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Research Paper

Ocimum sanctum leaf extracts attenuate human monocytic (THP-1) cell activation

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ABSTRACT

Ethnopharmacological relevance: *Ocimum sanctum* (OS), commonly known as Holy basil/*Tulsi*, has been traditionally used to treat cardiovascular diseases (CVD) and manage general cardiac health. The present study is designed to evaluate the antiinflammatory effect of *O. sanctum* and its phenolic compound and eugenol (EUG) in human monocytic (THP-1) cells and validate its traditional use for treating cardiovascular diseases.

Materials and methods: The phytochemical analysis of alcoholic and water extracts of OS-dry leaves (OSAE and OSWE) was done using LC–QTOF–MS. A phenolic compound, EUG was quantified in both OSAE and OSWE by an LC–MS technique using a mass hunter work station software quantitative analysis system. The effect of both OSAE, OSWE, pure compound EUG and positive control imatinib (IMT) was investigated in THP-1 cells by studying the following markers: lipopolysaccharide (LPS) induced tumor necrosis factor alpha (TNF-α) secretion by ELISA, gene expression of inflammatory markers (TNF-α, IL-6, MIP-1α and MCP-1) by real time PCR and translocation of nuclear factor kappa B (NF-κB) by confocol microscopy. Furthermore, the effect of the extracts, EUG and IMT, was studied on phorbol-12-myristate-13-acetate (PMA) induced monocyte to macrophage differentiation and gene expression of CD14, TLR2 and TLR4.

Results: The LC–MS analysis of OSAE and OSWE revealed the presence of several bioactive compounds including eugenol. Quantitative analysis revealed that OSAE and OSWE had EUG of 12 ng/mg dwt and 19 ng/mg dwt respectively. OSAE, OSWE (1 mg dwt/mL) pure compound EUG (60 μg/mL) and positive control IMT (20 μg/mL) showed marked inhibition on LPS induced TNF-α secretion by THP-1 cells. At the selected concentration, the plant extracts, EUG and IMT inhibited gene expression of cytokines and chemokines (IL-6, TNF-α, MIP-1α, MCP-1) and translocation of NF-κB-p65 to the nuclei. In addition, they showed significant inhibition on PMA induced monocyte to macrophage differentiation and the gene expression of CD14, TLR2 and TLR4 markers.

Conclusion: The result of the present study validated traditional use of *Ocimum sanctum* for treating cardiovascular disease for the first time by testing antiinflammatory activity of *Ocimum sanctum* in acute inflammatory model, LPS induced THP-1 cells. The plant extracts showed significant antiinflammatory activity, however, further to be evaluated using chronic inflammatory animal models like diabetic or apolipoprotein E-deficient mice to make it evidence based medicine. The phenolic compound eugenol (60 μg/mL) showed significant antiinflammatory activity. However the amount of eugenol present in 1 mg of OSAE and OSWE (12 ng/mg and 19 ng/mg dwt respectively) used for cell based assays was very low. It suggests that several other metabolites along with eugenol are responsible for the efficacy of the extracts.

1. Introduction

Ocimum sanctum Linn. (Lamiaceae) widely known as 'Holy basil/*Tulsi*' is a herbaceous sacred plant found across India. Extracts of this plant, particularly of leaves, have been traditionally used for their cardioprotective purposes (Joseph and Nair, 2013; Kumar et al., 2012;

Abbreviations: AE, Alcoholic extract; EUG, Eugenol; IMT, Imatinib; LPS, Lipopolysaccharides; PMA, Phorbol-12-myristate-13-acetate; WE, Water extract

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Pandey and Madhuri, 2010; Pattanayak et al., 2010; Rashid et al., 2013). Of recently, scientific investigations have found out that *Ocimum sanctum* (OS) possesses wide range of pharmacological benefits such as hypolipidemic (Hussain et al., 2001), hepatoprotective (Akilavalli et al., 2011) and neuroprotective (Yanpallewar et al., 2004) properties. Its anticoagulant (Khan et al., 2011), antioxidant, antiinflammatory (Kalabharathi et al., 2011; Kath and Gupta, 2006) and immunomodulatory (Mediratta et al., 2002) effects have been reported using animal models. All these pharmacological activities of OS are attributed to its phytoconstituents, one such compound which has been comprehensively investigated is eugenol. Eugenol is a phenolic compound which is a major constituent of *Ocimum sanctum* (Singh et al., 2013) and other aromatic plants, *Eugenia aromaticum* (Raghavendra and Naidu, 2011), *Cinnamomum verum*, *Pimenta racemosa*, etc. (Jaganathan et al., 2011). In last few decades several studies have been carried out globally by scientific community to suggest the role of eugenol in therapeutic potentials of *Ocimum sanctum* (Prakash and Gupta, 2005). Antioxidant (Nagababu et al., 2010), antiinflammatory (Daniel et al., 2009) and antiplatelet aggregatory (Raghavendra and Naidu, 2011) effects of eugenol have been demonstrated using the animal model. Further, its vasorelaxing action suggested its therapeutic importance as a vasodilator (Nishijima et al., 1999).

