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Immunomodulatory activity of black tea extract in rheumatoid arthritis: An *in-vitro* study

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Abstract
Introduction: Rheumatoid Arthritis (RA) is an autoimmune disorder affecting approximately 1% of the population. The hallmark of RA is synovial inflammation along with bony erosion. Inflammation and associated oxidative stress are primary foci to design therapeutic strategies against RA. Black tea (BT) is a popular beverage. The Constituents of BT, mainly catechins & theaflavins, have been reported to have anti-oxidant, anti-inflammatory effects & used in various diseased conditions like cancer, Alzheimer's disease etc. There is very scanty data on its effect on Rheumatoid arthritis. The study was designed to see the anti-inflammatory effect and anti-oxidative effect of black tea in RA
Methods: Sixty Patients aged between 18-60yrs attending OPD, Department of Rheumatology, fulfilling ACR 1987 criteria with active disease and no prior use of steroids and anti-oxidants were recruited. Ten healthy controls were also recruited for the study. Levels of oxidative stress markers (total ROS, NO etc.) were measured in healthy controls and RA pt. Levels of inflammatory parameters (TNFα, IL6) were measured by standard ELISA kits.
Results: Markers of oxidative stress and inflammation showed strong positive correlation in RA patients with their disease progression and severity. BT extract significantly down regulated ROS generation (GMFC mean ± SD: 413.7±267.5 in untreated cells, 146.4 ±122.2 for cells treated with BT 250ng/ml, 166.8±120.9 for cells treated with BT 500ng/ml) and super oxide radical generation from activated neutrophils compared to BT untreated samples.BT extract significantly decreased NO production from activated PBMCs and also down regulated pro-inflammatory cytokine (TNFα, IL6) production from PBMCs as compared to BT untreated samples.
Conclusions: Black tea contributes significantly in lowering most of the oxidative stress and inflammatory parameters in RA patients. Thus BT may be used as a potent anti-oxidative therapeutic modality to control the oxidative stress and inflammation in RA patients.

Keywords: Rheumatoid arthritis, oxidative stress, black tea extract, catechin gallate

1. Introduction
Rheumatoid Arthritis is a common autoimmune disorder that is associated with progressive disability, systemic complications and early death [1]. Recent researches have shown a potential role for oxidative stress and subsequently an imbalance in redox homeostasis in the pathogenesis of the disease. Oxidative stress, a resultant of uncontrolled release of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is associated with an increased release of numerous pro-inflammatory factors like cytokines and prostaglandins leading to tissue and joint damage in patients with RA.
The treatment modalities for RA, mainly intended for relief of pain as well as controlling damage, include non-steroidal anti-inflammatory drugs (NSAIDS), disease modifying anti-rheumatic drugs (DMARDS), glucocorticoids, and anti-cytokine agents [2]. Although these agents are effective in controlling disease to certain extent, they have significant toxicities and often are costly. For this reason, there is always a hunt for making the treatment modalities less expensive and relatively safe for all.
Plant-derived bio-molecules are recently emerging as an important alternative medicine because of their low cost, availability and comparatively less toxicity. Black Tea is the most consumed beverage in Indian subcontinent. The chemical constituents of tea leaf include polyphenols (catechins and flavonoids), alkaloids (e.g., caffeine, theobromine, theophylline),

volatile oils, polysaccharides, amino acids, lipids, vitamins (e.g., vitamin C), and inorganic elements (e.g., aluminium, fluorine and manganese). Of these, the polyphenols are mainly responsible for the beneficial properties of tea [3]. Studies have documented various biological properties of tea, including antibacterial, antiviral, anti-oxidative, anti-inflammatory, antitumor, anti-mutagenic and anti-carcinogenic activities [4].

Considering the strong anti-inflammatory activity of black tea and the contribution of inflammation in RA pathogenesis, this study was undertaken to evaluate the potential anti-oxidative and anti –inflammatory effect of polyphenol enriched black tea extract upon peripheral blood mononuclear cells and neutrophils of patients with RA.

2. Materials and Methods

2.1 Reagents

All chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA) except 4, 5-diaminofluorescein diacetate (DAF-2DA, Cayman Chemical Company, Ann Arbor, Michigan, USA), Hisep1073 and Granulosep 1119 (Himedia, Mumbai, India), TCA and TBA (Loba chemie), FBS, Pen-Strep (Invitrogen), TNF-α,IL-6Human Elisa Kits from (Ray Biotec).

