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Research Article
Anti-Inflammatory Activity of (2E)-7-hydroxy-3,7-dimethyl-2-octenyl-β-D-Glucopyranoside, Isolated from *Sanguisorba officinalis* L. in Toll-Like Receptor 9-Stimulated Immune Cells

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Abstract

Background and Objective: Herbal medicine has wide application in the treatment of inflammatory diseases. (2E)-7-Hydroxy-3,7-dimethyl-2-octenyl-β-D-glucopyranoside (Comp) are isolated from *Sanguisorba officinalis* L. The present study aimed to assess the possible influence of Comp on immune cells. **Materials and Methods:** To evaluate the effect of Comp on cell viability, Dendritic Cells (DCs) and RAW 264.7 cells were treated with different doses of Comp (0-10 μM) and cytotoxicity were measured by MTT. DCs were also treated with different doses of Comp and stimulated with CpG-DNA (1 μM). The concentration of pro-inflammatory cytokines in the culture supernatant was measured by ELISA. The effect of Comp (10 μM) on Mitogen Activated Protein Kinases (MAPKs), nuclear factor kappa B (NF-κB) pathways and iNOS expression were evaluated by western blot. Finally, the effect of Comp on Nitric Oxide (NO) production in TLR9-stimulated RAW 264.7 cells was also measured. **Results:** Comp showed dose-dependent inhibition on interleukin (IL)-12p40, IL-6 and TNF-α production in DCs with IC_{50} values of 0.69±0.07, 7.10±1.6 and 2.47±0.03 μM, respectively. This also significantly suppressed the activation of MAPKs and NF-κB pathways. Moreover, pre-treatment with Comp exhibited dose-responsive inhibition of NO production and iNOS expression in RAW 264.7 cells. **Conclusion:** In brief, these results demonstrate that Comp has considerable anti-inflammatory properties.

Key words: CpG-DNA, cytokines, mitogen-activated protein kinases, nuclear factor kappa B, anti-inflammatory

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The immune system is the primary protective mechanism of all living organisms against external pathogens and stimuli that exist in the environment. An appropriate immune response is key for the healthy living of an organism. There is two subdivision of the immune response in all vertebrate named innate and adaptive immune response. The innate immune response is evolutionary the most primitive protection system of plants and animals. DCs are major immune cells of the mammalian and works as a key antigen-presenting cells. The innate immune response depends on a few reserved receptors of host immune cells known as Pathogen Recognition Receptors (PRRs). Those receptors can interact with different molecules derived from several micro-organisms and damaged tissue, which is unlike that of the host[1]. Toll-like Receptors (TLRs) are an example of such PRRs that is a key mediator of inflammation and immune response. TLRs can identify the invading foreign pathogen by sensing the presence of conserved Pathogen-associated Molecular Patterns (PAMPs) of a wide range. Activation of TLRs (e.g., TLR9) by microbial product (e.g., unmethylated CpG of microbial DNA) initiates activation of downstream signalling pathway i.e., MAPK and NF-κB which induces production of pro-inflammatory cytokine[2].

MAPKs are protein kinases with a crucial role in cell proliferation, migration, survival and death[3]. MAPKs consist of three different kinases, which include extracellular signal-regulated kinase (ERK), the p38 family of kinases (p38) and the c-Jun N-terminal kinases (JNKs)[4]. Activation of ERK leads to phosphorylation of various structural and functional proteins, including activator protein-1 (AP-1)[5]. JNKs has involved in stress signalling pathways[6] and p38 is crucial for cellular differentiation, death, autophagy and inflammatory response[7]. The nuclear factor kappa B (NF-κB) protein family consists of five members that form dimers (homo or hetero) and regulate the expression of the pro-inflammatory cytokines gene. NF-κB pathway activation is regulated by the I κB family of inhibitor proteins[8]. Therefore, NF-κB activation can be indirectly measured by the phosphorylation and subsequent degradation of IκB.

Nitric Oxide (NO) is a tiny free radical with a short life span secreted by endothelial and nerve cells. It influences inflammation and acts as a neurotransmitter in nerve junctions as well. NO synthesis by vascular and nervous tissue has been catalyzed by an enzyme named inducible nitric oxide synthase (iNOS). This enzyme potentiates the conversion of L-arginine to citrulline, a crucial step of nitric oxide synthesis[9]. In normal physiological conditions, NO promotes vasodilation, prevents leukocytes and platelet adhesion to endothelium and prevents the exaggerated proliferation of vascular smooth muscle cells. These effects ultimately prohibit inflammation and thrombosis which is crucial for healthy livingorganism[10]. But in pathological conditions, endotoxin and pro-inflammatory mediators of inflammation induce iNOS over expression which leads to enormous production NO. This massive production of NO causes tissue damage and ultimately develops sepsis[10].

