Alleviative effects of green and black tea aqueous extracts on cellular oxidative stress and anemia in rat adjuvant-induced arthritis

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The aim of this study was to evaluate and compare the alleviative effects of two doses (0.5 and 1.0 gm/kg body weight) of green and black tea aqueous extracts (GTE and BTE, respectively) on articular/extra-articular complications in rat adjuvant-induced arthritis (AIA). Arthritic rats received distilled water as vehicle, indomethacin (1.0 mg/kg body weight; a non-steroidal/anti-inflammatory drug), or tea aqueous extracts orally/daily for 28 days started from the day of arthritis induction (day 0). Other arthritic rats received tea aqueous extracts orally/daily for 14 days started from the day of arthritis onset (day 15). Both tea aqueous extracts significantly suppressed (but with different degrees) the arthritis severity/complications in AIA rat model especially at the high dose and when the treatment started from day 0. Only the high dose of GTE (from day 0) significantly alleviated, as indomethacin (53.32 ± 15.41 and 48.35 ± 17.09, respectively), all complications shown in arthritic rats including body weight loss, anemia, arthritic score, and synovial/hepatic tissues lipid peroxidation (P<0.05-0.001) through significantly increasing food intake (P<0.001) and cellular antioxidants (P<0.05-0.001): reduced glutathione level and catalase, glutathione peroxidase and superoxide dismutase activities. Therefore, tea (especially green tea) may be useful in the management of rheumatoid arthritis complications.

Keywords: Anemia, Antioxidant, Camellia sinensis (L.) Kuntze, Experimental arthritis, Indomethacin

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Rheumatoid arthritis (RA) is a systemic inflammatory and destructive joint disease that affects 1-2% of the adult population worldwide causing significant disability and consequent reduction in quality of life, which have a substantial socio-economic impact¹. Extra-articular complications (outside the locomotor system) are frequent in RA patients, including weight loss, chronic fatigue, oxidative tissue damage, and hematological disorders⁵. Anemia is the most common extra-articular complication in RA and is often attributed to anemia of inflammation³. Prevalence of the extra-articular complications is considered as a predictor of mortality in patients with RA; they are mostly related to excessive inflammation and cellular oxidative stress induced by inflammatory mediators and free radicals, respectively, which are produced throughout disease onset and progression⁴. Free radicals especially reactive oxygen and nitrogen species (ROS and RNS, respectively) are able to destroy macromolecules as membrane lipids, proteins, DNA, and cartilages either directly by their degradative ability or indirectly via activation of proteolytic enzymes such as matrix metalloproteinases (MMPs) causing vital organs damage such as liver besides synovial joints damage⁴–⁶. Therefore, scavenging free radicals by antioxidants may reduce the arthritis severity and its complications.

Most drugs that are currently used to control RA symptoms/pain including non-steroidal/anti-inflammatory drugs (NSAIDs) have poor efficacy and potential toxic effects with chronic use. Therefore, scientists have recently been paying serious attention to herbal therapies that have anti-inflammatory and antioxidant properties with minimum side effects in the treatment of RA, especially after the withdrawal of many Food and Drug Administration-approved anti-inflammatory drugs⁴,⁷–⁹. Tea (Camellia sinensis), one of the most popular beverages consumed
worldwide, is rich in antioxidant polyphenols (∼30% of dry weight) mainly catechins (epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate) and flavonols (kaempferol, quercetin, and myricetin glycosides) that possess various pharmacological effects\textsuperscript{10,11}. During the manufacture of black tea (fully fermented tea), polyphenol oxidase in fresh tea leaves oxidizes catechins (tea tannins) into quinones, which condense to form theaflavins and thearubigins. This fermentation process is inactivated by steam or pan firing treatment of freshly harvested tea leaves to produce unfermented green tea\textsuperscript{10}. Tea has recently received much attention in alternative/complementary medicine against inflammatory diseases owing to its various biological properties, especially anti-inflammatory and antioxidant activities\textsuperscript{12-14}. Therefore, the present study aimed to evaluate and compare the alleviative effects (from the day of arthritis induction and arthritis onset) of two different doses of green tea aqueous extract (GTE, rich in non-oxidized catechins) and black tea aqueous extract (BTE, rich in oxidized catechins: theaflavins and thearubigins) on articular/extra-articular complications (especially with reference to cellular oxidative stress and anemia) in rat adjuvant-induced arthritis (AIA). AIA rat model is a well-developed, non-expensive, rat model that shares several features with human RA including weight loss, oxidative tissue damage and inflammatory infiltration of synovial membrane in association with joints swelling and destruction\textsuperscript{4,7}. Indomethacin (one of NSAIDs) was used as a reference drug in the present study.

