

Cat's whiskers flavonoid attenuated oxidative DNA damage and acute inflammation: its importance in lymphocytes of patients with rheumatoid arthritis

Asis Bala · Purbajit Chetia · Narayan Dolai ·
Bidita Khandelwal · Pallab Kanti Haldar

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Abstract *Cleome gynandra* L. (Capparidaceae) is one of the vegetables commonly known as 'Hurhur' and 'Karaila' in India, 'Pe Hua Tsai' in China and "Cat's whiskers" in English. Present study was aimed to characterize previously isolated Cat's whiskers flavonoid as 5-hydroxy-3, 7, 4'-trimethoxyflavone (5HTMF) and to evaluate its effect on carrageenan-induced acute inflammation in rats and hydrogen peroxide induced DNA damage in mouse macrophages. The ex vivo effect of 5HTMF upon generation of free radicals in the mononuclear lymphocytes of patients with rheumatoid arthritis (RA) was also evaluated. 5HTMF not only reduce the swelling of hind paw in rats from 1 to 3 h of carrageenan injection but also decreased serum nitric oxide (NO) production. Toxic hydrogen peroxide induced oxidative DNA damage that was significantly decreased by 5HTMF. Though oxidative stress is a potential biomarker for determining disease activity in patients with RA, surprisingly 5HTMF inhibited the superoxide, hydroxyl and NO radicals in the isolated peripheral blood mononuclear lymphocytes of patients with RA. From the above study, it may be concluded 5HTMF attenuated acute inflammation

by inhibiting NO and by protecting the oxidative DNA damage due to hydrogen peroxide scavenging property. It was also equally effective in scavenging the free radicals in lymphocytes of patients with RA. Collectively, our results indicate that 5HTMF as well as leafy vegetable of Cat's whiskers may be a promising nontoxic food alternative in attenuating the oxidative stress, meriting further studies on other human inflammatory cells.

Keywords *Cleome gynandra* · Capparidaceae · 5-hydroxy-3, 7, 4'-trimethoxyflavone · Inflammation · Superoxide · Hydroxyl · Nitric oxide · Rheumatoid arthritis

Introduction

Cleome gynandra L./*Gynandropsis gynandra* (L.) Briq. (Capparidaceae) is one of the vegetables, which grows as a weed in most topical countries. It is commonly known as 'Hurhur' and 'Karaila' in India, 'Pe Hua Tsai' in China and 'Cat's whiskers' in English (Borgio et al. 2008; Hever and Veenter 2007). The methanol extract of *C. gynandra* possesses free radical scavenging and antioxidant properties (Bala et al. 2009). Stems are used as analgesic and anti-inflammatory agents (Gupta and Chakravarty 1957). The potential anticancer activity of *C. gynandra* is already reported in Ehrlich's ascites carcinoma cell bearing mice, where a dose-dependent antitumor effect of methanol extract was reported (Bala et al. 2010). Free radical-induced oxidative stress in haematopoietic cells is the main factor in cell damage and dysfunction. Therefore, elevation of body's antioxidant defense is one of the weapons to fight against cancer (Dolai et al. 2012). Recently, it has been published that the antioxidant activity of *C. gynandra* flavonoid fractions

A. Bala (✉) · N. Dolai · P. K. Haldar
Department of Pharmaceutical Technology, Jadavpur University,
188B Raja SC Mullick Road, Kolkata 700032,
West Bengal, India
e-mail: asisbala_ju@yahoo.co.in