Theaflavin enriched Black tea extract (cat no: T5550; ≥ 80% Theaflavin) and Catechin Gallate (cat no: C0692; ≥98%,from green tea) were purchased from Sigma Aldrich (St. Louis, MO, USA). Both the extracts were dissolved primarily in DMSO to prepare primary stock from which desired treatment dosage (cytotoxicity assay and dose determination was done before starting experiments; data not shown) were achieved by diluting stock with PBS.

2.2 Study population

Sixty patients with Rheumatoid arthritis fulfilling American College of Rheumatology 1987 criteria for diagnosing RA [5], were recruited from the outpatient clinic of Department of Rheumatology, Institute of Postgraduate Medical Education and Research and SSKM Hospital, Kolkata. All the recruited patients were of age between 18-60yrs and receiving methotrexate (DMARDs) as conventional therapy. We excluded patients having other co-morbid conditions, smokers and patients receiving any type of glucocorticoid therapy and anti-oxidants as supportive medications.

We recruited 10 age and sex matched healthy controls to compare the oxidative status between healthy individuals and diseased persons. Controls were not taking any antioxidants and all were non- smoker.

The study protocol received prior approval from the Institutional Ethics Committee and written informed consent was obtained from all participants.

2.3 Study material

Overnight fasting (12 hour) blood samples were obtained from patients and healthy individuals. All experiments were performed with peripheral blood samples as the study was designed as an *in-vitro* study.

2.4 Baseline Evaluation of subjects

Demographic features like age, sex, height, weight were documented for the patients. Patients' evaluation included disease duration, tender joint count (TJC), swollen joint count (SJC), Health Assessment Questionnaire–Disability Index (HAQ-DI) score [6], and visual analog scale (0-100 scale). A composite Disease Activity Score (DAS28) was calculated using 4 variables: SJC- 28, TJC-28 [7], VAS (0-100 scale), and erythrocyte sedimentation rate (ESR) by Westergren method. The low disease activity is defined by DAS28≤3.2, moderate disease activity as DAS28 = 3.3 to 5.3, and high disease activity as DAS28 ≥ 5.4. Furthermore, values for C-reactive protein (CRP), Rheumatoid Factor (RF) and anti-cyclic citrullinated protein (anti-CCP) were also noted down.

2.5 Isolation of neutrophils and monocytes from peripheral blood

Neutrophils were isolated from peripheral blood using HiSep 1077 and GranuloSep GSM 1119 according to manufacturer's protocol. In short, a double gradient was created by layering an equal volume of HiSep 1077 onto GranuloSep GSM 1119 layer. Blood was layered over the upper HiSep 1077 medium and centrifuged at 3000 rpm for 40 minutes at room temperature. After centrifugation Neutrophils appeared at the 1077/1119 interphase layer and were collected, washed twice in RPMI 1640 PR^- medium, centrifuged (1800 rpm for 10 minutes) and re-suspended in RPMI 1640 PR^- medium; cell viability was determined using trypan blue (- 95%).

Monocytes were isolated from peripheral blood using monocyte isolation medium (HiSep LSM 1073), according to manufacturer 's protocol; briefly, equal volume of blood was layered over LSM1073 and centrifuged at 3000 rpm for 40 minutes at RT. After centrifugation cells were collected and re-suspended in RPMI 1640 PR^- medium supplemented with penicillin (100 U/ml), streptomycin (100 µ g/ml), and 10% heat-inactivated fetal bovine serum (FBS), viability was confirmed using trypan blue (-95%).

2.6 Determination of reactive oxygen species (ROS)

Neutrophils isolated from blood were seeded in an eppendorf at a count of $5x10^5$ cell/ml in 500µl PBS. Initially the cells were incubated with theaflavin enriched Black tea extract (doses: 250ng/ml and 500ng/ml) and catechin gallate (dose: 2.5µg/ml) for 1hr at 37 °C. After that cells were loaded with dichlorodihydrofluorescein diacetate (H_2DCFDA, 0.5 µM), and again incubated for 30 minutes, at 37 °C. Cells were then washed twice with phosphate buffered saline (PBS, 0.02M, pH 7.2) at 4000rpm for 4min, finally resuspended in PBS and acquired in a flow cytometer.