Cardioprotective property of OS and its metabolite EUG against isoproterenol induced myocardial infarction in rats has been demonstrated (Choudhary et al., 2006; Sharma et al., 2001). However, pharmacological action of OS as well as EUG against atherosclerosis is poorly addressed so far. Atherosclerosis, a type of cardiovascular disease (CVD) develops in the large arteries due to complexed interaction between activated peripheral blood monocytes and vascular endothelium (Boos and Lip, 2006; Libby, 2002; Ross, 1999). In view of validating traditional use of OS-leaves, the present study was designed to investigate the antiinflammatory effect of alcoholic and water extracts of OS-leaves and pure compound EUG on lipopolysaccharide (LPS) and/or phorbol-12-myristate-13-acetate (PMA) induced activation of human monocytic (THP-1) cells.

2. Materials and methods

2.1. Chemicals

Power SYBR Green PCR Master Mix was obtained from Applied Biosystems (Carlsbad, CA, USA), BD OptEIA human TNF-α ELISA kit was purchased from BD Biosciences (USA), iScript cDNA synthesis kit was from Bio-Rad (Hercules, CA), Alexa Fluor 594 goat anti-rabbit IgG (H+L), fetal bovine serum (FBS), L-glutamine, pen-strep, prolonged gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI), RPMI 1640, and trizol reagent were purchased from Invitrogen. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrezolium bromide (MTT) was obtained from Merck, imatinib (IMT) from Natco Pharma Limited, eugenol, lipopolysaccharide-*Escherchia coli* (LPS), phorbol-12-myristate-13-acetate (PMA), trypan blue, and DEPC treated water were purchased from Sigma-Aldrich (Germany). NF-κB-p65 polycolonal rabbit antibody was obtained from Thermo Scientific (USA).

2.2. Plant material collection and preparation of OS dry leaf extracts

The seeds of the *Ocimum sanctum* plants (voucher #46803) were obtained from CIMAP, Hyderabad and authenticated by Prof. B. R. Rao, Scientist in charge, CIMAP. The plants were grown in plant house located in UoH campus. The leaves were collected from one year old plants and shade dried for at least 7 to 10 days and stored at room temperature in dark until further use.

2.3. Preparation of OS dry leaf extracts

The protocol followed for the preparation of alcoholic and water extracts from *Ocimum sanctum* dried leaves was quite similar with the one as mentioned by Kokkiripati et al. (2011). Alcoholic extract was prepared by soaking the fine powder obtained from 190 mg of leaves in 20 mL of 80% (v/v) ethanol. This process was followed by continuous stirring at 40 °C for 5–6 h until the volume reached approximately to 1/5th of the initial volume. Water extract (OSWE) was prepared by soaking the same amount of the powder in 4 mL of distilled water for 12–16 h at room temperature. Later on, the extracts were centrifuged at 10,000 rpm for 10 min and supernatants were collected and stored until further use at 4 °C and used within 3 days. The respective extracts were dried completely under the vacuum to determine the yields of OSAE and OSWE. The alcoholic and water extracts yielded 4% (w/w) and 4.5% (w/w) based on the amount of dried leaves taken to prepare the extracts. Commercially available EUG was procured from Sigma-Aldrich and stock solution was prepared (60 mg/mL) in absolute alcohol and stored at −20 °C until further use. IMT of 10 mg/mL was solubilized in DMSO and subsequently diluted in a cell growth medium. Vehicle concentration, in case of OSAE, OSWE, EUG and IMT did not exceed to 1% of cell culture volume in all cell based experiments. The effect of respective vehicle volume was tested in each and every set of experiment.

2.4. ESI–LC–MS/MS analysis

Phytochemical analysis of the OSAE and OSWE was done using a 6520 Accurate Q-TOF (Agilent Santa Clara, CA) mass spectrometer coupled to HPLC. Metabolites of the extracts were separated using HPLC according to Dutta et al. (2007) on reverse phase column (Zorbax Eclipse XDB-C_{18}, 4.6 × 50 mm, 1.8 μ). Solvent A and solvent B of mobile phase were water with acetic acid (0.1% v/v) and methanol with acetic acid (0.1%) respectively. A controlled flow rate of 0.2 mL/min was maintained for the following linear gradient elution in terms of solvent B: (i) 20–55% from 0 to 15 min, (ii) 55–90% from 15 to 20 min, (iii) 90% isocratic from 20 to 23 min, (iv) 90–20% from 23 to 26 min, and (v) isocratic 20% from 26 to 35 min. Sample volume injected was 2 μL. ESI parameters: both on negative and positive ion modes; mass range 100–1700; spray voltage 4 kV; gas temperature 325 °C; gas flow 10 L/min; Nebulizer 40 psi. Quantification of eugenol in OSAE and OSWE was done and details are given in Supplementary materials.

2.5. Cell culture and treatments

Human monocytic (THP-1) cell line was obtained from National Centre for Cell Sciences, Pune, India. The cells were cultured at 37 °C in 5% CO_2 in RPMI media supplemented with 10% fetal bovine serum (FBS) and 100 μg/mL pen-strep. Thereafter the experiments were conducted at a cell density of 5×10^5 cells/mL. An acute inflammatory stimulus was given to the cells by incubating them with 0.5 μg/mL of LPS for 3 h at 37 °C in 5% CO_2 (Kokkiripati et al., 2011). In order to stimulate the differentiation of THP-1 cells to macrophages, the cells were treated with 5 ng/mL of PMA for 48 h at 37 °C in 5% CO_2 as described in Tsuchiya et al. (1982). The images of the cells were taken under an inverted microscope.