P. K. Haldar
e-mail: pallab_haldar@rediffmail.com

P. Chetia
Department of Pharmacology, Himalayan Pharmacy Institute,
Majhitar, Rangpo, East Sikkim 737136, Sikkim, India

B. Khandelwal
Department of Medicine, Sikkim Manipal Institute of Medical
Science, 5th Mile Tadong, Gangtok 737102, Sikkim, India

is responsible for its anticancer activity (Bala et al. 2011). A number of flavonoid fractions like 5-hydroxy-3, 7, 4'-trimethoxyflavone (5HTMF), kaempferol-3, 4', 7-trimethyl ether and 5,7-dihydroxychromone derivative have been isolated and reported (Jain and Gupta 1985; Bala et al. 2012a, b). The active Cat's whiskers flavonoid (CWF) was isolated and the antioxidant activity on some reactive oxygen species (ROS) and reactive nitrogen species (RNS) generating inflammatory cells were established where a concentration-dependent free radicals scavenging activity was observed in mouse erythrocytes, macrophages and lymphocytes cells (Bala et al. 2012a, b).

The present study was aimed at characterizing the isolated CWF as 5HTMF by high-performance liquid chromatography (HPLC) and its effect on acute inflammation and toxic hydrogen peroxide (H_2O_2)-induced oxidative DNA damage in mouse peritoneal macrophages. Furthermore, ex vivo effect of 5HTMF upon generation of free radicals in the isolated peripheral blood mononuclear lymphocytes of patient with rheumatoid arthritis (RA) in terms of inhibiting the free radicals like superoxide (O_2^-), hydroxyl (OH^-) and nitric oxide (NO) radicals was evaluated.

Materials and methods

Chemicals and reference drugs

All the chemicals were purchased from Himedia (Mumbai, India) and except hydrogen peroxide (H_2O_2) from Merck ($\geq 30\%$ purity by HPLC, Mumbai India), Aspirin ($\geq 99\%$ pure by HPLC), *N*-acetyl cysteine (NAC, $\geq 99\%$ pure powder by HPLC) from Sigma Aldrich (St. Louis, MO, USA), 5-hydroxy-3,7, 4'-trimethoxyflavone (5HTMF; $\geq 97\%$ pure by HPLC) from ChromoDex (Irvine, CA, USA). *N*-1 naphthyl ethylenediamine dihydrochloride from Loba Chemie Pvt. Ltd. Mumbai, India ($\geq 90\%$ pure by HPLC), deoxyribose (DOR, $\geq 99\%$ pure by HPLC), thiobarbituric acid, sulphanilamide and trichloroacetic acid (TCA) from Sisco Research Laboratories (Mumbai, India).

Plant materials

The plant materials were collected from Eastern Himalayan region of Jalpaiguri, West Bengal, India in January 2009, identified by Botanical Survey of India, Botanic Garden, Howrah-711103 India. A voucher specimen (No. CNH/I-I/(293)/2009/Tech.II/335) has been preserved at Phytotherapy and Pharmacology Research Lab., Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India.

Extraction, isolation and HPLC characterization of 5HTMF

CWF was isolated from the defatted methanol extract of *C. gynandra* and characterized by high-performance liquid chromatography (HPLC: Shimadzu, Japan; Pump: LC-20AT; Detector: SPD 20A, Analysed by Luna C18 Colum; 250 mm \times 4.6 mm, particle size 5 μ m, Phenomenax) using previously described methods (Bala et al. 2012a, b). Targeted lead flavonoid 5HTMF was also analyzed with same HPLC systems using acetonitrile : water (1:1, flow rate 1 μ L/min, sample concentration: 5 mg/mL, run time 7 min, injection volume 50 μ L) as solvent system and peak detected at 268 nm to identify the active flavonoid peak present in defatted methanol extract. 5HTMF was isolated from the defatted methanol extract by column chromatography (Silica gel 60–120 mesh, pH 6.5–7.5, Merck, Mumbai, India) using both isocratic and subsequently with gradient elution $CHCl_3$ (50 \times 10 mL) and 1, 5, 10, 25 and 50 % $CHCl_3$ in MeOH (250 mL each). 5HTMF was finally collected from elute of 50 % $CHCl_3$ in MeOH and used for further experiments. Nontoxic concentrations were used based on previous studies where concentrations of 25 and 50 mg/kg effectively showed in vivo antioxidant activity (Bala et al. 2012b).