### Methodology

#### Chemicals and tea preparations

Complete Freund’s adjuvant (CFA) containing 1.0 mg of dry, heat-killed Mycobacterium tuberculosis (strain H37Ra) per 1.0 mL sterile, non-metabolizable oils (0.85 mL paraffin oil and 0.15 mL of manniee monooate) was purchased from Sigma-Aldrich (St Louis, MO, USA). Ethylenediamine tetra-acetic acid (EDTA), Na\textsubscript{2}HPO\textsubscript{4} and NaH\textsubscript{2}PO\textsubscript{4} powders and phosphate buffered saline (PBS) were also purchased from Sigma-Aldrich. Indomethacin (C\textsubscript{15}H\textsubscript{18}Cl\textsubscript{2}O\textsubscript{4}; molecular weight = 357.79 Da) powder was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). Pure (100%) unflavored green and black teas [Camellia sinensis (L.) Kuntze (Theaceae)] were purchased from R. Twining & Co. Ltd (London, UK). GTE and BTE were prepared as described previously\textsuperscript{10,15}, by dissolving amounts equivalent to 0.5 and 1.0 gm of tea/kg body weight (b.w) in glassware containing 1.0 mL boiling distilled water (equivalent to 2.5 and 5 tea-cups for a 60-kg adult human, respectively), then covered and let stand for 10 min at room temperature. After that the extracts were filtered and given fresh to the animals. GTE contains 30-42% non-oxidized catechins, 5-10% flavonols, 2-4% other flavonoids, 7-9% xanthine alkaloids, 6-8% minerals, 4-6% amino acids, 4-6% organic acids, and 1-2% ascorbic acid of extracted solids; while BTE contains 3-10% non-oxidized catechins, 12-18% thearubigins, 3-6% theaflavines, 6-8% flavonols, 10-12% phenolic acids and depsides, 8-11% xanthine alkaloids, 10% minerals, and 13-15% amino acids of extracted solids\textsuperscript{11,16}.

#### Animals

Adult male Wistar albino rats weighing 120-130 gm were obtained from the National Research Centre, Giza-Egypt. Animals were housed in suitable cages and acclimatized to laboratory conditions for a period of one week before the commencement of the experiments. Rats were fed standard rodent food pellets (Agricultural-Industrial Integration Company, Giza, Egypt) and distilled water. The commercial food pellets were containing wheat-bran, dried clover, maize, bean-hay, methionine, molasses, salt, and mineral/vitamin mixes. The amount of crude proteins, fats and fibers in the food pellets were 12.0, 2.4 and 14.0%, respectively. The energy content of the standard diet was 920.48 kJ/100 gm. All animals were humanely treated in accordance with WHO guideline for animal care and the study design was approved by the Ain Shams University Research Ethics Committee.

#### Experimental design and treatment schedule

Animals were randomly divided into 11 groups of seven animals each: 10 arthritis groups and one healthy control group. Arthritis was induced by a single intra-dermal injection of 0.1 mL of CFA into the palmar surface of the left hind paw after the rats were subjected to light diethyl ether anesthesia\textsuperscript{4,7}. Articular rats received orally (by gavage) 1.0 mL distilled water as vehicle (arthritis control group), indomethacin (1.0 mg/kg body weight), 0.5 gm/kg b.w (low dose) of either GTE or BTE, or 1.0 gm/kg b.w (high dose) of either GTE or BTE for...
28 consecutive days started from the day of arthritis induction (day 0). Other arthritic rats received either low or high dose of tea aqueous extracts orally for 14 consecutive days started from the day of arthritis onset (day 15). Rats in the healthy control group were subjected to light diethyl ether anesthesia, as in arthritis groups, and injected with a single dose of 0.1 mL of physiological saline into the palmar surface of the left hind paw and received 1.0 mL distilled water orally for 28 consecutive days.

