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Anti-oxidant and anti-inflammatory effects of Cinnamaldehyde and Eugenol on mononuclear cells of rheumatoid arthritis patients

Somaiya Mateen\textsuperscript{a}, Md Tabish Rehman\textsuperscript{b}, Sumayya Shahzad\textsuperscript{a}, Syed Shariq Naeem\textsuperscript{c}, Abul Faiz Faizy\textsuperscript{d}, Abdul Qayyum Khan\textsuperscript{d}, Mohd. Shahnawaz Khan\textsuperscript{e}, Fohad Mabood Husain\textsuperscript{f}, Shagufta Moin\textsuperscript{e}

\textsuperscript{a} Department of Biochemistry, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

\textsuperscript{b} Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia 11451

\textsuperscript{c} Department of Pharmacology, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

\textsuperscript{d} Department of Orthopaedic Surgery, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

\textsuperscript{e} Protein Research Chair, Department of Biochemistry, College of Science, King Saud University, Riyadh-11451, Saudi Arabia

\textsuperscript{f} Department of Food Science and Nutrition, College of Food and Agriculture, King Saud University, Riyadh-11451, Saudi Arabia

smateen.amu@gmail.com
mrehman@ksu.edu.sa
sumayyashahzad3@gmail.com
syedshariq1@gmail.com
abul.faizy@gmail.com
drabdulqayyum@rediffmail.com
moskhan@ksu.edu.sa
fhussain@ksu.edu.sa
Abstract

Rheumatoid arthritis (RA) is an autoimmune disorder affecting joints and frequently characterized by initial local and later systemic inflammation. The present study was conducted with the aim to determine the anti-inflammatory and antioxidant effects of cinnamaldehyde and eugenol in the peripheral blood mononuclear cells (PBMC) of RA patients. PBMCs obtained from RA patients were treated with varying concentrations of cinnamaldehyde and eugenol. The levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were monitored in the 24-hours culture supernatant of PBMCs. Reactive oxygen species formation, biomolecular oxidation and the activities of antioxidant enzymes were also determined. FTIR analysis was done to determine structural alterations in the PBMCs. Molecular docking was performed to gain an insight into the binding mechanism of eugenol and cinnamaldehyde with pro-inflammatory cytokines. The levels of pro-inflammatory cytokines and markers of oxidative stress were found to be elevated in the PBMC culture of RA patients as compared to the healthy controls. Cinnamaldehyde and eugenol have significantly reduced the levels of cytokines. Reactive oxygen species formation, biomolecular oxidation and antioxidant defense response were also ameliorated by treating PBMCs with both the compounds. FTIR results further confirms cinnamaldehyde and eugenol mediated protection to biomolecules of PBMCs of RA patients. Molecular docking results indicates interaction of cinnamaldehyde and eugenol with key residues of TNF-α and IL-6. Cinnamaldehyde and eugenol were found to exert potent anti-inflammatory and antioxidant effects on the PBMC culture of RA patients. So, these compounds may be used as an adjunct in the management of RA.
1. Introduction

Rheumatoid arthritis (RA), a severe disabling arthritis is characterized by synovial infiltration and hyperplasia, thus leading to progressive destruction of articular tissues (Vivar and Van Vollenhoven, 2014). It affects around 1-2% of the world population, with women being 3 times more susceptible than man, probably due to the effect of female hormones on the activity of immune cells (Ngo et al., 2014). Although the etiology of RA is still not completely understood but some reports confirm the role of macrophages, T cells and B cells in the destruction of bone and cartilage (Hu et al., 2013). This autoimmune disorder is characterized by the presence of rheumatoid factor (antibody against IgG/IgM) and anti-citullinated peptide antibodies (ACPA) (Rantapää-Dahlqvist et al., 2003). Activation of CD4+T cells by the arthritogenic agent (microbial or self-antigen) drives the autoimmune attack in the joints (Ahmed et al., 2017). Pro-inflammatory cytokines produced by activated T cells cause macrophages to release inflammatory mediators which in turn leads to secondary inflammatory injuries in arthritis (Chun et al., 2016).
The inflammatory cascade is also associated with the activation of oxidant generating enzymes (NADPH oxidase, xanthine oxidase, myeloperoxidase) thus promoting the production of reactive oxygen (ROS) and nitrogen (RNS) species (Fay et al., 2006). These reactive species are involved in cellular damage in joint tissues via various independent mechanisms such as depletion of antioxidants, lipid peroxidation, protein oxidation and DNA damage (Mahajan and Tandon, 2004). Thus inflammatory mediators and reactive species have direct or indirect effects on the pathophysiology of RA.