Effects of 5HTMF on carrageenan-induced acute inflammation (Guo et al. 2011)

Rats (Male Wister weighing 200 ± 20 g) were randomly allocated into four groups ($n = 6$), each with six rats and treated as follows: first group served as control, second and third group received 5HTMF (25 and 50 mg/kg b.w., p.o. by oral gavage) daily for three consecutive days, respectively. Fourth group aspirin (250 mg/kg b.w., p.o. by oral gavage) served as controls. On day 3, all the groups received 0.1 mL of carrageenan (1 %) subcutaneously in the right hind paw of rats and after 1–2–3 h, the paw volume were measured by plethysmometer. All the animals in each group were sacrificed by ether anaesthesia and intracardiac blood sample was taken for measurement of NO present in serum using previously described and modified methods (Ansari et al. 2007; Mukhopadhyay et al. 2011). Experimental method described was reviewed and approved by the Institutional Animal Ethics Committee (2011/60/IAEC/110; Dated 25.10.2011).

Effect of 5HTMF on H_2O_2 -induced DNA damage in mouse macrophages

Mouse (Male Swiss albino mice weighing 20 ± 2 g) peritoneal macrophages were lavaged aseptically using ice cold phosphate buffer saline (PBS: 0.02 M pH-7.4) (Bala

et al. 2012c). After centrifugation (3,000 rpm \times 10 min) at 4 °C the pellet were resuspended in PBS and cell viability was confirmed by trypan blue exclusion method (Bala et al. 2012c). Isolated mouse peritoneal macrophages (2×10^6 /mL) were preincubated with the different concentrations of 5HTMF (5–20 μ g/mL) for 1 h and further incubated with H₂O₂ (10 mM) for 2 h. DNA was isolated by trichloroacetic acid (TCA) precipitation methods and oxidative DNA damage was estimated spectrophotometrically using diphenylamine reaction and deoxyribose as standard (Burton 1955; Das et al. 2010). Experimental method described was reviewed and approved by the Institutional Animal Ethics Committee (2011/60/IAEC/111; Dated 25.10.2011).

In vitro H₂O₂ scavenging assay

H₂O₂ scavenging activity was measured by conventional spectrophotometric method (Chaulya et al. 2010). Briefly, 200 mM concentration of H₂O₂ was incubated with different concentrations of 5HTMF (0–100 μ g/mL) and finally the remaining H₂O₂ concentration was measured at 230 nm. Percentage of inhibition was calculated by the formula $[(C - T)/C \times 100]$ where C = absorbance of H₂O₂ and T = absorbance of the “test” tubes. Finally, 50 % inhibitory concentration was calculated from the interpolation graph of concentration vs. % inhibition by using Grap pad Prism software version 3 (GraphPad Software Inc, San, Diego, CA).

Effect of 5HTMF on isolated lymphocytes of patients with rheumatoid arthritis (RA)

Study population: The study population included normal healthy human volunteers ($n = 10$) and patients with RA ($n = 10$, Table 1) who fulfilled the American College of Rheumatology criteria for RA (Arnett et al. 1988). The RA patients were recruited from the outpatient unit of Central

Table 1 Study populations

| Parameters | Rheumatoid arthritis (RA) | Normal human volunteer (N) |
|------------------------|---------------------------|----------------------------|
| Number of participants | 10 | 10 |
| Age | Between 19–50 years | Between 21–48 years |
| Sex: female : male | 3:2 | 1:4 |
| Duration of illness | 3 month—20 years | – |
| Blood collected | 5–10 mL | 5–10 mL |
| DMARDS received | Yes | NO |
| Steroid received | No | No |
| Antioxidant received | No | No |
| ACR criteria fulfilled | Yes | No |

Referral Hospital, a constituted hospital by Sikkim Manipal Institute of Medical Science Tadong, Gangtok, Sikkim, India. Patients with psoriatic arthritis, scleroderma, reactive arthritis, viral polyarthritis, systemic lupus erythematosus, renal and/or hepatic impairment and also patients with RA receiving steroids were excluded from this study. The study protocol received prior approval from the Institutional Ethics Committee (SMIMS/IEC/2011-M.Ph-1. Dated: 23/11/2011) and informed consent was obtained from all participants both patient and normal human. The Institutional Ethics Committee permission letter and all the respective consent forms are preserved at the Department of Pharmacology, Himalayan Pharmacy Institute, Gangtok, Sikkim, India.