Sanguisorba officinalis L. is a herbaceous plant, belongs to the family of Rosaceae and is found in Asia, North America, part of Europe and Africa[11]. Its dried roots used as conventional medicine in Eastern Asia from ancient times because of their analgesic and astringent properties. This herbal medicine has wide application in the treatment of dermatitis, burns, intestinal infections and hemorrhagic condition like hematemesis and melena[12]. As a part of ongoing research, this study evaluated the possible anti-inflammatory activities of (2E)-7-hydroxy-3, 7-dimethyl-2-octenyl-β-D-glucopyranoside (Comp) isolated from *Sanguisorba officinalis* L. in TLR9-stimulated immune cells.

MATERIALS AND METHODS

Study area: This study was conducted from January, 2019 to June, 2020 at the Department of Microbiology and Immunology, College of Medicine, Jeju National University, South Korea.

Chemicals: The following reagents were purchased: antibodies for phospho-ERK1/2, phospho-p38, phospho-JNK1/2, phospho-I κBα, horseradish peroxidase-linked anti-rabbit IgG (Cell Signaling, USA), β-actin (Santa Cruz Biotechnology, USA), CpG-DNA (Genotech, South Korea), dimethyl sulfoxide (DMSO, Amresco, USA), enzyme-linked immunosorbent assay (ELISA) kit (BD PharMingen, USA), fetal bovine serum (FBS, Gibco, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT, Sigma-Aldrich, USA), penicillin-streptomycin (Gibco, USA), PRO-PREP lysis buffer (iNtRON Biotechnology, South Korea), Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Gibco, USA) and western blot detection kit (iNTRON Biotechnology, South Korea).

Preparation of (2E)-7-hydroxy-3,7-dimethyl-2-octenyl-β-D-glucopyranoside: Dried roots of *S. officinalis* (2.4 kg) were collected from Korea Institute of Oriental Medicine and subjected to solvent extraction with 8.0 L of methanol. Then methanol extract was mixed with water followed by partitioned with ethyl acetate and n-butanol to yield

respective fractions. This n-butanol fraction was subjected to silica gel Column Chromatography (CC) with $CHCl_3$-MeOH-H_2O gradient to yield eight fractions. One fraction was segregated via $CHCl_3$-MeOH-H_2O gradients CC to obtained (2E)-7-Hydroxy-3,7-dimethyl-2-octenyl-β-D-glucopyranoside (Comp).

Cell culture and cytokines production measurement: Bone marrow cells obtained from C57BL/6 female mice (6 weeks) were cultured to grow DCs as described previously[13]. Experimental procedures have been authenticated by the Institutional Animal Care and Use Committee (#2019-0045). Briefly, RPMI 1640 medium with 3% GM-CSF and 10% FBS were used to culture DCs. On day 7 of culture, DCs were seeded in a 48-well culture plate (2×10^5 cells mL) and pretreated with Comp of particular concentrations for 1 hr before CpG-DNA stimulation (1 μM). After 18 hrs of incubation, supernatants were collected and pro-inflammatory cytokines concentration was determined with an ELISA kit by following the manufacturer's instruction.

Cell viability analysis: MTT assay was used to observe cell viability in response to Comp as described previously[13]. Briefly, on the 7th day of culture, DCs were seeded as per the above description and then incubated for 18 hrs with particular concentrations of Comp. RAW 264.7 cells were also cultured, seeded (2×10^5 cells mL^{-1}) and then incubated with indicated doses of Comp for 18 hrs. In both experimental procedures, cells were centrifuged and supernatants were removed. 250 μL of DMSO was used to dissolve formazan crystal and microplate absorbance was calculated at 540 nm wavelength. The viability of cells was expressed as the percentage of Comp non-treated viable cells.

Western blot assay: DCs were allocated to 35 mm culture plates at 410^6 cells/plate and incubated with and without Comp (10 μM). After 1 hr of incubation cells were stimulated with CpG at a specific interval of times. Then cells lysis was done with a lysis buffer to extract proteins. Protein samples were segregated by 10% SDS-PAGE gel and were blotted to a PVDF membrane. The membrane was treated with specific primary and secondary antibodies and desired protein bands were analyzed by the method described previously[14].