2.7 Estimation of superoxide radical

Following preincubation with catechin gallate (dose: 2.5µg/ml) and Black tea extract (250ng/ml and 500ng/ml, 1 h, 37 °C), $1x10^6$/ml neutrophils incubated with cytochrome C (1.0 mg/ml) for an additional 30 minutes at 37 °C. The tubes were placed on ice, centrifuged (6000 rpm for 5 minutes at 4 °C) and supernatants were collected. Absorbances of the supernatants were measured at 550 nm (OD_{550}), which were the representative of the reduced cytochrome c ($A_{superoxide}$). The amount of superoxide generated in 1 ml of the reaction mixture was calculated as follows:

O_2^- (nmol) = 47.7 × $A_{superoxide}$ (OD_{550}) [8]

2.8 Measurement of hydroxyl radical

The hydroxyl radical scavenging activity of BT extract was assessed using the deoxyribose assay described by

Gutteridge JMC *et al.* [9] with slight modification. Briefly, neutrophils at a count of $1x10^6$ cells/ml were pre-incubated with catechin gallate (dose: 2.5µg/ml) and Black tea extract (250ng/ml and 500ng/ml, 1 h, 37 °C). To this 500µl of 2-deoxy ribose (10 mM) and 500µl of PBS (pH 7.0) was added, the mixture was incubated for another 2hours at 37 ° C, the mixture was centrifuged at 4000rpm for 5 min; the supernatant was mixed with an equal volume of 2.8% TCA (500µl) and 1%TBA (500µl). After incubating the mixture at 100 ° C boiling water bath for 10 min, it was brought to room temperature under running tap water and absorbance measured at 520 nm; the results were expressed as percentage scavenging as follows:
(1-Sample absorbance/Blank sample absorbance) X 100%

2.9 Measurement of intracellular nitric oxide (NO)
Intracellular NO was measured using diaminofluorescein diacetate (DAF-2DA), a non-fluorescent dye that fluoresces on reaction with NO [10]. Briefly PBMCs at a count of $1x10^6$ cells/ml were pre-incubated with catechin gallate (dose: 2.5µg/ml) and Black tea extract (250ng/ml and 500ng/ml) for 18 h at 37 ° C, 5% CO_2, after that cells were scraped down and were washed at 4000 rpm for 4mins, resuspended in PBS. Cellular suspensions were then incubated with DAF-2DA (2 µ M) for 30 min at 37 °C. Cells were gated on the basis of the characteristic forward scatter and side scatter and acquired in a flow cytometer.

2.10 Flow cytometry
Cells from different experimental groups were assessed for their intracellular fluorescence using a flow cytometer (FACS Calibur, Becton Dickenson, San Jose, CA) equipped with an argon-ion laser (15 mW) tuned to 488 nm. The fluorescence of DCF and DAF-2T were collected in the FL1 channel, equipped with a 530/30 nm band pass filter. Fluorescence was measured in the log mode using Cell Quest Pro software (BD Biosciences, San Jose, CA) and represented as geometrical mean fluorescence channel (GMFC). Cells were gated on the basis of their typical morphology, i.e. forward scatter and side scatter of neutrophils, macrophages and lymphocytes. Acquisitions were performed on 10000 gated events, while data analysis was carried out with Cell Quest Pro software (BD Biosciences).

2.11 Cell Culture
PBMCs were isolated as described earlier. Cells were washed twice with RPMI-1640 incomplete media and counted in haemocytometer. Cells were then seeded in a six well plate at a count of $2x10^6$ cells /1ml complete media and autologous serum. The cells were allowed to adhere for 1hr at 37 °C CO_2 incubator. After 1hr whole media was discarded and the cells were washed once with incomplete media. After that again 1ml complete media was added and respective drugs were also added. Then it was incubated with the drugs for another 1hr at 37 °C CO_2 incubator, later on LPS (1µg/ml) was added to it and kept for 18hrs at 37 °C CO_2 incubator. Next day supernatant collected and centrifuged at 1800rpm for 4mins. Clear supernatant stored for ELISA.

2.12 Expression levels of pro-inflammatory cytokines
Levels of pro-inflammatory cytokines like TNF-α, IL-6 and IL-1β were measured by an enzyme-linked immunosorbent assay from cell culture supernatant using ready to use kits (Ray Bio® Human TNF-alpha ELISA Kit Catalog #: ELH-TNFa, Human IL-6 ELISA kit Catalog#: ELH-IL6, Human IL1-beta ELISA kit Catalog #: ELH-IL1-Beta). ELISA was done according to the manufacturers protocol.

2.13 Statistical analysis
All data were tested for normality by Shapiro-Wilk Test. Data were represented as mean values with standard deviations (SDs) for parametric or normal data or as median ± IQR for non-normally distributed ones. Comparisons between two groups were also done by unpaired t test and Mann-Whitney U test as applicable for the data sets. Between-group differences were calculated using one-way ANOVA with post hoc tests by Bonferroni correction or Dunns multiple comparison test for normally distributed and skewed data, respectively. All the data were analysed using Graph Pad Prism software, version 5.0 (Graph Pad Software Inc, San Diego, CA); $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Study population
The age of patients ranged from 19 – 59 years, and female: male ratio is 53:7. All the patients were with active disease with mean DAS 6.55, CRP value 1.2 (0.6-5.0) mg/l and positive for both RF and Anti-CCP. Among healthy individuals age ranges from 21-48yrs and female: male is 4:1.