Isolation of lymphocytes and evaluation of 5HTMF

Blood was collected directly from the patient with RA and transferred into a heparin-containing vial. Lymphocytes (mononuclear cells) were isolated according to the manufacturer's instruction (Hisep™ LSM 1073, Himedia, Mumbai, India) and cell viability was confirmed by trypan blue exclusion method (Bala et al. 2012c). Ex vivo assay were performed to evaluate the antioxidant activity of the 5HTMF on the lymphocyte cells of the patients with RA. In brief, isolated lymphocyte cells (2×10^6 /mL) were incubated in Krebs–Henseleit buffer in presence of different concentrations (1–20 μ g/mL) of 5HTMF or *N*-acetyl cysteine (NAC, 25 μ g/mL) for 2 h and according to the requirement the cells were lysed in 2 mL of 0.25 % sodium dodecyl sulfate in phosphate buffer (0.02 M, pH 7.4) with a subsequent three times freeze thawing process (–20 °C). Protein concentration was measured by the standard method (Lowry et al. 1951) and the resultant preincubated cells and cell lysate were used for the evaluation (Kundu et al. 2011). Most toxic free radicals like O₂[–] and NO radicals were determined by previously described nitroblue tetrazolium and Griess assay methods, respectively (Bala et al. 2012c; Mukhopadhyay et al. 2011). OH[–] radicals scavenging activity was measured by the deoxyribose method described by Kundu et al. (2011).

Statistical analysis

Results are expressed as mean \pm SEM. Statistical significance (p) was calculated using Grap Pad Prism version 4.03 and GrapPad InStat software (GraphPad Software Inc, San Diego, CA, USA) and one-way ANOVA followed by Dunnett's post hoc test of significance where $*p < 0.05$ and $**p < 0.01$ are considered to be significant and highly significant, respectively, when test group is compared with respective control group.

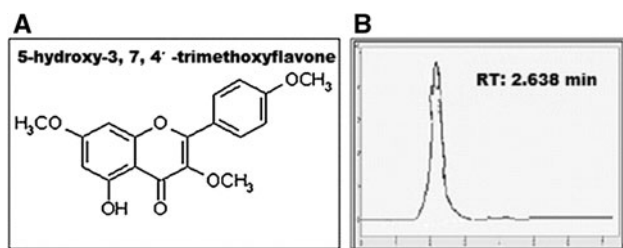


Fig. 1 Isolated 5HTMF were analyzed by HPLC (Shimadzu, Japan; Pump: LC-20AT; Detector: SPD 20A) using Luna C18 Column (250 mm × 4.6 mm, particle size 5 μm, Phenomenax) and acetonitrile/water (1:1, flow rate 1 μL/min, sample concentration: 5 mg/mL, run time 7 min, injection volume 50 μL) as solvent system. Peak detected at 268 nm. 5HTMF (a) shows a characteristic single peak at a retention time of 2.638 min in the same HPLC system (b)

Table 2 Effect of 5HTMF on carrageenan-induced acute paw inflammation in rats

| Groups | % Increase of paw volume | | | NO in serum (μM) N = 8.21 ± 1.21 |
|-------------|--------------------------|--------------|--------------|-------------------------------------|
| | 1 h | 2 h | 3 h | |
| Control | 66.99 ± 2.08 | 80.07 ± 3.21 | 89.61 ± 3.03 | 47.72 ± 2.32 |
| 5HTMF 25 | 31.30 ± 1.99 | 24.4 ± 2.32 | 17.92 ± 1.46 | 25.23* ± 2.22 |
| 5HTMF 50 | 23.13 ± 1.58 | 10.20 ± 1.15 | 8.96 ± 1.75 | 14.45** ± 1.34 |
| Aspirin 250 | 15.21 ± 2.50 | 8.31 ± 3.01 | 6.61 ± 1.23 | 10.43** ± 1.99 |