Non-steroidal anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs) used for the treatment of RA are associated with various adverse drugs reaction, for example gastrointestinal ulcers, cardiovascular complications, and emergence of opportunistic infections due to immunosuppressants (Desai et al., 2014). Moreover, biologics such as TNF-α inhibitors, IL-1β inhibitors, IL-6 inhibitors are also used in the treatment of RA. However their cost, side effects and interference in immune defense response limit their use (Li et al., 2013). Therefore, there is a need to evaluate anti-arthritis efficacy of natural compounds which are relatively safe, tolerable and easily accessible.

Cinnamaldehyde is a bioactive compound isolated from the bark of Cinnamomum cassia (Hanci et al., 2016). It has been used in the manufacturing industry as a spice and flavoring agent in beverages, sweets, ice-creams and chewing gums (Roth-Walter et al., 2014). Eugenol is a phenylpropanoid compound present in honey and essential oil of spices such as Syzygium aromaticum, Cinnamomum verum and Pimenta racemosa (Jaganathan et al., 2011). Both these compounds have been used in traditional medical practices (local analgesic and for the treatment of disturbances in blood circulation, dyspepsia, inflammation, gastritis) due to their anti-fungal, anti-bacterial, anti-inflammatory, anti-mutagenic and anti-oxidant effects (Jaganathan et al., 2011; Roth-Walter et al., 2014).

In our previous publication we have shown that cinnamaldehyde and eugenol have ameliorated oxidative stress and inflammation in collagen induced arthritis in rats (Mateen et al., 2019). In the present study keeping into consideration that RA is a systemic disorder, PBMCs obtained from RA patients were treated with various concentrations of cinnamaldehyde and eugenol in order to determine their effect on oxidant-antioxidant status and secretion of pro-inflammatory cytokines.

2. Material and Methods
2.1 Human subjects

The study protocol was approved from the Institutional Ethical Committee of Jawaharlal Nehru Medical College, A.M.U. Informed consent was obtained from all the participants of the study. Blood was obtained from healthy controls (n=10) and RA patients (n=20) who attended the Orthopaedics Out Patient Department (OPD) of Jawaharlal Nehru Medical College and Hospital, Aligarh, India. Patients were diagnosed according to the European League Against Rheumatism (EULAR) 2010 classification criteria for RA. The clinical and demographical data of healthy controls and RA patients has been given in Table 1 (data is represented as mean±S.D.). Smokers, alcoholics and patients suffering from chronic diseases were excluded from the study. Furthermore, patients receiving NSAIDs, DMARDs and steroids were also excluded from the study.

Table 1. Demographical and clinical data of healthy controls and RA patients.

<table>
<thead>
<tr>
<th></th>
<th>Healthy Controls</th>
<th>RA Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>2/8</td>
<td>3/17</td>
</tr>
<tr>
<td>Age</td>
<td>39±8.7</td>
<td>40±9.6</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>161±6.4</td>
<td>162±4.5</td>
</tr>
<tr>
<td>Weight (Kg)</td>
<td>59±4.7</td>
<td>57±5.1</td>
</tr>
<tr>
<td>Body mass index (BMI)</td>
<td>22.2±1.5</td>
<td>22.1±1.4</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate (ESR, mm/hr)</td>
<td>11.5±6.3</td>
<td>34.6±5.8</td>
</tr>
<tr>
<td>28-Joint count disease activity score (DAS28)</td>
<td>-</td>
<td>4.1±4.6</td>
</tr>
</tbody>
</table>

2.2 Isolation of PBMCs

Histopaque was used to isolate peripheral blood mononuclear cells from healthy controls and RA patients. PBMCs were collected and washed thrice with phosphate buffered saline (pH 7.4). The isolated PBMCs were checked for cell viability by trypan blue exclusion assay.

2.3 Cell culture and treatment
After isolation, PBMCs (1×10^6 cells/well) were divided into following groups and were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2mM L-Glutamine-streptomycin solution and cultured for 24 h at 37°C, 5% CO₂.

**Group I:** PBMCs obtained from healthy control

**Group II:** PBMCs obtained from RA patients

PBMCs obtained from RA patients were also divided into the following groups:

**Group III:** PBMCs treated with 10 µM of cinnamaldehyde

**Group III':** PBMCs treated with 10 µM of eugenol

**Group IV:** PBMCs treated with 20 µM of cinnamaldehyde

**Group IV':** PBMCs treated with 20 µM of eugenol

**Group V:** PBMCs treated with 40 µM of cinnamaldehyde

**Group V':** PBMCs treated with 40 µM of eugenol

2.4 Cell viability assay

The effect of varying doses (10, 20 and 40 µM) of cinnamaldehyde and eugenol on the viability of PBMCs were determined by performing MTT assay as described earlier. The percentage of viable cells were calculated by the previously described formula and the results are expressed as “Viable cells (% of control cells)” (Islam et al., 2000).