2.6. Plant extracts dosage determination

Cell viability of THP-1 cells in the presence of various amounts of OSAE/OSWE, EUG and IMT was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Mosmann (1983). An aliquot of 500 μL cell suspension (5×10^5 cells/mL) was distributed in each well of 24 well plates and then incubated for 24 h in the presence or absence of various concentrations of OSAE, OSWE, pure compound EUG, and positive control IMT. Effect of respective vehicle solvents was tested. Vehicle concentration was limited to $<1\%$ of cell culture volume. MTT (5 mg/mL in PBS) reagent of 20 μL was added to each well and incubated for an additional 4 h at 37 °C. Subsequently, the formed precipitate containing purple-blue formazan was dissolved in 100 μl of DMSO and the optical density was measured at 570 nm using a micro-plate reader (Tecan, Germany).

2.7. Quantification of secreted TNF-α

In order to carry out this experiment, cells were seeded in 24-well plates at a density of 5×10^5 cells/mL and pretreated with or without plant extracts (1 mg dwt/mL), EUG (6 and 60 μg/mL) and IMT (5 and 20 μg/mL) at 37 °C in 5% CO_2 for 12 h. Subsequently the cells were exposed to 0.5 μg/mL of LPS for 3 h. Cells were centrifuged, collected supernatant stored at −80 °C until further use. TNF-α present in the supernatants was determined using BD OptEIA human TNF-α ELISA kit (BD Biosciences) as per the manufacturers protocol.

2.8. Transcript analysis of inflammatory markers

After required treatments with LPS or PMA in the presence or absence of plant extracts (1 mg dwt/mL), EUG (60 μg/mL) and IMT (20 μg/mL), total RNA from the cells was isolated using trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Subsequently, 1 μg of RNA was used for c-DNA synthesis (Bio-Rad synthesis kits). Then the transcripts were quantitated by real time PCR using specific set of primers which are listed in Supplementary Table 1.

2.9. Nuclear translocation of NF-κB

The NF-κB-p65 in THP-1 cells was detected by indirect immunofluorescence assay using confocal microscopy (Jeong et al., 2010). THP-1 cells were cultured directly on glass coverslips. After the respective treatments of plant extracts (1 mg dwt/mL), EUG (60 μg/mL) and IMT (20 μg/mL), the cells were fixed using 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 (w/v), and blocked with 3% bovine serum albumin (Sigma-Aldrich, Germany). Subsequently polyclonal antibodies against NF-κB-p65 (1:250) were applied for 12 h followed by 3 h incubation with anti-rabbit IgG conjugated to Alexa fluor 594 antibody (1:250). Excitation and emission maxima used were 543 nm 617 nm respectively. The nuclei were visualized by staining with DAPI (excitation wavelength, λ_{ex} 358 nm and emission wavelength, λ_{em} 400 to 500 nm).

2.10. Statistical analysis

To analyze the obtained data, Sigma Plot 11 software was used. All the data obtained were analyzed by one way analysis of variance (ANOVA) test using statistical package for the Life Sciences (SPLS version 11). Data obtained from all the experiments were expressed as mean ± S.D of three individual experiments. *p*-values lesser than 0.001 were considered as statistically significant.

3. Results

3.1. Metabolite profiling of OSAE and OSWE

Negative ion and positive ion modes of LC–QTOF–MS detected 16 and 7 compounds in OSAE; 4 and 6 compounds in OSWE. MS–MS fragmentation spectra of the compounds are shown in Fig. 1 and other details including *m/z* values, retention time, etc. are shown in Supplementary Tables 2 and 3. Majority of the identified compounds belong to either phenolics or terpenoids. Vicenin, luteolin-7-O-glucuronide, apigenin-7-O-glucuronide, methyl chavicol, methyl cinnamate, eugenol, fatty acid, and linoleic acid were commonly detected in both OSAE and OSWE. Rosmarinic acid, orientin, germacrene D, α-pinene, carvacrol, apigenin, linalool, cirsimaritin, cirsilineol, sinapic acid and ascorbic acid were exclusively detected in OSAE, while methyl eugenol and stearic acid were detected from OSWE. Eugenol, which has been detected in both modes of extracts, was investigated for its antiinflammatory activity in THP-1 cells. Quantitative analysis of EUG in plant extracts revealed that OSAE and OSWE contained 12 ng/mg dwt and 19 ng/mg dwt respectively.

3.2. Effect of OSAE, OSWE and EUG on LPS induced TNF-α secretion

TNF-α is a potent proinflammatory cytokine secreted by activated monocytes, subsequently activate other cell sepsis in the blood vessel and thus initiates/promotes the progression of atherosclerosis. As shown in Fig. 2, LPS stimulates THP-1cells to secrete 22 fold more amount of TNF-α compared to control unstimulated cells. Pretreatment of cells with 0.5 mg and 1 mg dwt/mL of OSAE brought down to 18 and 9 fold and OSWE to 19 and 6 folds respectively. EUG at 6 μg/mL reduced to 14 fold, whereas 60 μg/mL completely attenuated LPS induced stimulation. The cells secreted similar to unstimulated control cells (Fig. 2). Pretreatment of cells with positive drug control IMT at 5 and 20 μg/mL brought down to 19 and 9 fold respectively in the presence of LPS (Fig. 2). The rest of the cell based assays were done using pretreatments with 1 mg dwt/mL of the plant extracts, 60 μg/mL of EUG and 20 μg/mL of IMT.