**Macroscopic evaluation of arthritis**

Disease severity and progression were evaluated weekly by arthritic scoring as previously described. Briefly, erythema and edema (signs of inflammation) that occurred in the left hind paw were macroscopically and blindly scored as 0–no macroscopic changes, 1–mild, 2–moderate and 3–severe inflammation.

**Blood and tissues sampling**

Animals were killed on day 29. The blood was collected into clean test-tubes with anticoagulant (EDTA) to determine anemia by Hemat 8 analyzer (SEAC, Freiburg, Germany). Immediately after killing the animal, the liver was quickly perfused in situ (via the hepatic portal vein) with ice-cold PBS (to remove erythrocytes and clots) and separated out of the body. Left ankle joint was also amputated and quickly stripped of skin and connective tissue. Liver (after the gall bladder was dissected away) and left ankle joint of each animal were separately homogenized in 5 mL cold buffer (0.5 gm of Na$_2$HPO$_4$ and 0.7 gm of NaH$_2$PO$_4$ per 500 mL deionized water, pH 7.4) per gram tissue. Then, the homogenates were centrifuged in a cooling centrifuge (IEC centra-4R; International Equipment Co., Needham Heights, MA, USA) for 15 min at 4000 rpm and 4°C; and the obtained supernatants were divided into samples and preserved at -80°C until used for evaluating the oxidant and antioxidant parameters.

**Measurements**

The body weight gain (for each animal) and food intake (on a per-group basis) were measured weekly after arthritis induction by using a Sartorius LP2200S balance (Göttingen, Germany). Nitric oxide (NO), malondialdehyde (MDA) and reduced glutathione (GSH) levels as well as catalase (CAT), glutathione peroxidase (GPx, selenium-dependent) and superoxide dismutase (SOD) activities were determined by commercial kits (Bio-Diagnostic, Giza, Egypt).

**Statistics**

Statistical analysis was performed with analysis of variance (ANOVA), and the differences among groups were determined by Bonferroni's multiple comparison test using GraphPad Prism version 4.03 for Windows (GraphPad software Inc., San Diego, CA, USA). P values of <0.05, <0.01 and <0.001 were considered statistically significant, highly significant and very highly significant, respectively.

**Results and discussion**

Modulatory effects of GTE and BTE on body weight loss and arthritic score of AIA rat model

Body weight gain and food intake were significantly decreased (P<0.001) by 70.5% and 23.1%, respectively, in arthritic rats that received vehicle compared with the healthy control rats (Figs. 1a & b), which may be due to appetite loss, the decrease in intestinal absorption capacity (arising from intestinal oxidative damage), and/or metabolic disorders resulting from excessive release of systemic pro-inflammatory mediators and free radicals. In addition, redness (erythema) and swelling (edema) were the inflammatory signs that developed over a 24 hrs period and increased thereafter in the present study in left hind paws injected with CFA. On the other hand, both doses of GTE and the high dose of BTE (from day 0 only) significantly alleviated (P<0.05-0.001) the body weight loss of arthritic rats during the treatment course (Fig. 1a). The improvement of body weight gain of arthritic rats by tea aqueous extracts from day 0 was concomitant with the significant increase shown in food intake (P<0.001, compared with the arthritic rats that received vehicle; Fig.1b). In addition, arthritic score (hind paws inflammation) was significantly decreased (P<0.001) in arthritic rats treated orally from day 0 with tea aqueous extracts compared with those receiving vehicle (Fig. 1c). Both the improvement of body weight gain and the decrease in the arthritic score are considered good and simple indicators for arthritis recovery during the treatment course in AIA rat model. The highest suppressive effect on the body weight loss and arthritic score of arthritic rats, which was equivalent to that of indomethacin (P>0.05), was induced by the high dose of GTE from day 0 (Figs. 1a & c).
Fig. 1—Body weight gain (A), food intake (B) and arthritic score (C) of arthritic rats (CFA-treated rats) given vehicle, indomethacin (indom), green tea aqueous extract (GTE), or black tea aqueous extract (BTE). Values are means, with their standard errors represented by vertical bars. b.w: body weight, CFA: complete Freund's adjuvant. ***P<0.001: compared with the healthy control group; †P<0.05, ††P<0.01, †††P<0.001: compared with the arthritis group that received vehicle; §P<0.05, §§P<0.01, §§§P<0.001: compared with the arthritis group that received indomethacin; ‡‡P<0.01, ‡‡‡P<0.001: compared with the arthritis group that received tea aqueous extracts from day 0 (Repeated Measures ANOVA with Bonferroni's multiple comparison test, n=7).