2.5 Level of pro-inflammatory cytokines

The levels of TNF-α and IL-6 in the culture supernatant of PBMCs were determined by using a commercially available ELISA kits from Diaclone, France. Results are expressed as pg/ml.

2.6 Measurement of nitric oxide (NO) level

Griess reagent was used to determine the NO level (nitrite level; stable end product of NO) in the 24 h culture supernatant of the treated and untreated PBMCs (Miranda et al., 2001).

2.7 Measurement of intracellular reactive oxygen species
DCF-DA dye was used to assess the intracellular reactive oxygen species generation in the PBMC by following the method described by Keller et al. (Keller et al., 2004). Spectrofluorometer was used to determine the fluorescence intensity with an excitation and emission wavelength of 485 and 530 nm, respectively. Simultaneously, a parallel set of treated cells were analyzed by using an inverted fluorescence microscope to visualize intracellular fluorescence of cells.

2.8 Determination of reduced glutathione level

After incubation, cells were centrifuged to remove the medium. Cells were suspended in 4% sulphosalicylic acid and centrifuged at 1200 g for 15 min. Supernatant was used to determine the level of reduced glutathione in terms of μM GSH/mg protein (Jollow et al., 1974). Protein content was determined by using standard method of protein estimation of Lowry et al (Lowry, 1951).

2.9 Preparation of cell homogenates

After incubation PBMCs were centrifuged at 750 g for 20 min to remove the medium. Pellets were washed with PBS and homogenized using lysis buffer (pH 8; containing 10 mM Tris, 20 mM EDTA, 0.25% Triton X 100). The homogenate was then centrifuged at 750 g for 20 min at 4°C to remove cell debris. The cell lysate was stored at -20°C until further analysis.

2.10 Assay of enzymatic antioxidants

The activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) were determined by following the methods described by Marklund and Marklund, Aebi, Flohe and Gunzle respectively (Aebi, 1984; Flohé and Günzler, 1984; Marklund and Marklund, 1974).

2.11 Markers of oxidative stress

Markers of lipid peroxidation and protein oxidation were evaluated in terms of the levels of malondialdehyde and protein carbonyl groups respectively (Buege and Aust, 1978; Levine et al., 1990).

2.12 Measurement of DNA damage

Comet assay was performed to determine DNA damage by a method described by Singh et al. Tail length in terms of migration of DNA from nucleus was used to assess DNA damage (Singh et al., 1988).
2.13 Fourier Transform Infrared Spectroscopic (FTIR) analysis

FTIR analysis gives the measurement of wavelength as well as intensity of absorption of infrared radiation. FTIR analysis of the PBMCs were performed on the Perkin Elmer FTIR spectrophotometer between the 1000-3000 cm\(^{-1}\) range.

2.14 Preparation of proteins

The X-ray crystal structure of TNF-\(\alpha\) (PDB Id: 1TNF; 2.6 Å resolution) and IL-6 (PDB Id: 1ALU; 1.9 Å resolution) were retrieved from RCSB protein data bank (https://www.rcsb.org/). The three-dimensional structure of TNF-\(\alpha\) is a homotrimer of three chains (A, B, and C) without any bound ligand (Eck and Sprang, 1989). Likewise, the three-dimensional structure of IL-6 comprises a single chain without any bound heteroatom (Somers et al., 1997). AutoDock Tool (ADT) was used to prepare proteins suitable for molecular docking by adding polar hydrogen atoms, assigning Kollman united atom charges and defining solvation parameters (Morris et al., 2009).

2.15 Preparations of ligands

The two-dimensional structures of cinnamaldehyde (CID: 637511) and eugenol (CID: 3314) were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/). These structures were converted into three-dimensional structures in PDB format using OpenBabel software (O’Boyle et al., 2011). ADT was used to prepare ligands suitable for molecular docking by adding Gasteiger partial charges, hydrogen atoms, and defining rotatable bonds. The energies of the ligands were minimized using Universal Force Field (UFF).

2.16 Molecular docking

Autodock Vina (Trott and Olson, 2010) was employed to gain an insight into the binding mode of eugenol and cinnamaldehyde to TNF-\(\alpha\) and IL-6 as described earlier (Rehman et al., 2016; Rehman et al., 2014). Molecular docking was performed after creating a grid box of 62.7 × 63.9 × 61.4 Å centered at 20 × 50 × 40 Å, and 45.3 × 50.1 × 40.1 Å centered at 3 × -20 × 9 Å for TNF-\(\alpha\) and IL-6 respectively. Spacing between the grids was maintained at 1.00 Å. The docking results were analyzed and final images were generated in Discovery Studio 2016.

2.17 Statistical analysis
Data is expressed as mean±S.D. Statistical analysis was carried out by using one-way ANOVA and it was followed by Tukeys’ post hoc test (GraphPad Prism 7.1). p value of less than 0.05 was considered statistically significant.