Toxicity of plant extracts or pure compound EUG and positive control IMT, if any, on THP-1 cells was determined based on MTT assay. No cell death was observed up to 3 mg dwt/mL of OSAE and OSWE and the data is shown up to 1 mg dwt/mL in Supplementary Fig. 2. Similarly cell viability in the treatments with EUG (up to 60 μg/mL) and IMT (up to 20 μg/mL) was $>95\%$ (Supplementary Fig. 2). Vehicles solvents (alcohol or water of $<1\%$) had no effect on cell viability as well as TNF-α secretion. Volume of DMSO did not exceed 0.5% and had no effect on cell viability.

3.3. Effect of OSAE, OSWE and EUG on LPS induced inflammatory markers

The effect of the plant extracts, EUG and positive drug control IMT on LPS induced gene expression of inflammatory markers including TNF-α was tested to examine if the effect was at transcriptional or posttranscriptional level. The transcript analysis by real-time quantitative PCR showed that LPS stimulated the expression of IL-6 (2-fold increase), TNF-α (5-fold increase), macrophage inflammatory protein (MIP-1α) (2.15-fold increase) and MCP-1 (36-fold increase) as shown in Fig. 3. Here it is noteworthy that such stimulation of proinflammatory gene expression was markedly inhibited in the cells pretreated with OSAE/OSWE (1 mg dwt/mL). OSWE showed stronger effect than OSAE in attenuating the MCP-1 marker. EUG (60 μg/mL) and IMT (20 μg/mL) showed significant inhibition of all the selected markers. At these concentrations, EUG showed higher inhibitory effect