Fig. 2—Nitric oxide (NO) concentration in synovial joints (A) and malondialdehyde (MDA) concentration in synovial joints and hepatic tissues (B and C, respectively) of arthritic rats (CFA-treated rats) given vehicle, indomethacin (indom), green tea aqueous extract (GTE), or black tea aqueous extract (BTE). Values are means, with their standard errors represented by vertical bars. Liver NO concentration did not significantly change (P>0.05) among all groups. b.w: body weight, CFA: complete Freund's adjuvant. **P<0.01, ***P<0.001: compared with the healthy control group; †P<0.05, ††P<0.01, †††P<0.001: compared with the arthritis group that received vehicle (One-Way ANOVA with Bonferroni's multiple comparison test, n=7).

The alleviative activity of tea aqueous extracts (from day 0) on body weight loss and hind paws inflammation shown in arthritic rats may be, in part, due to inhibiting the release of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β; since they cause appetite loss and augment the release of free radicals and other inflammatory mediators such as NO and prostaglandin (PG)E₂ via enhancing expression of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 enzymes, respectively. In addition, COX-2 converts arachidonic acid into PGE₂, which greatly potentiate exudates by inducing relaxation of arteriolar smooth muscles and increasing the blood supply to synovial tissues causing erythema and edema. Indeed our previous study showed a significant decrease (P<0.05-0.001) in TNF-α and IL-1β in sera of arthritic rats that received tea aqueous extracts compared with those receiving vehicle.

Modulatory effects of GTE and BTE on synovial/hepatic tissues oxidative stress in AIA rat model

The uncontrolled production of ROS (especially superoxide anion and hydroxyl radicals) by phagocytic cells due to the inflammatory surge in RA leads to a decrease in cellular antioxidants as a consequence of their supersaturation and consumption during oxidative stress and loss through cellular lysis, which resulted from membrane lipid peroxidation (LPO) and lysosomal destruction. In the present study, the concentration of NO (a gaseous free radical) in synovial joints and the concentration of MDA (an indicator of LPO) in synovial and hepatic tissues were significantly increased by 333.4% (P<0.001), 50.4% (P<0.001), and 6.9% (P<0.01), respectively, in arthritic rats that received vehicle compared with the healthy control rats (Fig. 2). In contrast, the concentration of GSH (a non-enzymic...
antioxidant) and the activity of enzymic antioxidants (CAT, GPx and SOD) in synovial and hepatic tissues were significantly decreased \( (P<0.05-0.001) \) by 36.18 ± 11.49% in arthritic rats that received vehicle compared with the healthy control rats (Table 1). On the other hand, only the high dose of GTE from day 0 significantly modulated \( (P<0.05-0.001) \) both synovial/hepatic tissues LPO and the release of synovial tissues NO in arthritic rats by improving the cellular antioxidant defense system (SOD, CAT and GPx activities in addition to GSH level) (Fig. 2 and Table 1). In addition, the modulatory effect of the high dose of GTE from day 0 on GSH concentration in synovial joints of arthritic rats significantly exceeded \( (P<0.05) \) that of indomethacin (Table 1). All of the above results proved the antioxidant activity of GTE over BTE in arthritic rats. Other studies reported that tea catechins (particularly EGCG) showed strong free radical scavenger activity that directly scavenged superoxide anion, NO and peroxynitrite and protected human chondrocytes from oxidative damage\(^{13,19}\). After the oxidation of tea catechins due to their reaction with free radicals, a dimerised product was formed that had higher catechins due to their reaction with free radicals, a
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Modulatory effects of GTE and BTE on anemia of AIA rat model