3. Results

3.1 Effect of cinnamaldehyde and eugenol on cell viability

To examine the effect of cinnamaldehyde and eugenol on the viability of PBMCs, MTT assay was performed. As shown in Fig 1[A], 10, 20, 40 μM of cinnamaldehyde and eugenol caused no significant change in the viability of PBMCs. Therefore, for further experiments 10, 20 and 40 μM of cinnamaldehyde and eugenol were used.

3.2 Effect of cinnamaldehyde and eugenol on cytokine level

The level of pro-inflammatory cytokines TNF-α and IL-6 [Fig 1B and C] were monitored in the 24-hour culture supernatant of PBMCs. RA patients showed significantly higher levels of both TNF-α and IL-6 as compared to the normal healthy controls. PBMCs treated with 10, 20 and 40 μM of cinnamaldehyde and eugenol showed a significant dose-dependent decline in the levels of TNF-α and IL-6.

3.3 Effect of cinnamaldehyde and eugenol on intracellular reactive oxygen species production

Using DCF-DA probe, we found a prominent increase in the reactive oxygen species formation (higher green fluorescence) in PBMCs obtained from RA patients as compared to the PBMCs obtained from healthy controls. However, cinnamaldehyde and eugenol (10, 20, 40 μM) treated PBMCs showed much lower reactive oxygen species formation (Fig. 2A and B).

3.4 Effect of cinnamaldehyde and eugenol on nitric oxide level

Nitric oxide level was found to be increased in the culture supernatant of RA patients as compared to the healthy controls (Fig 2C). There occurred a significant decrease in the level of nitric oxide on treatment with cinnamaldehyde (20, 40 μM) and eugenol (10, 20, 40 μM).

3.5 Effect of cinnamaldehyde and eugenol on reduced glutathione level
The intracellular level of reduced glutathione was found to be significantly lower in RA patients as compared to the normal healthy controls (Fig 2D). Cinnamaldehyde and eugenol caused significant dose dependent elevation in the glutathione level.

### 3.6 Effect of cinnamaldehyde and eugenol on enzymatic antioxidants

The antioxidant status of RA patients was evaluated in terms of three antioxidant enzymes viz SOD, GPx and catalase. As depicted in Fig 3A, B, C, the activities of SOD, GPx and catalase were found to be compromised in RA patients as compared to the healthy controls. However, there occurred a significant rise in the activity of SOD on treatment with 10, 20, 40 µM of cinnamaldehyde and eugenol. Catalase activity also showed elevation at 20 and 40 µM of cinnamaldehyde and eugenol. However, 10 µM of cinnamaldehyde and eugenol were ineffective in causing significant increase in catalase activity. Similarly, the GPx activity was also found to be significantly enhanced at all the three tested concentrations of cinnamaldehyde. However only 20 and 40 µM of eugenol caused a significant amelioration in the activity of GPx.

### 3.7 Effect of cinnamaldehyde and eugenol on markers of oxidative stress

Lipid peroxidation was assessed in terms of its end product, malondialdehyde (MDA). Upon comparison with healthy controls, RA patients showed a significant increase in the MDA level (Fig 4A). 10, 20 and 40 µM of cinnamaldehyde and eugenol caused a prominent dose-dependent decrease in the lipid peroxidation. The level of protein carbonyl groups were determined for the assessment of protein oxidation. RA patients showed a significant enhancement in carbonyl groups as compared to the healthy controls (Fig 4B). Upon incubation of PBMC with 10, 20 and 40 µM of eugenol, there occurred a significant reduction in protein carbonyl groups. 20 and 40 µM of cinnamaldehyde produced a significant decrease in the level of carbonyl groups. DNA damage was assessed in terms of the tail length (Fig 4C and D). Upon comparison with the healthy controls, RA patients showed an appreciable increase in the tail length. There occurred a significant decrease in the tail length on treatment with 10, 20 and 40 µM of cinnamaldehyde and eugenol.