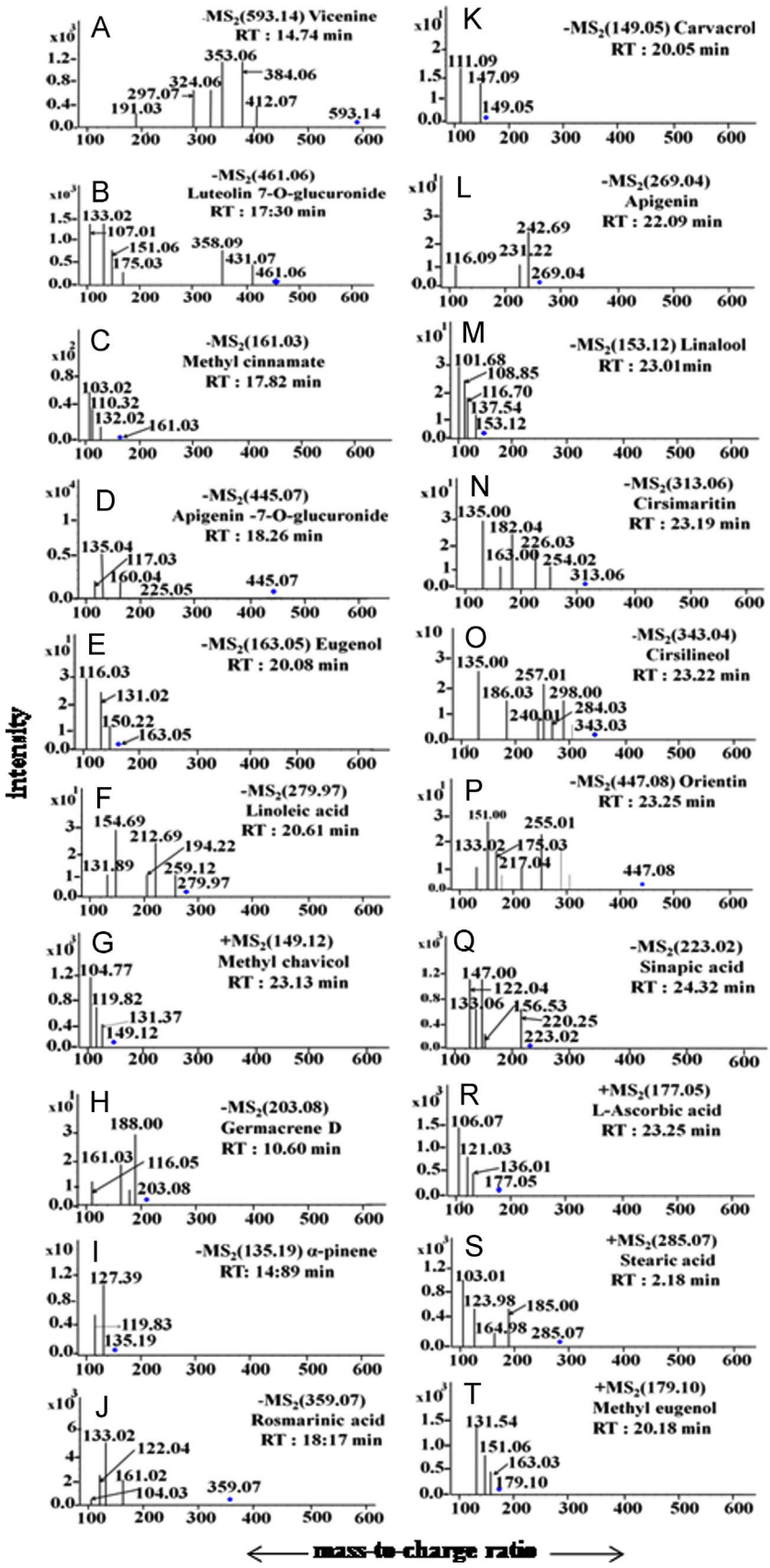

Fig. 1. Negative ion (−) and positive ion (+) ESI/MS/MS of alcoholic and water extracts of OS-dry leaves. Labeled peaks are corresponding to the fragments.

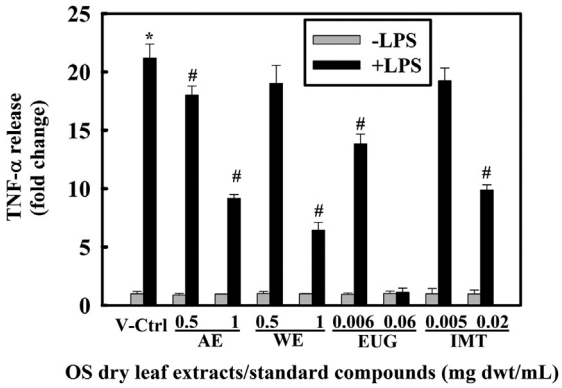

Fig. 2. Effect of OS-dry leaf extracts, EUG and IMT on LPS-mediated TNF-α secretion in THP-1 cells. Cells were seeded at 5×10^5 cells/mL. Data represents fold change in TNF-α release into the media by the cells treated with LPS (0.5 μg/mL for 3 h) compared to untreated cells. OSAE, OSWE, EUG and IMT added to the cells 12 h prior to incubation with LPS. Vehicle solvents of alcohol/water (was limited to < 1%) and of DMSO used (was limited to < 0.5%) had no effect. Both types of solvents had no effect on TNF-α secretion; mean value is represented as V-control. Data represent $n=6$ mean of six measurements. *Statistical significance of $p < 0.001$ within the control groups i.e. cells+LPS vs cells−LPS. #$p < 0.001$ compared between cells treated with LPS in the presence of OSAE/OSWE, pure compound EUG and positive control IMT in their absence.

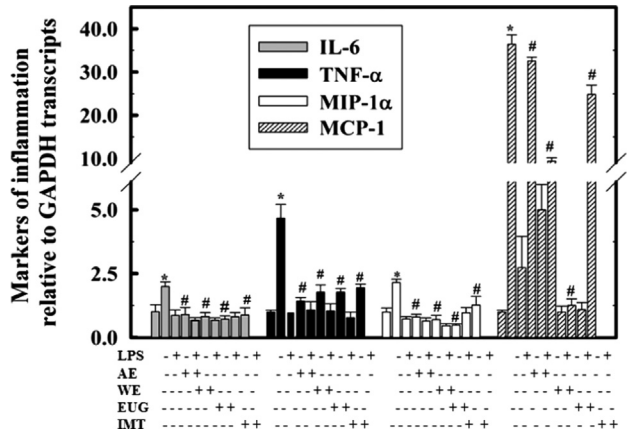

Fig. 3. Effect of OS-dry leaf extracts (1 mg dwt/mL), EUG (60 μg/mL) and IMT (20 μg/mL) on gene expression of IL-6; TNF-α; MIP-1α and MCP-1 in control vs LPS induced THP-1. Vehicle volume of alcohol or water (did not exceed 1%) and DMSO (< 0.2%) had no effect. Experiments were performed at least in triplicate and the results are expressed as the mean ± S.D. *$p < 0.001$ for comparison between cells+LPS vs cells-LPS. #$p < 0.001$ compared between cells treated with LPS in the presence of plant extracts, pure compound and positive control IMT vs in their absence.

than IMT on MIP-1α and MCP-1. IMT at 50 μg/mL stimulated proinflammatory effect in LPS unstimulated cells (data not shown).

3.4. Effect of OSAE, OSWE and EUG on the subcellular localization of NF-κB-p65

In order to further elucidate, the molecular mechanism by which the plant extracts modulate gene expression in THP-1 cells, the effect of OSAE/OSWE and EUG on transcriptional factor NF-κB was investigated. The transcription factor NF-κB plays a crucial role in the regulation of gene expression of several proinflammatory markers including TNF-α, interleukins and chemokines. Results presented in Fig. 4 clearly indicate that the plant extracts and pure compound EUG inhibit LPS induced translocation of NF-κB in THP-1 cells. The confocol images of Fig. 4A–C (1st panel) showed that NF-κB-p65 was mostly sequestered in the cytoplasm of normal uninduced cells, whereas it was predominately located in the nuclei of the LPS stimulated cells (Fig. 4D–F). Such LPS dependent nuclear translocation of NF-κB-p65 was blocked in the cells pretreated with OSAE (Fig. 4G–I), OSWE (Fig. 4J–L) or EUG (60 μg/mL) (Fig. 4M–O). These results demonstrated that OSAE, OSWE or EUG elicited antiinflammatory activity in THP-1 cells by modulating subcellular localization of transcriptional factor NF-κB at their tested concentration. Positive drug control, IMT (20 μg/mL) showed similar response (Fig. 4P–R).