Blood erythrocytes count, hemoglobin (Hb) content, and hematocrit (HCT) value were significantly decreased \( (P<0.001) \) by 23.4-28.9% in arthritic rats that received vehicle compared with the healthy control rats (Table 2). In contrast, mean corpuscular Hb (MCH), mean corpuscular Hb concentration (MCHC), and mean corpuscular volume (MCV) as well as red blood cell distribution width (RDW) values did not significantly change \( (P>0.05) \) in arthritic rats that received vehicle compared with the healthy control rats (Table 2). The hematological changes in arthritic rats that received vehicle indicated normocytic normochromic anemia without anisocytosis (unequal size of red blood cells), which may be due to gastrointestinal bleeding (resulted from oxidative damage of gastrointestinal cells), the decrease in erythrocytes life span (resulted from oxidative damage of erythrocytes), and/or inhibition of bone marrow erythropoiesis (resulted from the deficiency in erythropoietin synthesis by pro-inflammatory cytokines, especially TNF-\( \alpha \), IL-1\( \beta \) and IL-6)\(^{13,23}\). Anemia may lead to other clinical problems in RA patients such as chronic fatigue, dizziness, reduction of exercise tolerance, angina and heart failure\(^3\).

GTE only, especially at the high dose and when the treatment started from day 0, significantly alleviated \( (P<0.01-0.001) \) the normocytic normochromic anemia shown in arthritic rats (Table 2). Moreover, the modulatory effects of the high dose of GTE from day 0 on the Hb content and HCT value of arthritic rats significantly exceeded that of indomethacin \( (P<0.05; \) Table 2). The obvious anti-anemia activity of GTE shown in arthritic rats may be due to its higher antioxidant/anti-inflammatory activity compared with BTE. Indeed our previous study showed that GTE reduced the release of systemic TNF-\( \alpha \) more than BTE in AIA rat model\(^{15}\). The suppressive effects of green tea catechins on TNF-\( \alpha \) gene expression and TNF-\( \alpha \) induced production of IL-1\( \beta \), IL-6 and MMPs in RA were mediated by the inhibition of nuclear factor-\( \kappa \)B/activator protein-1 pathway\(^{24}\). Another study reported that green tea catechins protected erythrocyte from damage by oxidative stress\(^{25,26}\).

Tea is a safer anti-inflammatory/antioxidant agent compared with indomethacin

The alleviative activity of tea aqueous extracts from day 0 on altogether complications shown in AIA rat model exceeded that in arthritic rats receiving tea aqueous extracts from day 15 (39.79 ± 5.31 and 16.61 ± 1.86, respectively), and was in the following order: the high dose of GTE = indomethacin (53.32 ± 15.41 and 48.35 ± 17.09, respectively) > the low dose of GTE = the high dose of BTE (40.04 ± 12.54 and 38.39 ± 12.11) > the low dose of BTE (27.42 ± 8.24). The anti-inflammatory activity of indomethacin was mediated through selective inhibition of COX-1 and COX-2 over lipoxygenases. Inhibition of COX-1 induced adverse effects shown in indomethacin-treated patients such as gastrointestinal
Table 1—Reduced glutathione (GHS) level and the activity of enzymatic antioxidants (CAT, Gpx and SOD) in synovial joints and hepatic tissues of arthritic rats (CFA-treated rats) given vehicle, indomethacin (Indom), green tea aqueous extract (GTE), or black tea aqueous extract (BTE).