### 3.8 Effects of cinnamaldehyde and eugenol on RA induced alterations in the macromolecules of PBMCs

(FTIR analysis)
FTIR is an effective and non-destructive technique for studying the cellular alterations in various diseases. The region between 1000-1300 cm\(^{-1}\) corresponds to DNA. The band at 1082 and 1224 cm\(^{-1}\) corresponds to symmetric and asymmetric phosphate group stretching in DNA (Fig 5A) (Movasaghi et al., 2008). There occurred a shift in the band position in the PBMCs of RA patients (Table 2). However, cells co-incubated with cinnamaldehyde/ eugenol ameliorated this shift in the band position. The region between 1600-1700 cm\(^{-1}\) and 1500-1600 cm\(^{-1}\) corresponds to amide I and amide II band of proteins respectively (Fig 5B). Amide I band corresponds to stretching vibrations of CO, CN bonds and bending of NH bond of peptide linkages. The amide II band originates mainly from NH bending and CN stretching vibrations (Movasaghi et al., 2008). The amide I and amide II band of healthy controls appeared at 1645 cm\(^{-1}\) and 1548 cm\(^{-1}\) (Fig 5B). The amide I and amide II band position of PBMCs of RA patients and those treated with cinnamaldehyde and eugenol has been given in Table 2. The amide II peak remains unaltered in RA patients, however change in the intensity of both amide I and amide II has been found. Absorption between 2800-3000 cm\(^{-1}\) occurs mainly due to stretching of CH\(_2\) and CH\(_3\) groups present in the lipid acyl chains (Movasaghi et al., 2008). The band at 2853 and 2924 cm\(^{-1}\) corresponds to asymmetric stretching of CH\(_2\) group of lipids (Fig 5C). The change in band position of various groups has been given in Table 2.

### Table 2. FTIR absorption bands (cm\(^{-1}\)) for DNA, proteins and lipids in PBMCs of healthy controls, RA patients and those treated with cinnamaldehyde (40µM) and eugenol (40µM).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>RA Patients</th>
<th>Cinnamaldehyde</th>
<th>Eugenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (cm(^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1082</td>
<td>1082</td>
<td>1082</td>
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<td>1082</td>
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<tr>
<td>1224</td>
<td>1231</td>
<td>1226</td>
<td>1223</td>
<td></td>
</tr>
<tr>
<td>Amide I (cm(^{-1}))</td>
<td>1645</td>
<td>1652</td>
<td>1648</td>
<td>1647</td>
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<tr>
<td>1548</td>
<td>1548</td>
<td>1548</td>
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<td></td>
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<tr>
<td>Amide II (cm(^{-1}))</td>
<td>2853</td>
<td>2853</td>
<td>2853</td>
<td>2852</td>
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<td>Lipids (cm(^{-1}))</td>
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<td>2931</td>
<td>2926</td>
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</table>

### 3.9 Molecular docking analysis

Here, we performed molecular docking to gain an insight into the binding mechanism of cinnamaldehyde and eugenol with TNF-\(\alpha\) and IL-6. The results indicate that for each ligand-receptor pair, there were nine docking poses
ranked on the basis of their binding energies (Table 3). The binding energies of TNF-α with cinnamaldehyde and eugenol were within the range of -4.6 to -5.4 and -5.0 to -5.5 kcal/mol respectively. Similarly, the binding energies of IL-6 with cinnamaldehyde and eugenol were within the range of -4.2 to -5.0 and -4.5 to -5.2 kcal/mol respectively (Table 3).

Table 3: Molecular docking of cinnamaldehyde and eugenol with TNF-α and IL-6 using AutoDock Vina.

<table>
<thead>
<tr>
<th>Target</th>
<th>Docking pose</th>
<th>Binding energy (kcal mol⁻¹)</th>
<th>Distance from the best docking mode</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>RMSD lower bound</td>
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<tr>
<td>Cinnamaldehyde</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>1</td>
<td>-5.4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-5.2</td>
<td>3.386</td>
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<tr>
<td></td>
<td>3</td>
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<tr>
<td></td>
<td>4</td>
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<td>19.01</td>
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<tr>
<td>IL-6</td>
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<tr>
<td></td>
<td>8</td>
<td>-4.2</td>
<td>30.437</td>
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</table>
The analysis of the best docking pose revealed that eugenol binds at the center of cone-shaped binding pocket of TNF-α (Fig. 6 A and B, Table 3). The eugenol-TNF-α complex was stabilized by one hydrophobic interaction with Cys101 of chain A and three hydrogen bonds (two with Gln102 of chain B and one with Glu116 of chain C (Fig 6 A and B, Table 4). Other residues that also interact with eugenol were Pro100 and Gln102 of chain A, Ser99, Pro100 and Cys101 of chain B, and Pro100 and Gln102 of chain C. Conversely cinnamaldehyde binds at the top of the cone-shaped binding pocket of TNF-α formed as a result of interaction between different chains of TNF-α (Fig. 6 C and D). The cinnamaldehyde-TNF-α complex was stabilized by one hydrogen bond with Gln61 of chain C, and one
hydrophobic interaction with Pro117 of chain B. The amino acid residues such as Lys 98, Pro117, Ile118 and Tyr119 of chain A, Ala96, Lys98, Ile118 and Tyr119 of chain B, and Lys98, Pro117, Ile118 and Tyr119 of chain C were also involved in stabilizing the cinnamaldehyde-TNF-α complex. The binding free energies of cinnamaldehyde and eugenol towards TNF-α were -5.4 kcal/mol and -5.5 kcal/mol which corresponded to a binding affinities of the order of $0.65 \times 10^4 \text{ M}^{-1}$ and $1.08 \times 10^4 \text{ M}^{-1}$ respectively.