3.2 Association between inflammation and free radical generation in RA patient
Our result (Fig 1A) indicated that there existed a positive association between ROS generation and development of inflammatory stress (r=0.8143, p= 0.0002 of Total ROS & TNFα) in RA patients might state the generated free radicals might be the reason for inflammation and disease progression.
We observed markers of disease activity in RA (DAS28 score), inflammation (C-reactive protein (CRP)), and Anti-CCP values to correlate with accumulated ROS (Fig 1B). Our result indicated strong correlation between inflammation and total ROS (r=0.8821, $p<0.0001$). Similar observation also evident in disease activity score and generation of ROS (r= 0.6716, p=0.0006). Auto-antibody i.e. Anti-CCP values also positively correlated with ROS accumulation in RA patients (r=0.5827, p=0.02)

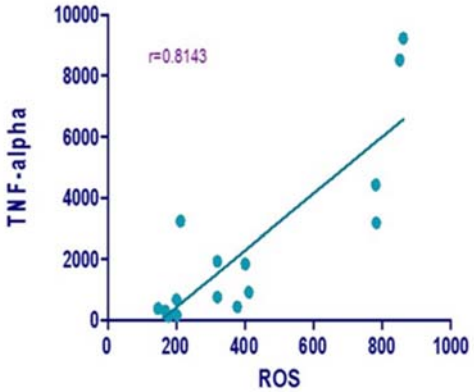

Fig 1A : Association of TNF-alpha and total ROS in RA patients. They exhibited strong positive correlation with r=0.8143, p=0.0002

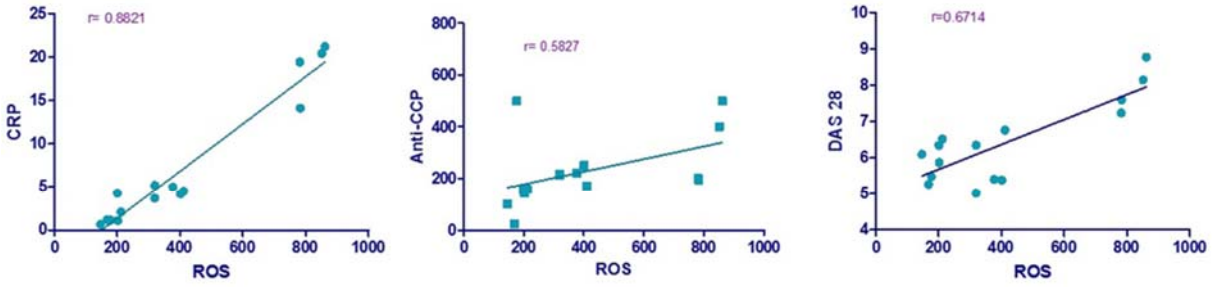

Fig 1B: Correlation between oxidative stress and disease related parameters of RA patients. ROS was positively correlated with CRP (r=0.8821),Anti-CCP (0.5827),DAS-28 (r=0.6714)

3.3.a Generation of free radicals in the neutrophils isolated from RA patients

The prevalent oxidative burden in RA patient was reasoned by analysing the intracellular ROS accumulation using a membrane-permeable compound H_2DCFDA. Results (Fig. 2A) indicated there was a sharp in increase in DCF fluorescence showing a significant amount of ROS accumulation in neutrophils of RA patients than healthy counterparts (ROS generation in healthy 78.50±18.25, RA Pt. 387.5±249.0, p <0.0001). The accumulated ROS in the neutrophils prompted us to look into the source of its production under chronic inflammatory disorder. Result (Fig. 2B) showed a significant accumulation of $O_2^{\cdot-}$ (super oxide radical in healthy 14.03±3.149, RA pt. 40.91±4.535, p<0.0001) in neutrophils RA patients compared to control patients demonstrating there might be concomitant accumulation of $O_2^{\cdot-}$radicals under oxidative stress.