After 3 days pretreatment of 5HTMF at 25 mg/kg (5HTMF 25), 50 mg/kg (5HTMF 50) and Aspirin 250 mg/kg (ASPIRIN 250) carrageenan (0.1 mL each, 1 %) was injected in the hind paw of all groups of rats except normal (N). Paw volume was measured with a plethysmometer 1, 2 and 3 h after carrageenan injection and finally the % increase of paw volume was calculated. After the last measurement of paw thickness, rats of all groups were sacrificed and collected blood serum NO content was measured by the procedures described in “Materials and methods” and finally compared with 5HTMF untreated (CONTROL) group. Values are represented as mean ± SEM ($n = 6$) where ** $p < 0.01$ and * $p < 0.05$ were considered as highly significant and significant, respectively, with respect to the control group

Results

Previously CWF was isolated and analyzed by HPLC using acetonitrile/water (1:1, flow rate 1 μL/min) as solvent system and peak detected at 268 nm to confirm the purity. CWF showed a characteristic single peak at a retention time of 2.638 min with AUC of 103.011 mV [10]. The lead flavonoid 5HTMF was also analyzed with same HPLC systems to identify the active flavonoid peak present in defatted methanol extract of *C. gynandra*. 5HTMF (Fig. 1a) also shows a characteristic single peak at a retention time of 2.638 min in the same HPLC system (Fig. 1b).

In present study isolated flavonoid, 5HTMF and Aspirin significantly decreased carrageenan-induced rat paw

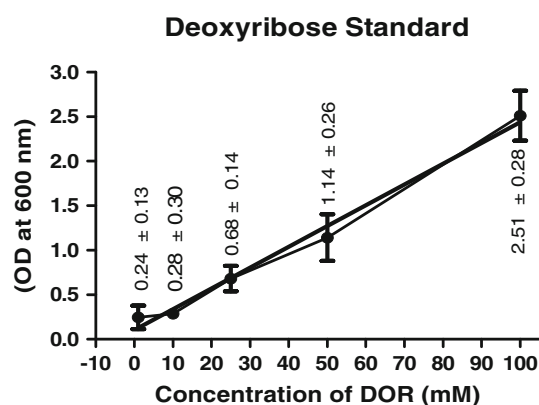


Fig. 2 A representative profile of DNA standard curve prepared by the reaction of deoxyribose (DOR) (0–100 mM) and diphenylamine in same reaction conditions. Exact concentrations of DNA present in the 5HTMF-treated or untreated cells were calculated using this standard curve using Grap Pad Prism software version 4.03. *Straight line* indicated the best fitting line of standard curve prepared by linear regression analysis. And curve-shaped one reflected the actual experimental plot

oedema in an acute inflammation model. 5HTMF at a dose of 25 and 50 mg/kg significantly reduced the swelling of hind paw in inflammatory rats from 1 to 3 h of carrageenan injection (Table 2). To explore the mechanism of action of 5HTMF, it was found that 5HTMF decreased (* $p < 0.05$ and ** $p < 0.01$) the serum NO production 3 h after carrageenan injection.