Similarly, cinnamaldehyde and eugenol binds at the same binding site on IL-6 (Fig. 7). The eugenol-IL-6 complex was stabilized by three hydrogen bonds with Thr43, Arg104 and Thr163 along with two hydrophobic interactions with Arg104 and Phe105. Other amino acid residues that stabilized the eugenol-IL-6 complex were Glu42, Lys46, Ser47, Glu106, ser107 and Asp160 (Fig. 7 A and B, Table 4). Likewise, cinnamaldehyde-IL-6 complex was stabilized by two hydrogen bonds with Thr43 and Arg104. Other residues such as Glu42, Phe105, Ser107, Ser108 and Asp160 also played significant role in the formation of a stable cinnamaldehyde-IL-6 complex (Fig. 7 C and D, Table 4). The binding free energies of cinnamaldehyde and eugenol towards IL-6 were -5.0 kcal/mol and -5.2 kcal/mol which corresponded to a binding affinities of the order of $0.46 \times 10^4 \text{ M}^{-1}$ and $0.91 \times 10^4 \text{ M}^{-1}$ respectively.

### Table 4. Molecular interactions between eugenol and cinnamaldehyde with TNF-α and IL-6.

<table>
<thead>
<tr>
<th>Target</th>
<th>Hydrogen bonds</th>
<th>Hydrophobic interactions</th>
<th>Other interacting residues</th>
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</thead>
<tbody>
<tr>
<td>Cinnamaldehyde</td>
<td></td>
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</tr>
</tbody>
</table>
4. Discussion

Rheumatoid arthritis is an inflammatory autoimmune disorder. Reactive oxygen species and pro-inflammatory cytokines have been shown to play an important role in the pathophysiology of RA (Mateen et al., 2016; Mateen et al., 2017). Various natural compounds have been shown to impart their antioxidant and anti-inflammatory effects in
the management of many diseases (Umar et al., 2013; Umar et al., 2012; Wang et al., 2017). The results of the present study showed heightened inflammatory state in RA patients due to the over-production of pro-inflammatory cytokines; TNF-α and IL-6. Furthermore, compromised antioxidant status and elevation in the markers of oxidative stress were found in the PBMC culture of RA patients. Cinnamaldehyde and eugenol treated PBMC showed amelioration in the levels of pro-inflammatory cytokines and markers of oxidative stress. FTIR analysis further confirms the protection offered by cinnamaldehyde and eugenol to the macromolecules of PBMCs of RA patients. This study for the first time demonstrates the antioxidant and anti-inflammatory effects of cinnamaldehyde and eugenol in the PBMC culture of RA patients.

Although, chemically synthesized inhibitors of TNF-α and IL-6 are available in the market, they display serious side effects. Thus, the quest to explore compounds from natural sources as novel scaffold for the inhibition of TNF-α and IL-6 has taken a momentum. Natural products offer lesser toxicity or side effects and they are cost effective. In the present study, we have identified two natural products (cinnamaldehyde and eugenol) possessing antioxidant anti-inflammatory potential in RA patients. TNF-α plays a key role in RA as it is involved in formation, differentiation and activation of osteoclasts which ultimately results in the erosion of bone and cartilage (Yuan et al., 2012). IL-6 apart from promoting differentiation and activation of osteoclasts, also enhances the vascular permeability of synovial tissue by stimulating the production of vascular endothelial growth factor (Yao et al., 2014). IL-6 is also involved in promoting the differentiation of Th17 cells while it inhibits the activity of T regulatory cells which are involved in the secretion of anti-inflammatory cytokines such as IL-10 (Yoshida and Tanaka, 2014). Our results demonstrated higher TNF-α and IL-6 levels in 24-hour culture supernatant of PBMC of RA patients. Previous reports have also suggested that the levels of pro-inflammatory cytokines in serum and synovial fluid are elevated in RA patients (Mateen et al., 2017; Umar et al., 2012). Cinnamaldehyde and eugenol were found to suppress the secretion of pro-inflammatory cytokines thus exerting anti-inflammatory effects on PBMCs of RA patients.

