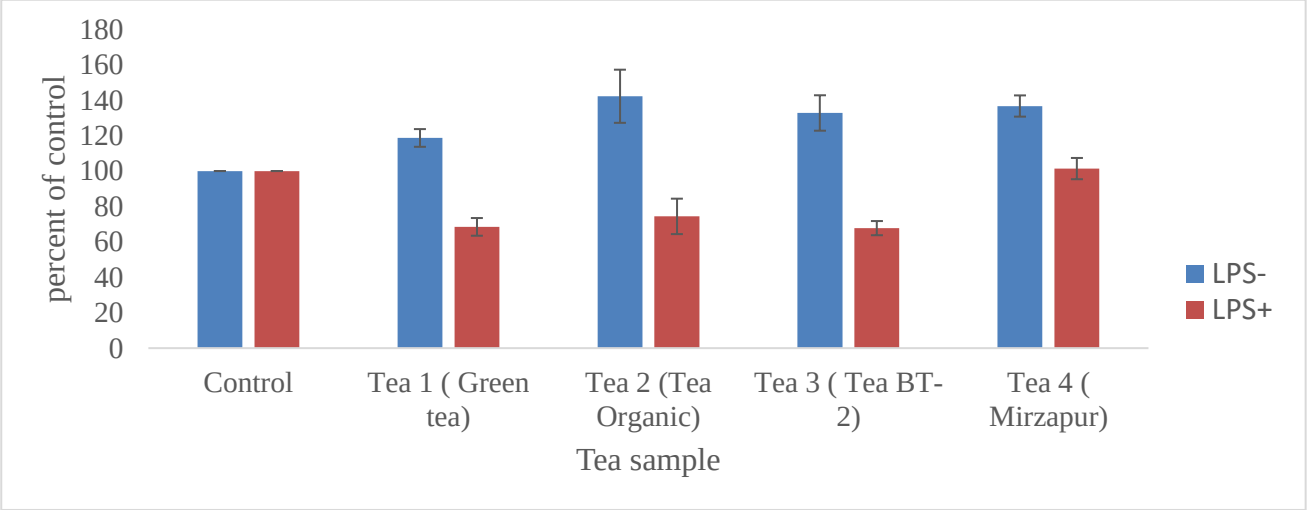

Figure 2: Anti-inflammatory activity (Percent Production of TNF-α with and without LPS in J774.1A Macrophage cells) in different types of tea.

Inflammation is the first response of the immune system to infection or irritation. Many researchers reported that different type of foods can cause anti-inflammatory as well as, pro-inflammatory effects and some of the polyphenols performed as potent anti-inflammatory agent. Nowadays, extensive research already established that polyphenols of foods, especially some flavonoids have inhibitory effects on TNF-α production in Lipopolysaccharide-stimulated Mouse macrophage cell lines J774.1 A [17]. TNF- α, an inflammatory mediator is produced by activated monocytes and macrophages and secreted during the early phase of acute chronic diseases such as asthma, rheumatoid arthritis, septic shock and allergic diseases.

In present study, preliminary screening for in vitro anti-inflammatory activity identified that all the

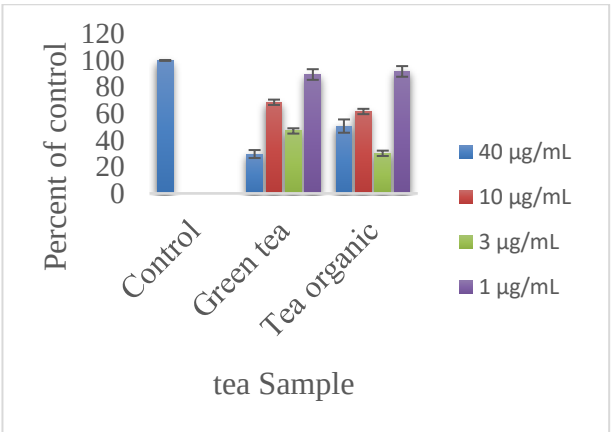

Figure 3: Positive dose response of green tea and organice tea extracts on the inhibition of LPS stimulated TNF-α production in J774.1A macrophage cells.

Mast cells that leads to the released of inflammatory substances, histamine, cytokines and other related metabolites or chemical mediators

which tigers allergic reactions. For the screening of anti-allergic effect of tea, rat basophilic leukemia (RBL-2H3) cell line was selected to estimate the degranulation inhibitors.

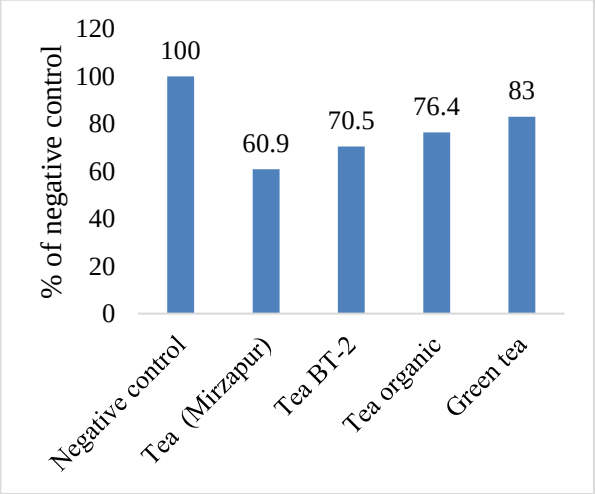

Figure 4: Anti-allergic activity (Antigen induced degranulation effect in RBL2H3 cells expressed as percent negative control) in different types of teas from Bangladesh

Anti-allergic activity (expressed as the percent inhibition of negative control) ranged from (60.9-83%) in four different type of tea samples (Figure 4) where green tea showing the highest anti-allergic activity and branded tea being the lowest anti-allergic potential. Present study findings are supported by the recent study [31] that reported polyphenolic compounds from tea inhibit histamine release from mast cells, thereby showing anti-allergenic activity.

CONCLUSION

This study contains key information regarding potential health beneficial role of four differently processed tea of Bangladesh. TPC and AA were found to be higher in green tea as compared to black tea. L-ORAC was negligible in all four tea samples while H-ORAC contributed most of the Total ORAC. A Considerable amount of Anti-inflammatory activity has been exhibited by all four tea samples where green tea being the most anti-inflammatory potential. Dose-response relationship of anti-inflammatory activity was observed in both green and branded (mirzapur) tea. Similarly, green tea showed highest anti-allergic activity while other showed the considerable amount. One of the most striking findings of this study is the effect of different brewing time on achieving highest antioxidant activity in boiling water extract by different tea samples. Green tea and organic tea showed highest ORAC value when brewed for 5 min in hot water extract while tea BT-2 and branded tea exhibited highest ORAC value for 10 min brewing. Present study is significant as first-time systematic attempt to quantify the quality of different types of tea (antioxidant, anti-inflammatory and anti-allergic activity) in Bangladesh. The findings of the present study suggest that analyzed selected tea samples have considerable antioxidant; anti-inflammatory, anti-allergic potential and therefore tea polyphenols extract may be used as nutraceutical to avoid the side effects of available anti-inflammatory and anti-allergic drugs. Additionally, tea extract specially green tea may be used as natural flavoring agent with antioxidant properties instead of commercially available chemical antioxidant used for the enhancement of self-life of lipid-bearing foods by delay in lipid peroxidation.

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Declaration of interest statement

The authors declare no financial and conflict of interest.

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