To assess the effect of CWF on H₂O₂-induced oxidative DNA damage in the mouse macrophages cells, we measured its initial toxicity on the cells at different concentrations and subsequently isolated DNA was estimated by deoxyribose standard prepared by the reaction of deoxyribose (0–100 mM) and diphenylamine in same reaction condition (Fig. 2). We have not found any toxicity up to 100 μg/mL concentration of 5HTMF (data not shown), but it was found that H₂O₂ obviously induced DNA fragmentation in 10 mM concentration which was significantly quenched by 5HTMF in concentration-dependent manner (Fig. 3) that correlates the in vitro H₂O₂ scavenging activity where 50 % inhibitory concentration was found to be 50.20 ± 2.25 μg/mL. Exact concentrations of DNA present in the CWF treated or untreated cells were calculated by using deoxyribose standard curve (Fig. 2) using Grap Pad Prism software version 4.03. During the incubations of H₂O₂ with the mouse macrophages cells, the oxidative damage of DNA was significantly quenched by 5HTMF dose dependently via its H₂O₂ scavenging activity (Fig. 3).

During ex vivo study, *N*-acetyl cysteine (25 μg/mL), a known free radical scavenger published earlier (Bala et al. 2012c; Chetia et al. 2012), was found to be significantly effective to decrease oxidative stress in lymphocyte cells of

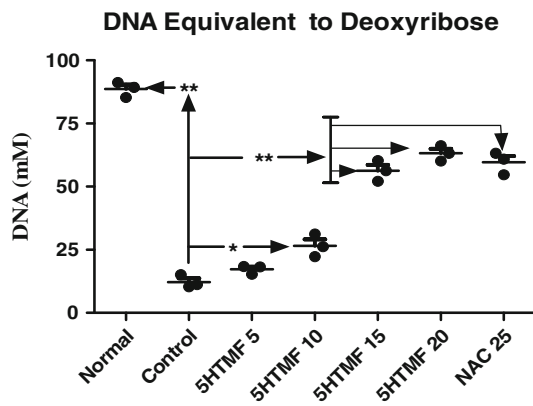


Fig. 3 Effect of 5HTMF on H_2O_2 -induced oxidative DNA damage in isolated mouse peritoneal macrophages cells. Isolated mouse peritoneal macrophages (2×10^6) (Normal) were preincubated with the 5HTMF 5 $\mu\text{g}/\text{mL}$ (5HTMF 5), 10 $\mu\text{g}/\text{mL}$ (5HTMF 10), 15 $\mu\text{g}/\text{mL}$ (5HTMF 15), 20 $\mu\text{g}/\text{mL}$ (5HTMF 20) and *N*-acetyl cysteine 25 $\mu\text{g}/\text{mL}$ (NAC 25) for 1 h and further incubated with H_2O_2 (10 mM) for 2 h and processed for DNA analysis described in the “Materials and methods” and compared with only H_2O_2 -treated cells (Control). Values are represented as mean \pm SEM of three individual experiments ($n = 3$) where $**p < 0.01$ and $*p < 0.05$ were considered as highly significant and significant, respectively

Table 3 Effect of 5HTMF on isolated peripheral blood mononuclear lymphocyte cells in terms of base line generation of superoxide (O_2^-), nitric oxide (NO) and hydroxyl (OH^-) radicals

| Groups | O_2^- (% of Control) | NO (μM of Nitrite) | OH^- (% Scavenging) |
|----------|---------------------------|-----------------------------------|--------------------------|
| Normal | 41.95 \pm 3.05 | 33.41 \pm 2.04 | – |
| RA | 100.00 \pm 0.00** | 99.04 \pm 4.61** | 00.00 \pm 0.00 |
| 5HTMF 5 | 92.19 \pm 0.83 | 86.11 \pm 3.15 | 10.24 \pm 2.21 |
| 5HTMF 10 | 73.94 \pm 3.10* | 78.30 \pm 4.71* | 20.75 \pm 4.21 |
| 5HTMF 15 | 63.90 \pm 2.65* | 74.07 \pm 3.00* | 23.42 \pm 2.61 |
| 5HTMF 20 | 56.42 \pm 1.87* | 66.23 \pm 2.87* | 60.67 \pm 5.55 |
| NAC 25 | 50.34 \pm 2.21* | 58.34 \pm 1.99* | 63.34 \pm 2.23 |