Measurement of nitrite production: RAW 264.7 cells were seeded (2×10^5 cells mL^{-1}) and incubated with indicated doses of Comp for 1 hr. Then cells were stimulated overnight with CpG-DNA and cell supernatants were harvested. Here we assessed nitrite concentration in supernatant which is a stable metabolic end product of NO[15]. Nitrite production is measured with the Griess reagent system in culture supernatants by following the manufacturer's instructions. Microplate absorbance was calculated at 540 nm wavelength.

Statistical analysis: All experimental results are stated as Mean±Standard Deviation (SD) of three independent experiments. One-way ANOVA was used to compare the comp treated group with the control group. Statistically, $p<0.05$ was considered significant.

RESULTS

Effect of Comp on DCs viability: The effect of Comp on the cell viability was evaluated by MTT assay as shown in Fig. 1. Absorbance at 540 nm wavelength was measured by using a microplate reader. Cell viability (%) was expressed as the percentage of Comp non-treated viable cells. At the highest concentration of Comp (10 μM) cell viability decreased to 90.2±2.5%. Therefore, the result revealed that, indicated concentrations of Comp (0.1-10 μM) has little influence (by 5-10% reduction) on the cell viability compared to Comp untreated control (Fig. 1).

Effect of Comp in the production of inflammatory cytokines by DCs stimulated with CpG-DNA: Activation of TLR9

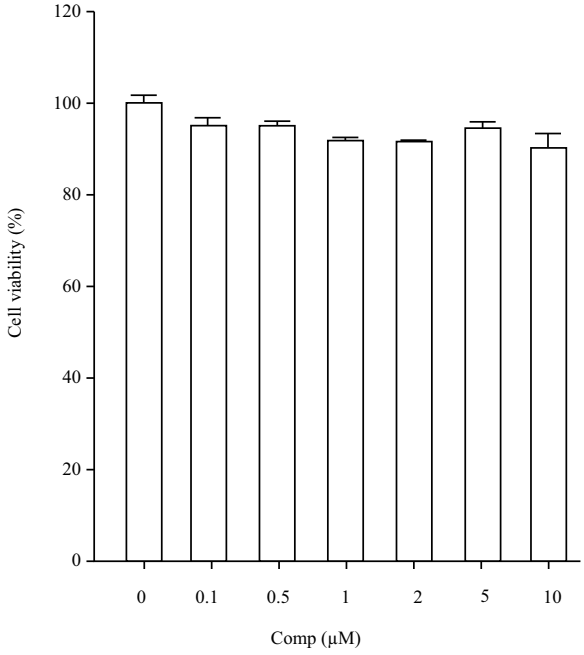

Fig. 1: Effect of Comp on DCs viability
DCs were pretreated with Comp at the indicated doses. The viability of cells was evaluated through MTT assay

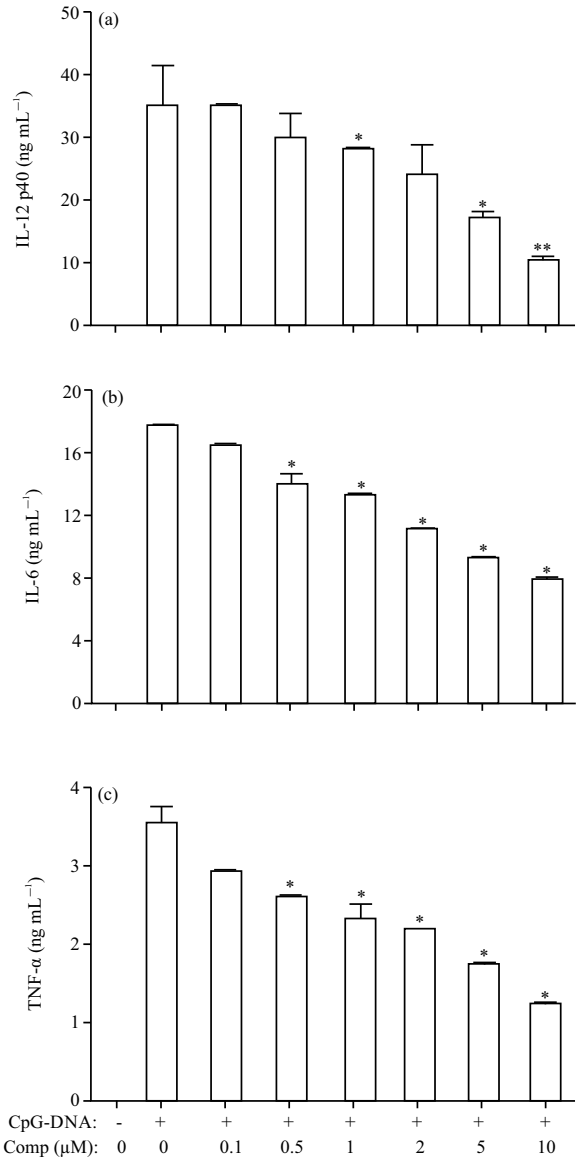

Fig. 2(a-c): Effect of Comp on cytokines production in TLR9-stimulated DCs level were measured by ELISA, (a) IL-12p40, (b) IL-6 and (c) TNF-α