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<th>CFA + Indom</th>
<th>CFA + Tea aqueous extracts from day 0</th>
<th>CFA + Tea aqueous extracts from day 15</th>
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<td></td>
<td>LD</td>
<td>HD</td>
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<tr>
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<td>250.3 ± 3.2</td>
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<td>(µmol/g tissue)</td>
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<td></td>
<td>CAT</td>
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<td></td>
<td>GPx</td>
<td>113.10 ± 8.25</td>
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<td></td>
<td>SOD</td>
<td>380.80 ± 0.85</td>
<td>365.20 ± 2.14</td>
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<td>Hepatic tissue</td>
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<td></td>
<td>CAT</td>
<td>65.64 ± 5.46</td>
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<td></td>
<td>SOD</td>
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Values are means, with their standard errors. CAT: catalase; CFA: complete Freund's adjuvant. GPx: glutathione peroxidase, HD: high dose (1.0 g of tea/kg body weight), LD: low dose (0.5 g of tea/kg body weight), SOD: superoxide dismutase. *P<0.05, **P<0.01, ***P<0.001: compared with the healthy control group; †P<0.05, ††P<0.01, †††P<0.001: compared with the arthritis group that received vehicle; $P<0.05, $$$P<0.001: compared with the arthritis group that received indomethacin; ††††P<0.001: compared with the arthritis group that received tea aqueous extracts from day 0 (One-Way ANOVA with Bonferroni's multiple comparison test, n=7).
Table 2—Hematological parameters of arthritic rats (CFA-treated rats) given vehicle, indomethacin (indom), green tea aqueous extract (GTE), or black tea aqueous extract (BTE)

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<td>6.09</td>
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<td>Hb (g/dL)</td>
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<td>± 0.30</td>
<td>10.93</td>
<td>± 0.26</td>
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<td>HCT (%)</td>
<td>41.90</td>
<td>± 1.05</td>
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<td>MCV (fL/RBC)</td>
<td>52.82</td>
<td>± 0.68</td>
<td>55.43</td>
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<td>MCH (pg/RBC)</td>
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<td>MCHC (g/dL)</td>
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<td>± 0.45</td>
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<td>RDW (%)</td>
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Values are means, with their standard errors. CFA: complete Freund's adjuvant, Hb: hemoglobin, HCT: hematocrit, HD: high dose (1.0 g of tea/kg body weight), LD: low dose (0.5 g of tea/kg body weight), MCH: mean corpuscular Hb, MCHC: mean corpuscular Hb concentration, MCV: mean corpuscular volume, RBCs: red blood corpuscles, RDW: red blood cell distribution width. **P<0.001: compared with the healthy control group; †P<0.05, ††P<0.01, †††P<0.001: compared with the arthritis group that received vehicle; §§P<0.05, §§§P<0.01: compared with the arthritis group that received indomethacin; ‡‡P<0.01: compared with the arthritis group that received tea aqueous extracts from day 0 (One-Way ANOVA with Bonferroni's multiple comparison test, n=7)
and renal injuries. In contrast, tea catechins (particularly EGCG) selectively and effectively inhibited COX-2 overexpression without affecting COX-1. The anti-inflammatory activity of tea was similar to that of BW755C, a dual inhibitor of COX-2 and 5-lipoxygenase enzymes. The daily dose of tea aqueous extracts (0.5 and 1.0 gm/kg) used in the present study corresponds 1/4 and 1/2, respectively, of the no observed-adverse-effect level dose of tea catechins used earlier for systemic toxicity study in rats following oral administration for 28 consecutive days. Indeed consumption of tea aqueous extracts did not induce any adverse effects in healthy rats. On the other hand, tea aqueous extracts (especially GTE) significantly increased and decreased (P<0.05) the concentration of hepatic GSH and LPO, respectively, in healthy rats (data not shown). All of these findings indicated that tea is considered as a safer anti-inflammatory/antioxidant agent. In conclusion, GTE was effective as indomethacin in alleviating the articular/extra-articular oxidative damage and anemia in AIA rat model, especially at the high dose (1.0 gm/kg body weight) and when the treatment started from the day of arthritis induction. The higher alleviative effects of GTE (12.62-14.93% more) versus BTE on arthritis severity/complications shown in AIA rat model may be due to the fact that GTE contains more non-oxidized catechins (particularly EGCG) that possess stronger free radical scavenging activity and act synergistically to alleviate tissues inflammation and oxidative damage compared with oxidized catechins found in BTE (e.g. theaflavins and thearubigins). These findings provide a scientific rationale for using green tea in the management of RA manifestations.

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