Both the isolated lymphocyte cells (2×10^6) from normal human (Normal) and patient with rheumatoid arthritis (RA) were preincubated with the 5HTMF 5 $\mu\text{g}/\text{mL}$ (5HTMF 5), 10 $\mu\text{g}/\text{mL}$ (5HTMF 10), 15 $\mu\text{g}/\text{mL}$ (5HTMF 15), 20 $\mu\text{g}/\text{mL}$ (5HTMF 20) and positive control NAC 25 $\mu\text{g}/\text{mL}$ (NAC 25) for 2 h and further processed for antioxidant assay described in the “Materials and methods”. Values are represented as mean \pm SEM ($n = 10$) where $**p < 0.01$ when untreated RA compared with normal but $*p < 0.05$ when 5HTMF-treated RA compared with untreated RA

patient with RA ($*p < 0.05$) in terms of scavenging reactive oxygen and nitrogen intermediates (Table 3). Baseline generation of O_2^- radicals in the lymphocytes of RA patient was higher ($**p < 0.01$) than of normal but when cells were incubated with 5HTMF (10–20 $\mu\text{g}/\text{mL}$), the superoxide generation decreased approximately 30–50 % ($*p < 0.05$) (Table 3). Although H_2O_2 is proposed to be a major ROS in lymphocytes, we wanted to check the effect

of 5HTMF on OH^- radical, measured by deoxyribose assay. Our results showed that 5HTMF (5–20 $\mu\text{g}/\text{mL}$) dose dependently decreased the baseline levels of OH^- radicals in the lymphocytes of RA patients. And interestingly, the effect of 5HTMF at concentration of 20 $\mu\text{g}/\text{mL}$ is equivalent to known scavenger *N*-acetyl cysteine at a concentration of 25 $\mu\text{g}/\text{mL}$. Elevated levels (threefold, $**p < 0.01$) of NO equivalent to nitrite were also observed in lymphocytes cells lysate of RA than normal human volunteers which was subsequently decreased up to 30 % by 5HTMF incubation for a period of 2 h (Table 3). But interestingly when the lymphocytes of normal volunteers were incubated with 5HTMF at same concentration and condition, no significant change of NO concentration was observed (data not shown) that confirmed again the etiology of high oxidative stress in patient with RA.

Discussion

Inflammation is a complex stereotypical response of the body to cell damage and vascularized tissues. Cyclooxygenase (COX) is an enzyme responsible for formation of important biological mediators called prostanoids (including prostaglandins, prostacyclin and thromboxane) (Sarkar et al. 2008; Magari et al. 2004). Synthesis of prostaglandins E_2 (PgE_2), an oxygenated product of arachidonic acid, is dependent on ROS- and NO-mediated activation of COX, thus several anti-inflammatory compounds also display antioxidant properties and vice versa (Mollace et al. 2005). In our previous study, isolated active CWF showed antioxidant activity on some ROS- and RNS-generating inflammatory cells where concentration-dependent free radical scavenging activity was established in mouse erythrocytes, macrophages and lymphocytes cells. Therefore, the anti-inflammatory activity of 5HTMF may be extrapolated.

The production of NO and oxidative stress are closely related with inflammation. Carrageenan is a pro-inflammatory agent which increases the production of NO in early and late phase of inflammation (Jocelyne et al. 2004; Daniela et al. 1996). 5HTMF decreases the carrageenan-induced paw edema in rats and subsequently suppressed the production of NO in the serum of carrageenan-treated rats. Attenuation of NO correlates the previously described free radical scavenging activity of 5HTMF in chronic inflammatory cells (Bala et al. 2012c).