Suppressive effect of cinnamaldehyde on the secretion of TNF-α, IL-1β, IL-6 have been shown to be related to the inhibition of ERK, JNK and p38 MAPKs phosphorylation in RAW264.7 murine macrophage cell line (Kim et al., 2018). Eugenol has been shown to downregulate the levels of TNF-α and IL-6 by inhibiting the activation of NF-κB pathway in acute lung injury model (Huang et al., 2015). The over production of reactive oxygen species and unbalanced glutathione redox status induces the activation of signal transduction pathways including NF-κB pathway, which in turn regulates the transcription of inflammatory cytokines (TNF-α, IL-1β, and IL-6) (Filippin et
Cinnamaldehyde and eugenol mediated reduction in the secretion of TNF-α and IL-6 might be due their ability to ameliorate the levels of reactive oxygen and glutathione, as a result of which lower activation of NF-κB pathway might have occurred. Moreover, our molecular docking studies also showed that eugenol and cinnamaldehyde were able to interact with the key residues of TNF-α and IL-6 and thereby could act as inhibitors of these proteins. The structural and biochemical analysis of IL-6 revealed that it contains three main binding sites (I-III). Site I comprises the amino acid residues involved in the interaction with IL-6 receptor (IL-6R). Amino acid residues forming Site II interact with gp130 only in the presence of IL-6R, while Site III amino acid residues interact with gp130 in the trimeric form of IL-6 (Somers et al., 1997). Our docking analysis revealed that cinnamaldehyde as well as eugenol bind at site III of IL-6 and thus could act as potential inhibitors of IL-6-gp130 mediated signaling pathway. In a previous study, a small molecule composed of trifluoromethylphenyl inode and dimethyl chromone linked together by dimethylamine has been shown to bind at the interior of the cone formed by TNF-α trimer and promote dissociation of subunits (He et al., 2005). Similarly, in this study, the docking of cinnamaldehyde and eugenol with TNF-α revealed that they were bound at the center of TNF-α trimer. We speculate that the binding of cinnamaldehyde and eugenol at the center of TNF-α trimer promotes dissociation of the three-dimensional structure of TNF-α which would indirectly affect the function of TNF-α. Since TNF-α regulates the activation of NF-κB pathway, therefore cinnamaldehyde and eugenol mediated inhibition in the secretion of TNF-α might also result in the suppression of NF-κB activation which would otherwise result in the transcription of downstream genes involved in cell proliferation, adhesion, inflammation and oxidative stress response (Filippin et al., 2008). In the past, many inhibitors of TNF-α and IL-6 have been isolated from natural sources (Paul et al., 2006). For instance, polyphenolic molecules such as naringenin, pelargonidin, and cyanidin have been shown to possess moderate potential to inhibit TNF-α. Luteolin, luteolin-7-glucoside, quercetin, and genistein have been found to inhibit lipopolysaccharide (LPS)-stimulated release of TNF-α and IL-6. (Herath et al., 2003). Further, hesperidin and resveratrol have been reported to exert anti-inflammatory effect by inhibiting the release of TNF-α (Bi et al., 2005). Curcumin has also been reported to exhibit the anti-inflammatory properties by inhibiting the release of TNF-α and IL-1β (Chan, 1995).

Nitric oxide production has been reported to be elevated in inflammatory diseases due to the activation of inducible nitric oxide synthase (iNOS) pathway (Van't Hof et al., 2000). Animal studies have shown the role of nitric oxide in joint inflammation and tissue damage and the severity of arthritis has been found to be decreased by the
administration of iNOS inhibitors (McCartney-Francis et al., 1993). Cinnamaldehyde and eugenol mediated decrease in the nitrite level might be due to the inhibition of iNOS mediated production of nitric oxide (Bezerra et al., 2017; Oktyabrsky and Smirnova, 2007). Reactive oxygen species plays a very crucial role in the pathophysiology of RA. They cause damage to lipids, nucleic acids and proteins in the joint tissues. These reactive species have the potential to damage cellular components in cartilage and extracellular matrix by upregulating inflammatory mediators (Hitchon and El-Gabalawy, 2004). Nitric oxide combines with superoxide anion to form highly toxic peroxynitrite which in turn leads to fast protonation and depletion of −SH groups and other antioxidants, DNA disruption, oxidation and deamination of DNA bases, oxidation and nitration of lipids (Halliwell and Gutteridge, 2015). As a result of unbalanced redox status, activation of NF-κB pathway occurs which in turn promotes the formation of inflammatory mediators. Reactive oxygen species mediated elevation in lipid peroxidation, protein oxidation and DNA damage were found in PBMC cultures of RA patients. Other studies have also reported in higher biomolecular oxidation in the plasma and synovial fluid of RA patients (Mateen et al., 2016; Seven et al., 2008). Cinnamaldehyde and eugenol were effective in decreasing the formation of reactive species due to their antioxidant or free radical scavenging action which in turn has reduced lipid peroxidation, protein oxidation and DNA damage.