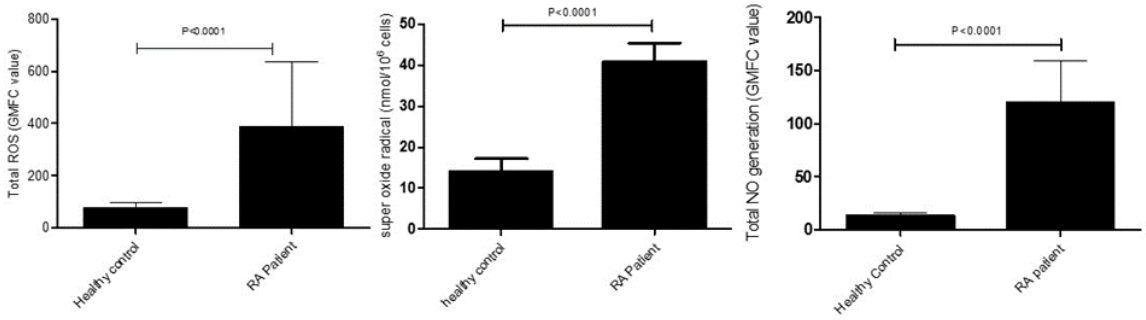

Fig 2A,B &C: 2A ROS accumulation in RA pts is higher than healthy controls(p<0.0001), 2B represents comparative superoxide radical generation in healthy controls & in RA pts.2C depicts NO generation from activated cells, which is lower in case of healthy than RA patients (p<0.0001).

3.3.b Raised intracellular NO level was observed in peripheral blood mononuclear cells(PBMCs) isolated from RA patient

For this reason, we measured NO level in macrophages (PBMCs). The intracellular NO level was measured indirectly by flow cytometric method based on conversion of DAF-2DA to 4,5-diaminofluorescein (DAF-2), which is subsequently oxidized by NO and produced the fluorescent triazolofluorescein (DAF-2T). Our result detected a significant rise in intracellular NO level (Fig 2C) in RA patients compared to healthy individuals (NO generation in healthy 13.56±2.827, RA pt. 119.8±39.42, p<0.0001) indicating increased NO level during inflammation might be triggered by inflammatory mediators.

3.4.a Effect of BT extract and Catechin gallate on intracellular ROS level from neutrophils of RA patients

Next, we decided to visualize and compare the effect of whole BT extract and *Catechin gallate* which is an active compound from green tea on the accumulated ROS burden in the neutrophils from peripheral blood of RA patients. We analyzed the total ROS level from different treatment groups of cells taken from RA patients by flow cytometry. Result showed (Fig 3A) a significant rise in ROS generation (GMFC mean ± SD: 413.7±267.5) in untreated cell group which was successfully ameliorated after administration of BT extract (146.4 ±122.2 for 250ng/ml), (166.8±120.9 for 500ng/ml) and *Catechin gallate* (163.0±103.9 for CG 2.5µg/ml). But the best result was obtained after treatment with BT extract at 250ng/ml.

Furthermore, spectrophotometric data indicated a concomitant increase in superoxide level in peripheral blood neutrophils (40.62± 4.411nM) which after incubation with different doses of BT extract (250ng/ml and 500ng/ml) and CG (2.5µg/ml) was significantly reduced to 23.64± 5.183 nM (P<0.0001), 24.60± 4.402 nM (p< 0.0001) and 24.32 ± 6.814 nM (p< 0.0001) respectively, very likely to that healthy counterparts (Fig 3B).

These findings prompted us to look inside status of hydroxyl radicals generated from neutrophils and assessed the scavenging activity BT and CG treatments since it is well known that accumulated H_2O_2 generally gets converted into hydroxyl radicals. Our findings (Fig 3C) demonstrated that BT extract (250ng/ml,500ng/ml) were more successful in scavenging hydroxyl radicals (94.44% and 89.88%) than CG (79.02%) with respect to untreated cells clearly indicating BT extract might have the property for extinguishing intracellular ROS levels.

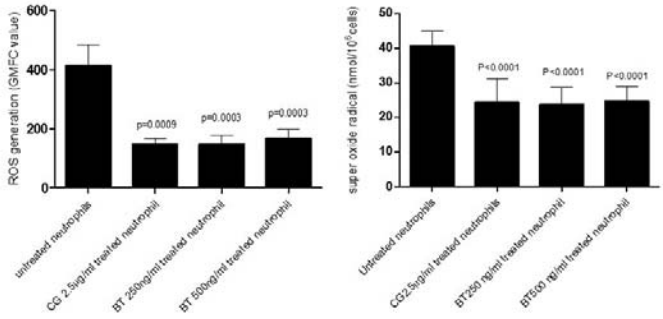

Fig 3A & B: 3A graphical representation of ROS accumulation in neutrophils from RA patients and the treatment groups with CG and BT extracts. 3B illustrates super oxide radical generation from activated RA neutrophils and from the treated neutrophils.