The most abundant ROS and RNS represent in the living inflammatory cells are O_2^- , OH^- , H_2O_2 , NO and toxic peroxynitrite radicals (Kundu et al. 2011). Oxidative stress refers to a situation where in the production of oxidants exceeds the capacity to neutralize them, leading to damage to cell membranes, lipids, nucleic acids, proteins and

constituents of the extracellular matrix such as proteoglycans and collagens (Hitchon and El-Gabalawy 2004). ROS causes various types of damage to DNA, including breaking of single and double strands, releasing of free nucleobases, chemical changes of nucleobases, and modification of sugar moieties. DNA damage may lead to abnormal functioning of the cell (Sarkar et al. 2013). H_2O_2 is one of the important ROS that destroyed the biomolecules via its oxidative property (Duthie et al. 1996). During incubation of cells with H_2O_2 , the oligonucleosome-sized fragments resulting from the oxidative cleavage of nuclear DNA are formed and this DNA fragmentation was estimated by diphenylamine reagent using deoxyribose as standard. To assess the effect of 5HTMF on oxidative DNA damage by H_2O_2 in the mouse macrophage cells, we measured its initial toxicity on the cells at different concentrations and subsequently isolated DNA was estimated by deoxyribose standard (Fig. 3). We have not found any toxicity in macrophage cells up to 100 $\mu\text{g/mL}$ concentration of 5HTMF (data not shown), but H_2O_2 (10 mM) significantly (** $p < 0.01$) caused DNA damage in mouse macrophages which was interestingly prevented by 5HTMF (10–15–20 $\mu\text{g/mL}$) accordingly. It has already been established that 5HTMF has a superior ability to scavenge free radicals (Bala et al. 2012b). This was again confirmed additionally by H_2O_2 scavenging assay that highlights the mechanism of action of attenuating oxidative DNA damage.

Currently, oxidative stress is a potential biomarker for determining disease activity in patients with RA (Kundu et al. 2012). In addition, oxidative stress plays a pivotal role in the pathogenesis of RA (Hitchon and El-Gabalawy 2004) and higher amounts of ROS and RNS have been reported in the synovial joints as well as peripheral blood inflammatory cells of the RA patients (Kundu et al. 2011). During *ex vivo* study, those who were included as patients with RA had not received any antioxidants before the experiments. The peripheral blood primarily comprises neutrophils, monocytes and lymphocytes, only the mononuclear lymphocyte cells were analyzed in the present investigation. Himedia (Hisep™ LSM 1073, Mumbai, India)-based differential migration accorded us the privilege of separating the mononuclear cell populations based on centrifugal migration to cells towards density gradient and the cell population contains more than 92 % lymphocytes (Bala et al. 2012c). Cell viability was confirmed by previously described trypan blue exclusion methods (Bala et al. 2012c). More than 95 % cell viability was accepted for the present study. The normal levels of oxidative stress in lymphocytes (Table 3) were higher in case of patient with RA than the levels generated in normal human lymphocyte cells. 5HTMF incubation surprisingly decreased both the oxidative load on base line O_2^- , OH^- and NO

radicals level in a short period of incubation but its ability to decrease phorbol myristate acetate (PMA) induced oxidative stress and to modulate the enzymatic and/or nonenzymatic antioxidant pathways needs to be established. Although the collection of large volume blood from both the normal and RA volunteers would pose an ethical problem limitation.

HPLC allows for the efficient analysis of a large number of compounds that give a characteristic single peak with specific retention time as it is an invaluable quality assessment tool for the evaluation of phytochemicals (Kalaivani et al. 2011). In our study, isolated CWF showed a similar peak with 5HTMF that ultimately concluded that 5HTMF is one of the key molecules for its previously reported antioxidant activity.

From the above study, it may be concluded that CWF as well as 5HTMF attenuated acute inflammation by inhibiting NO and protected the oxidative DNA damage due to H_2O_2 scavenging property. However, 5HTMF was equally effective in scavenging the free radicals in lymphocytes of patients with RA. Collectively, our results indicated that 5HTMF as well as leafy vegetable of Cat's whiskers may be a promising nontoxic food alternative in attenuating the oxidative stress, meriting further studies on other human inflammatory cells.

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Conflict of interest The authors declare no conflict of interest.

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