3.5. Effect of OSAE, OSWE and EUG on PMA induced cell differentiation markers

Human monocytic THP-1 cells committed to the monocytic cell lineage are suspension in nature and do not adhere to the plastic surfaces of the culture plates as shown in Supplementary Fig. 2A. PMA (5 ng/mL) stimulated differentiation of THP-1 cells to macrophages as indicated by their adherence to substratum of the culture dish (Supplementary Fig. 2B). The adhered cells showed morphological characteristics similar to macrophages. Such PMA induced differentiation process was inhibited by the plant extracts, pure compound EUG and positive control, IMT. As shown in Supplementary Fig. 3C–E, pretreatment with OSAE/OSWE (1 mg dwt/mL) and EUG (60 μg/mL) significantly attenuated the process of differentiation by PMA challenged THP-1 cells. In agreement with the literature, differentiated macrophages (PMA challenged THP-1 cells) showed upregulation in the expression of CD14 and toll like receptors (TLR2 and TLR4) by 3, 64 and 37 folds respectively (Fig. 5). Such upregulation of the markers was markedly attenuated by OSAE/OSWE (1 mg dwt/mL) and EUG (60 μg/mL) in PMA treated monocytes. Results are able to replicated by IMT (Supplementary Fig. 3F and Fig. 5).

4. Discussion

In recent years, the use of herbal products raised steeply due to increased interest in the developed and developing countries for their use as antioxidants and good health supplements (Mukherjee et al., 2010). In traditional medicine, *Ocimum sanctum* leaves are found to be used for a broad range of health benefits including cardiac health. Commercially available herbal products of *Ocimum sanctum* (e.g. *Tulsi* by Kirpal Export Overseases, India; Dry herbal extracts by Nikita Extracts, India; *Ocimum sanctum*-Beadlet by Borion Canada Inc., Canada) are known majorly for their cardiac health benefits. Eugenol has been extensively investigated while addressing the therapeutic potentials of *Ocimum sanctum*. However, molecular mechanism of action of OS-leaves as well as eugenol in offering cardiac health, specifically inhibiting atherogenesis, is not clearly understood. The present study investigated the antiinflammatory properties of *Ocimum sanctum* dry leaf extracts and its pure compound EUG using human monocytic (THP-1) cells. To the best of our knowledge, this is the first attempt that has been made to understand the cellular and molecular basis of atheroprotective activity of OS-dry leaf extracts using human monocytic (THP-1) cells.

In this study, the effect of *Ocimum sanctum* on the LPS induced secretion of TNF-α and gene expression of cytokines and chemokines like IL-6, TNF-α, MIP-1α and MCP-1 in THP-1 cells was tested because of their significance in inflammation. These cytokines and chemokines are shown to be produced by THP-1 cells up on stimulation with LPS (Weiss et al., 2004; Xue et al., 2006). TNF-α is a potent proinflammatory cytokine, and plays an important role in developing many chronic inflammatory diseases including atherosclerosis and rheumatism (Huo et al., 2012). TNF-α produced by