3.4.b Impact of BT extract and CG on intracellular NO level from neutrophils of RA patients

Down regulation of intracellular ROS levels after BT and CG administration in neutrophils from RA patients instigated us observe their effects on NO level from monocytes. Finding (Fig 3D) showed that there was a rise in intracellular NO level (112.0± 48.71) in cells from RA patients. This was significantly decreased after administration our respective treatment molecules; (CG2.5µg/ml: 31.40± 13.46, BT 250ng/ml: 59.07± 11.79 and BT500ng/ml: 68.23± 17.37).

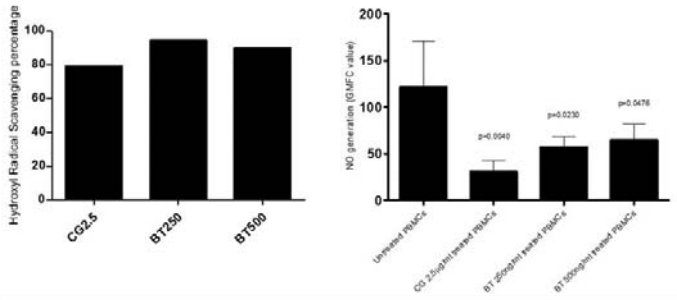

Fig 3C & D: 3C portrays hydroxyl radical scavenging percentage of CG and BT extracts of which BT250ng/ml has the best scavenging activity. 3D is the representation of NO generation from activated monocytes and from treated groups.

3.5 BT and CG extract reduced the level of pro-inflammatory cytokines released from PBMCs

Monocytes are the primary cells which generates various pro-inflammatory cytokines after activation within blood of RA patients. So, we decided to observe the level of expression of different pro-inflammatory cytokines like TNF-α and IL-6 (major cytokines in RA pathogenesis) upon treatment with our desired extracts in monocytes of RA patients. To conduct this experiment we exposed the untreated cells with LPS for cytokines stimulation (TNFα release after LPS stimulation 6657±5634 pg/ml) and found there was a significant reduction (Fig 4A) in TNF-α level after treatment with CG (CG 2.5µg/ml: 3996 ± 3628 pg/ml, p =0.0491) and BT extract (for BT 250ng/ml 3252 ± 3196 pg/ml p=0.0178, for BT 500ng/ml 3512 ± 2928,p=0.0314). The best result was obtained after BT administration at 250ng/ml. Very similarly we observed the level of IL-6 in monocytes form RA patients. Here also both BT extract and CG successfully reduced the levels of IL-6 in LPS stimulated monocytes from RA patients (Fig 4B) (LPS stimulated release of IL-6 is 2624±1303 pg/ml, CG 2.5µg/ml 2104±1057pg/ml p=0.0062). Amongst the treatments, BT extract at 250ng/ml (BT 250 ng/ml 1837 ± 989.8 pg/ml, p= 0.0012, BT 500 ng/m 1934±1022 pg/ml, *p*=0.0033) was most successful in downregulating the IL-6 levels compared to other treatments. Similar finding was observed in case of IL-1β production and concomitant treatment with our desired tea extract (data not shown).

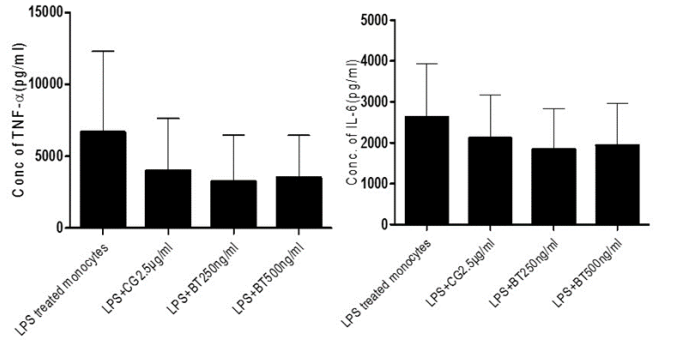

Fig 4A&B: Fig A represents TNF-α generation from LPS activated PBMCs and treatment groups with CG 2.5µg/ml, BT 250 ng/ml & BT 500 ng/ml. Fig B is the illustration of IL-6 generation from LPS stimulated cells and treatment study groups.