The results of DNA damage, protein oxidation and lipid peroxidation were further supported by FTIR analysis. The change in the intensity and position of phosphate stretching bands in the 1000-1300 cm⁻¹ indicates that alterations in the conformation of DNA occurs in RA patients which might be due to the observed DNA damage (strand cleavage and chromatin fragmentation) (Lipiec et al., 2014). Similarly, alterations in the band position in amide I region of proteins has been found in RA patients. Proteins has been reported to undergo various modifications due to the presence of excess free radicals in RA Patients (Islam et al., 2018). These modifications tend to cause structural perturbations in proteins which in turn have caused a change in the intensity of amide I and amide II bands. Similarly increased lipid peroxidation leads to change in membrane permeability which in turn has changed the absorption occurring due to the stretching vibrations of CH₂ groups (Fabian et al., 1995). Cinnamaldehyde and eugenol have ameliorated the structural perturbations in the proteins, lipids and DNA of cultured PBMCs of RA patients.
The formation of reactive oxygen is controlled by various enzymatic and non-enzymatic antioxidants present in the body. However impaired antioxidant defence system has been found in the PBMC culture of RA patients. This indicates compromised ability of PBMCs to protect themselves from damaging effects of oxidants. This enzyme inactivation might be due to reactive oxygen species mediated direct inhibition or complex formation between NO and bound Cu and Fe present in SOD and catalase respectively (Escobar et al., 1996). Alteration in the levels of antioxidants have also been reported by other authors as well (Khojah et al., 2016; Kundu et al., 2012; Seven et al., 2008). Superoxide dismutase is involved in the dismutation of the superoxide radical into hydrogen peroxide (H₂O₂). Glutathione peroxidase reduces H₂O₂ by oxidising glutathione. Catalase also provides protection from harmful effects of H₂O₂ by converting it to water and oxygen. Cinnamaldehyde and eugenol have increased the activities of antioxidant enzymes by neutralizing reactive oxygen/nitrogen species which can otherwise directly modify the enzyme molecules. Increase in the activities/level of antioxidants by cinnamaldehyde and eugenol indicates reversal in the impaired antioxidant defence response in RA patients.

**Conclusion**

Cinnamaldehyde and eugenol were effective in suppressing the secretion of pro-inflammatory cytokines from the cultured PBMCs of RA patients. These compounds have also reduced the neutralizing reactive oxygen/nitrogen species formation which in turn has ameliorated biomolecular oxidation and antioxidant defence response in the PBMC culture of RA patients. Thus these compounds have potential to be used as an adjunct in the management of RA by virtue of their free radical scavenging and anti-inflammatory effects.

**5. Author contributions**

Conceived and designed the experiments: SMateen, SMoin. Performed the experiments: SMateen, MTR, SS, SK, FMH. Analyzed the data: SMateen, MTR, SMoin. Contributed samples/reagents/materials/analysis tools: SMoin, SSN, AQK, MTR, SK, FMH. Wrote the paper: SMateen, MTR, SSN.

**6. Conflict of interest**

The authors declare that they have no conflict of interest.
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Author agreement:

The manuscript entitled ‘Anti-oxidant and Anti-inflammatory effects of Cinnamaldehyde and Eugenol on Mononuclear Cells of Rheumatoid Arthritis Patients’ was reviewed by all authors, agreed with its content and agreed to its submission in European journal of pharmacology.

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Fig. 1. [A] Percent cell viability of PBMCs of healthy controls co-cultured with varying doses of cinnamaldehyde and eugenol for 24 hrs; effects of varying doses of cinnamaldehyde and eugenol on the level of [B] TNF-α and [C] IL-6 on the culture supernatant of PBMCs of RA patients. * P<0.05

Fig. 2. [A] Photomicrographs showing intracellular reactive oxygen species generation by DCF-DA dye in PBMCs treated with varying concentrations of cinnamaldehyde and eugenol, [B] Bar graph representation of DCF-DA assay; effects of cinnamaldehyde and eugenol on the levels of [C] nitric oxide and [D] reduced glutathione in PBMCs of RA patients. * P<0.05

Fig. 3. Effects of varying concentrations of cinnamaldehyde and eugenol on the activities of [A] Superoxide dismutase, [B] Glutathione peroxidase, [C] Catalase in PBMCs of RA patients. * P<0.05

Fig. 4. Effects of varying concentrations of cinnamaldehyde and eugenol on the levels of [A] malondialdehyde, [B] protein carbonyl, [C] Representative images of comet assay, [D] Tail length in PBMCs of RA patients. * P<0.05

Fig. 5. Effects of cinnamaldehyde (40µM) and eugenol (40µM) on macromolecules in PBMCs of RA patients. [A] DNA, [B] Amide I and Amide II, [C] Lipids.

Fig. 6. Molecular docking analysis of interaction of eugenol and cinnamaldehyde with TNF-α. Molecular surface docked view representation of eugenol and cinnamaldehyde with TNF-α [A, C]; Fig B and D shows the residues of TNF-α interacting with eugenol and cinnamaldehyde.

Fig. 7. Molecular docking analysis of interaction of eugenol and cinnamaldehyde with IL-6. Molecular surface docked view representation of eugenol and cinnamaldehyde with IL-6 [A, C]; Fig B and D shows the residues of IL-6 interacting with eugenol and cinnamaldehyde.