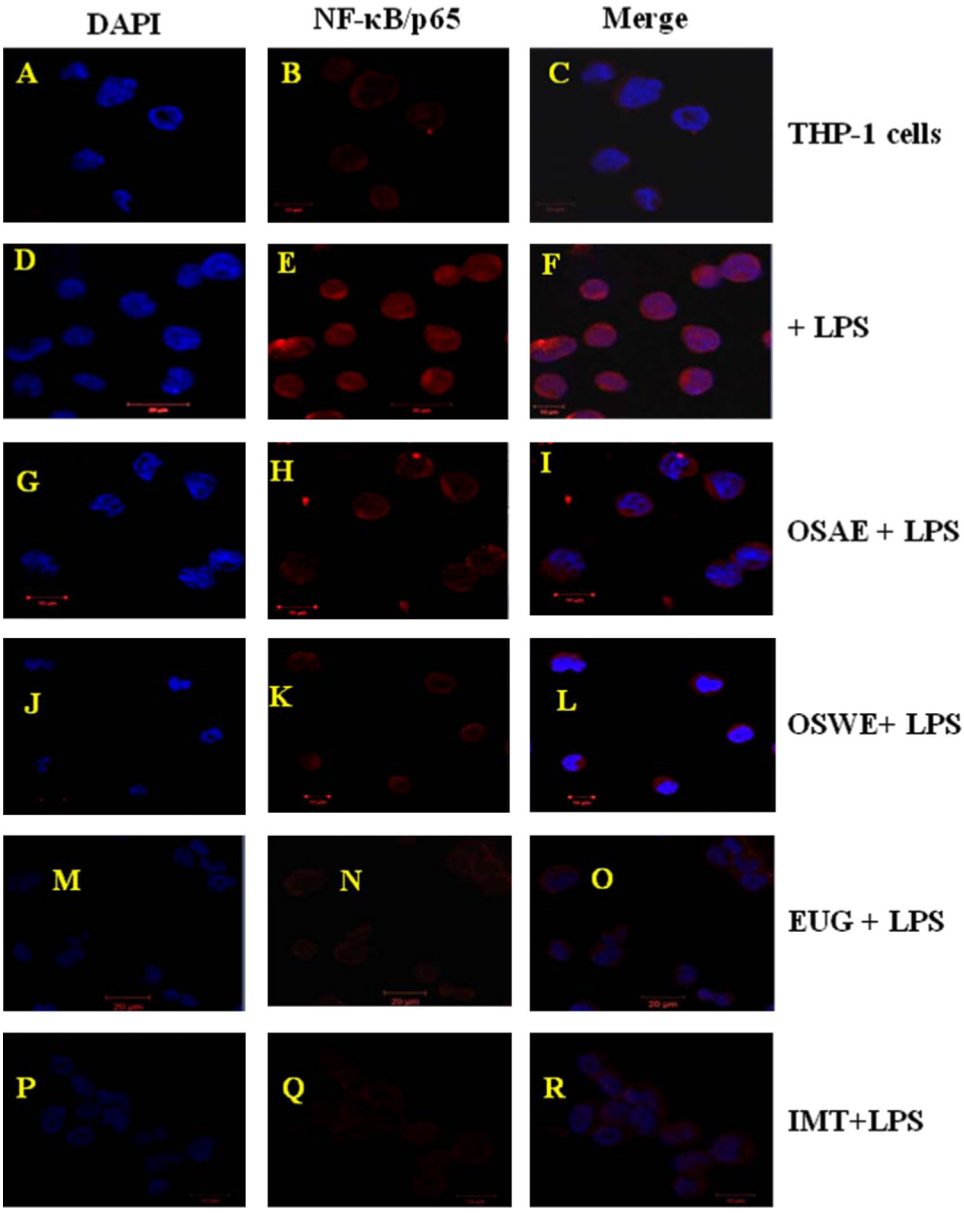

Fig. 4. Confocal microscopic images demonstrating the effect of OS-dry leaf extracts, pure compound EUG and positive control IMT on LPS-induced subcellular localization of NF-κB-p65 in THP-1cells. Images with vehicle control 0.5% (alcohol) cells are shown in panel 1. Vehicle controls of water (< 1%) and DMSO (0.2%) also had no effect (not shown). NF-κB translocation was analyzed by staining with NF-κB-p65, red (A); nucleus with DAPI, blue (B); merged image of NF-κB-p65 and DAPI (C). THP-1 cells treated with LPS (0.5 μg/mL for 3 h) shown in 2nd panel (D–F); cells pretreated with OSAE or OSWE (1 mg dwt/mL) and then treated with LPS shown in 3rd (G–I) and 4th panels (J–L). Panel 5 (M–O) and panel 6 (P–R) represent cells preincubated with EUG (60 μg/mL) and IMT (20 μg/mL) respectively, then challenged with LPS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

monocytes and macrophages (Mackenzie et al., 2002) activates other cell types like endothelial cells. Endothelial cells up on activation produce cell surface adhesion molecules and selectins, facilitating rolling, adherence and diapedsis of monocytes into subendothelial space where they differentiate into macrophages (Postea et al., 2006). IL-6 is a proatherogenic cytokine and is associated with fatty lesion development in aorta (Kishikawa et al., 1993). MIP-1α and MCP-1 belong to chemokine family and are also associated with atherosclerotic lesion development. High levels of MIP-1α along with other chemokines like MIP-1β, RANTES and cytokines IFN-γ, IL-2, IL-4, and IL6 are found in blood plasma of atherosclerotic patients (Cagnin et al., 2009). MCP-1 is established as one of the important chemokines involved in the development of atherosclerosis, specifically by playing a role in the monocyte recruitment and infiltration into the arterial wall (Reape and Groot, 1999; Takahashi et al., 1995). The present study showed inhibitory effect of *Ocimum sanctum* dry leaf extracts on LPS induced production of TNF-α and expression of some of the above mentioned inflammatory markers, IL6, MIP-1α and MCP-1. LPS activates expression of these markers via NF-κB pathway (Ci et al., 2010). In our study, *Ocimum sanctum* leaf extracts showed significant inhibition of nuclear translocation of NF-κB in LPS stimulated THP-1 cells. These results suggest that the plant extracts may have the potential to inhibit several other genes regulated by this pathway. In addition to the effects on LPS induced cytokine and chemokine expression, the extracts inhibited the expression of PMA induced cell differentiation markers CD14, TLR2 and TLR4 as well as morphological characteristics of macrophages. All these three markers are strongly associated with inflammation and atherosclerosis plaque development (Schlitt et al., 2004). All the inhibitory effects showed by plant extracts are comparable to the positive drug control IMT.