4. Discussion

Several studies have proved the implication of oxidative stress in the pathogenesis of RA [11, 12] and accumulation of higher ROS concentration in the synovial joints and blood have been reported in RA patients [13]. At the time of respiratory burst, cellular subsets like neutrophils, monocytes and macrophages generate ROS by membrane-bound NADPH oxidase, this enzyme catalyses a single electron transfer from the cytosolic NADPH which reduces extra-membranous molecular oxygen to highly active superoxide anion. In our study, the redox status was evaluated in peripheral blood neutrophils from healthy individuals and RA patients where RA patients exhibited raised level of total ROS than healthy individual clearly indicating accumulation of oxidative burden. NO is mostly released by activated monocytes and upon oxidative insult surplus intracellular NO gets converted into peroxynitrite which is extremely detrimental for cell. This phenomenon is well corroborated with our study with a significant rise in NO level in cells from RA patient.

Reports suggested that oxidative stress leads to generation of several pro-inflammatory cytokines. Availability of high level of inflammatory markers from the peripheral blood of the RA patients clearly suggested the participation of ROS in this act. We found increasing levels of ROS with a concomitant increase in inflammatory markers like TNF-α and IL-6. So, we decided to find out any relationship exists among these findings with disease associated parameters like DAS28 score, Anti-ccp and CRP values. Excessive generation of ROS in PBMC from RA patients exhibit high disease activity as well increased production of auto antibodies like Anti-CCP. This finding may corroborate the most proposed hypothesis of RA pathogenesis which indicates excessive oxidative stress induces cell activation and release of pro-inflammatory cytokines from activated cells. Hence, exacerbate the pro-inflammatory milieu of RA. All the patients who were taken for our study received DMARDs as a conventional medication. In spite of that they manifested high disease activity, PBMC exhibited high level of inflammatory markers and oxidative stress was also very high. Even the inflammatory status was not under control in spite of having basic treatment. This unexplained consequence prompted us to take up this study in which we can find out the effectivity of herbal agents in RA pathogenesis. Since herbal drugs are very effective in their target of actions and have minimal side effects. We have chosen whole black tea extract rich in theaflavin molecules and also in parallel to that we used CG which is one of the predominant active compounds of green tea. Theaflavins are polyphenolic moieties which have shown to be very effective in scavenging free radicals in different cell types [14-16] but ROS scavenging activity and anti-inflammatory properties have not yet been demonstrated in cells from RA. Henceforth, we decided to treat the cells with our desired tea extracts to find out if they have any role in controlling the oxidative stress and inflammation.

Treatment of neutrophils with BT effectively decreased generation of ROS from peripheral blood of RA patients. This ROS scavenging activity of BT extract can be attributed to its ability to modulate the anti-oxidant pathways (enzymatic and/or non-enzymatic) or simply by neutralizing ROS generation. As cells were incubated with BT extract for only 1 h, it may be concluded that BT extract was acting as an effective scavenger of ROS. On the other hand, CG was less efficient in reducing the total ROS levels in cells from RA patients compared to that of theaflavin rich BT extract. Downregulation in total ROS generation like superoxide anions, and hydroxyl radicals after BT treatment in PBMC from RA patients clearly indicate that there might be an active indirect modulation of BT extract on neutrophil level thereby reducing its activity in inflammatory zones. This action of BT was far better than CG administration alone clearly pointing the successful role of theaflavins in this regard.

As compared to the increase in ROS, the increased generation of a major pro-oxidant NO has also been reported in RA patients.NO is a very reactive inorganic free radical mostly generated by macrophages and contributes to cytotoxicity, inflammation and carcinogenicity [17, 18]. Having half-life shorter than 10secs because of its rapid oxidation to nitrites; it is very difficult to estimate the exact amount of NO released by activated monocytes. Our results showed that treatment with BT extract caused a dose dependent decrease in intracellular production of basal NO in blood monocytes. This may be due to the ability of BT extract to scavenge surplus NO *in vitro*. So in this context we can conclude that black tea extract is working as a potent anti-oxidative agent, as it is effectively down-regulating the stress related parameters in blood neutrophils of RA patients.

The inflammatory status of RA patients usually inclined to the pro-inflammatory state due to the excessive release of several pro-inflammatory cytokines. The major pro-inflammatory cytokine TNF-α, produced by activated macrophages and T-cells, exerts its pro-inflammatory effects by inducing the release of other pro-inflammatory cytokines, metalloproteinases, and free radicals. We treated the PBMCs with LPS (external stimuli) and assessed the cytokines release from the activated cells. BT extract is significantly down regulating the generation of TNF-α and IL-6 from activated PBMCs. This finding could attribute an important way out to control these cytokines in RA patients who are receiving conventional therapy and still exhibit a higher inflammatory status.