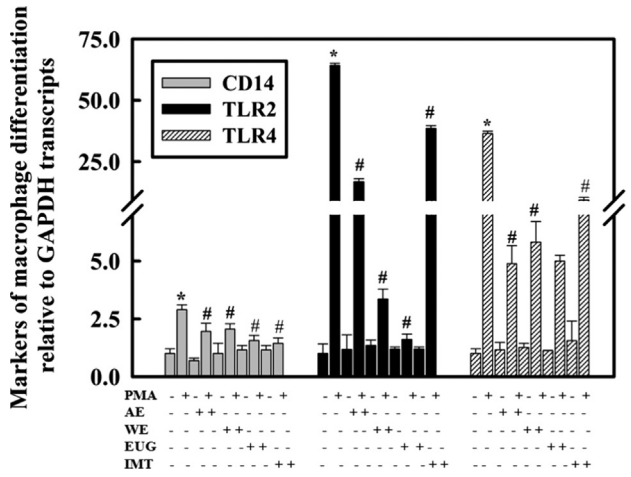

Fig. 5. Effect of OS-dry leaf extracts, pure compound EUG and IMT on PMA induced CD14, TLR2 and TLR4 mRNA transcripts in THP-1cells. THP-1 cells pretreated for 12 h with or without OSAE (1mg dwt/mL), OSWE (1mg dwt/mL), EUG (60 μg/mL) and IMT (20 μg/mL) and then incubated with PMA (5 ng/mL) for 48 h. After incubations, transcript levels by CD14, TLR2 and TLR4 were determined by RT-PCR as a measure of THP-1 cell differentiation. Vehicle control solvents, alcohol/water (were limited to < 1%) and DMSO (0.2%) had no effect. Data shown are mean value of 3 independent experiments ± S.D. $^{*}p < 0.001$, for comparison between treated (cells + PMA) and untreated groups (cells-PMA). $^{\#}p < 0.001$ compared between cells treated with PMA in the presence of OSAE, OSWE,EUG and IMT vs in their absence.

LC–MS analysis showed the presence of eugenol in both OSAE and OSWE along with many other bioactive compounds like rosmarinic acid, vicenin, orientin, apigenin, α-pinene, carvacrol, stearic acid and linoleic acid (Supplementary Tables 2 and 3). Some of these compounds like linoleic acid and α-pinene are shown for their antiinflammatory property in THP-1 cells (Zhao et al., 2005; Zhou et al., 2004). In the present study, we tested the effect of eugenol in LPS/PMA stimulated THP-1 cells, EUG at 60 μg/mL inhibited the expression of both LPS/PMA induced inflammatory cytokines, chemokines (IL-6, TNF-α, MIP-1α, MCP-1); macrophage differentiated markers (CD14, TLR2 and TLR4) and translocation of NF-κB protein into the nucleus in (THP-1) cells. However, LC–MS based quantification of EUG revealed for its presence in minute quantity in both the plant extracts (12 ng/mg dwt of OSAE and 19 ng/mg dwt of OSWE) suggesting that the antiinflammatory effect of the extracts could be due to synergistic/cumulative activity of several metabolites an addition to EUG present in the extracts. The efficacy of the plant extracts cannot be attributed to EUG alone. Earlier, Mahapatra et al. (2011) showed inhibitory activity of eugenol on NF-κB activation in murine macrophages. The present study suggests that bioactive compounds such as eugenol and others, which can attenuate monocytic activation, a key step in the atherogenesis, can be extracted easily from the leaves of *Ocimum sanctum* using nontoxic solvents like water, and it is commonly used for preparation of herbal extracts in traditional medicine. Therefore, *Ocimum sanctum* can be considered as a rich natural source for isolating drug molecules, which can be of use in treating inflammatory diseases like atherosclerosis, as these plants can be grown widely in tropical and subtropical regions of the world. With respect to traditional use of OS-leaves for treating cardiovascular diseases, the present study raised the question on dosage requirement. The amount of OS extracts (1 mg dwt/mL) required to elicit antiinflammatory in LPS/PMA stimulated THP-1 cells is towards on higher side, though the dosage was per se not cytotoxic to THP-1 cells. Thus the study strongly recommends further evaluation of medicinal use of OS-leaves for treating CVD. Future direction of this study is to test the efficacy of OS-leaves on their antiatherosclerotic property using chronic inflammatory models, e.g. diabetic or apoE-/-mice.

5. Concluding remarks

Critical cellular and biochemical investigation of the present study demonstrated antiinflammatory activity of alcoholic and water extracts of OS-dry leaves: (i) attenuated LPS induced proinflammatory gene expression in THP-1 cells by inhibiting nuclear translocation of NF-κB; (ii) inhibited PMA induced monocyte to macrophage differentiation and (iii) had bioactive compounds like phenolics, flavonoids, terpenoids and fatty acids. Pure compound, eugenol (60 μg/mL) a phenylpropanoid showed remarkable antiinflammatory activity in LPS/PMA induced THP-1 cells. LC–MS quantification of EUG from the plant extracts suggested that the efficacy of OSAE and OSWE is due to their richness with several bioactive compounds along with EUG. Thus the study supports the traditional use of OS-dry leaves for treating cardiovascular diseases, with leaving questions on dosage requirement for clinical use. It suggests, further testing the extracts, using chronic inflammatory models to make it evidence based on traditional use of *Ocimum sanctum* for treating cardiovascular diseases.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2014.03.049.

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