Oxidative stress, produced by activation of neutrophils and macrophages, is found to be the major contributor in the pathogenesis of RA. These oxidative stress molecules in turn induce the release of several pro-inflammatory cytokines in the disease milieu. Our *ex vivo* study confirmed the excellent potentiality of BT extract to attenuate these inflammatory mediators in the activated cells of RA patients. BT extract was found to be effective in down regulating several inflammatory mediators like including cyclooxygenase-2, prostaglandin E2 as well as the transcription factor, NF- κ B in other diseases [3]. Detailed studies on these parameters with samples of RA patients might provide useful insights into the upstream signalling pathways of inflammation in RA

Taken together, BT extract mediates its anti-inflammatory activity *via* suppressing the pro-inflammatory cytokines as well as by scavenging the oxidative stress parameters. It may be used as a promising non-toxic formulation in attenuating oxidative stress associated pathology in RA patients along with the conventional therapy. Patients those who fail biologic therapies can be considered to suggest use BT as support system, as it can modulate the generation of cytokine release and suppress inflammation. Furthermore, this study highlighted the importance of measurement of

oxidative stress and it could well be an effective marker for monitoring disease progression. This emphasizes the importance of anti-oxidants like black tea being included as an 'add on' therapy for improved management of rheumatoid arthritis.

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Competing Interests
The authors declare that they have no competing interests.

5. References

1. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature. 2003; 423:356-61.
2. Cobo-Ibá ñ ez T, Martin-Mola E. Etanercept: long-term clinical experience in rheumatoid arthritis and other arthritis. Expert Opin Pharmacother. 2007; 8:1373-1397.
3. Sharangi AB. Medicinal and therapeutic potentialities of tea (*Camellia sinensis* L.) – A review. Food Research International. 2009; 42:529-535.
4. Halil Erhan Eroğlu. The Cytogenetic Effects of Black Tea and Green Tea on Cultured Human Lymphocytes. 2011; 54(6):1159-1165. ISSN 1516-8913
5. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, *et al*. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988; 31:315-24.
6. Bruce B, Fries J. The Stanford health assessment questionnaire (HAQ): a review of its history, issues, progress, and documentation. J Rheumatol. 2003; 30(1):167-178.
7. Prevoo ML, van't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum. 1995; 38:44-48.
8. Markert M, Andrews PC, Babior BM. Measurement of O 2 _ production by human neutrophils. The preparation and assay of NADPH oxidase-containing particles from human neutrophils. Methods in Enzymol 1984; 105:358-365.
9. Gutteridge JM, Halliwell B. The deoxyribose assay: an assay both for 'free' hydroxyl radical and for site-specific hydroxyl radical production. Biochem J. 1988; 253:932-3.
10. Saha P, Bhattacharjee S, Sarkar A, Manna A, Majumder S, Chatterjee M. Berberine Chloride Mediates Its Anti-Leishmanial Activity via Differential Regulation of the Mitogen Activated Protein Kinase Pathway in Macrophages. PLoS One. 2011; 6(4):e18467. Published online 2011 Apr 5.
11. Hitchon CA, El-Gabalawy HS. Oxidation in rheumatoid arthritis. Arthritis Res Ther. 2004; 6:265-78.
12. Filippin LI, Vercelino R, Marroni NP, Xavier RM. Redox signalling and the inflammatory response in rheumatoid arthritis. Clin Exp Immunol. 2008; 152(3):415-422.
13. Remans PH, van Oosterhout M, Smeets TJ, Sanders M, Frederiks WM, Reedquist KA *et al*. Intracellular free radical production in synovial T lymphocytes from patients with rheumatoid arthritis. Arthritis Rheum. 2005; 52:2003-9.
14. Gramza, Pawlak-Lemañska K, Korczak J, W¹sowicz E, Rudzinska M. Tea Extracts as Free Radical Scavengers. Polish Journal of Environmental Studies. 2005, 14(6):861-867.
15. Gargi S, Biswajit B. Black tea as a part of daily diet: A boon for healthy living. International Journal of Tea Science. 2013; 9(2-3).
16. Min-Hsiung Pan a, Ching-Shu Lai a, Hong Wang b, Chih-Yu L, Chi-Tang H, Shiming Li e. Black tea in chemo-prevention of cancer and other human diseases. Food Science and Human Wellness. 2013; 2:12-21.
17. Nguyen T, Brunson D, Crespi CL, Penman BW, Wishnok JS, Tannenbaum SR. DNA damage and mutation in human cells exposed to nitric oxide *in vitro*. Proc Natl Acad Sci U S A. 1992; 89:3030-4.
18. Liu RH, Hotchkiss JH. Potential genotoxicity of chronically elevated nitric oxide: a review. Mutat Res. 1995; 339:73-89.