DCs were pretreated with different concentrations of Comp and then stimulated with CpG-DNA, *p<0.05, **p<0.01 vs. Comp untreated and CpG-stimulated DCs

signalling by CpG-DNA in DCs can result in IL 12 p40, IL 6 and TNF-α production and those cytokines have a significant role in inflammation. The level of cytokine production (ng mL^{-1}) in CpG-DNA stimulated DCs was evaluated by ELISA as shown in Fig. 2. This study evaluated the suppressive effects of Comp on cytokines production in TLR9-stimulated DCs. Without the stimulation of CpG-DNA concentration of IL-12p40, IL-6 and TNF-α in cell culture medium were 0.11±0.01, 0.20±0.01 and 0.05±0.001 ng mL^{-1}, respectively in Fig. 2(a-c). TLR9 stimulation by CpG-DNA causes more than 100-fold rise of above all three cytokines levels in DCs (IL-12p40, 35.1±3.5, IL-6, 18.2±0.2 and TNF-α, 3.80±0.01 ng mL^{-1}, respectively). Comp showed dose-dependent inhibition on IL-12p40, IL-6 and TNF-α production in DCs with IC_{50} values of 0.69±0.07 ($p<0.01$, Fig. 2a), 7.10±1.6 ($p<0.05$, Fig. 2b) and 2.47±0.03 μM ($p<0.05$, Fig. 2c), respectively. At the highest concentration of Comp (10 μM) IL-12p40, IL-6 and TNF-α production decreased to11.1±1.5, 8.0±0.1 and 1.3±0.001 ng mL^{-1}, respectively. Therefore, Comp pretreated DCs along with CpG-stimulation showed significant inhibition of pro-inflammatory cytokine production with dose-response (0.5-10 μM) (Fig. 2).

Effect of Comp on MAPKs phosphorylation in TLR9-stimulated DCs: Activation of TLR9 upon binding with its ligand CpG containing DNA triggers some downstream signalling pathways (NF-κB and MAPKs) and ultimately produces cytokines and chemokines like mediators of inflammation. This study examined the effect of Comp on phosphorylation of MAPKs (ERK1/2, JNK1/2 p38) in TLR9-stimulated DCs through western blot analysis in Fig. 3a and scanning densitometry in Fig. 3b-d for relative phosphorylation as shown in Fig. 3. CpG-DNA stimulation of TLR9 in DCs phosphorylates all MAPKs between 15-60 min (Fig. 3a). Specifically, CpG-DNA stimulation induces phosphorylation of ERK1/2, JNK1/2, p38 (relative phosphorylation, 1.80±0.01, 4.81±0.02 and 1.60±0.1 in Fig. 3b-d), respectively at 30 min. However, Comp treatment reduced phosphorylation of ERK1/2, JNK1/2, p38 (relative phosphorylation, 0.41±0.02, 0.52±0.001 and 0.80±0.01), respectively at 30 min. In other words, Comp pre-treated cells stimulated with CpG inhibited phosphorylation of MAPKs (p-ERK1/2, Fig. 3b, p-JNK1/2, Fig. 3c, p-p38, Fig. 3d) by 60-90% compared to Comp untreated control ($p<0.05$, Fig. 3b-d).

Effect of Comp on phosphorylation and degradation of IκBα in TLR9-stimulated DCs: NF-κB is a protein complex, which acts as a key transcription factor for the production of several inflammatory cytokines by immune cells. Activation of NF-κB depends on phosphorylation and degradation of IκBα, which is an inhibitor of NF-κB. Upon stimulation of TLR9 by CpG-DNA, IκBα gets phosphorylated and degraded, NF-κB moved into the nucleus and induces production of inflammatory cytokines. The current study examined the effect of Comp on the phosphorylation and degradation of IκBα in TLR9-stimulated DCs through western blot analysis in Fig. 4a

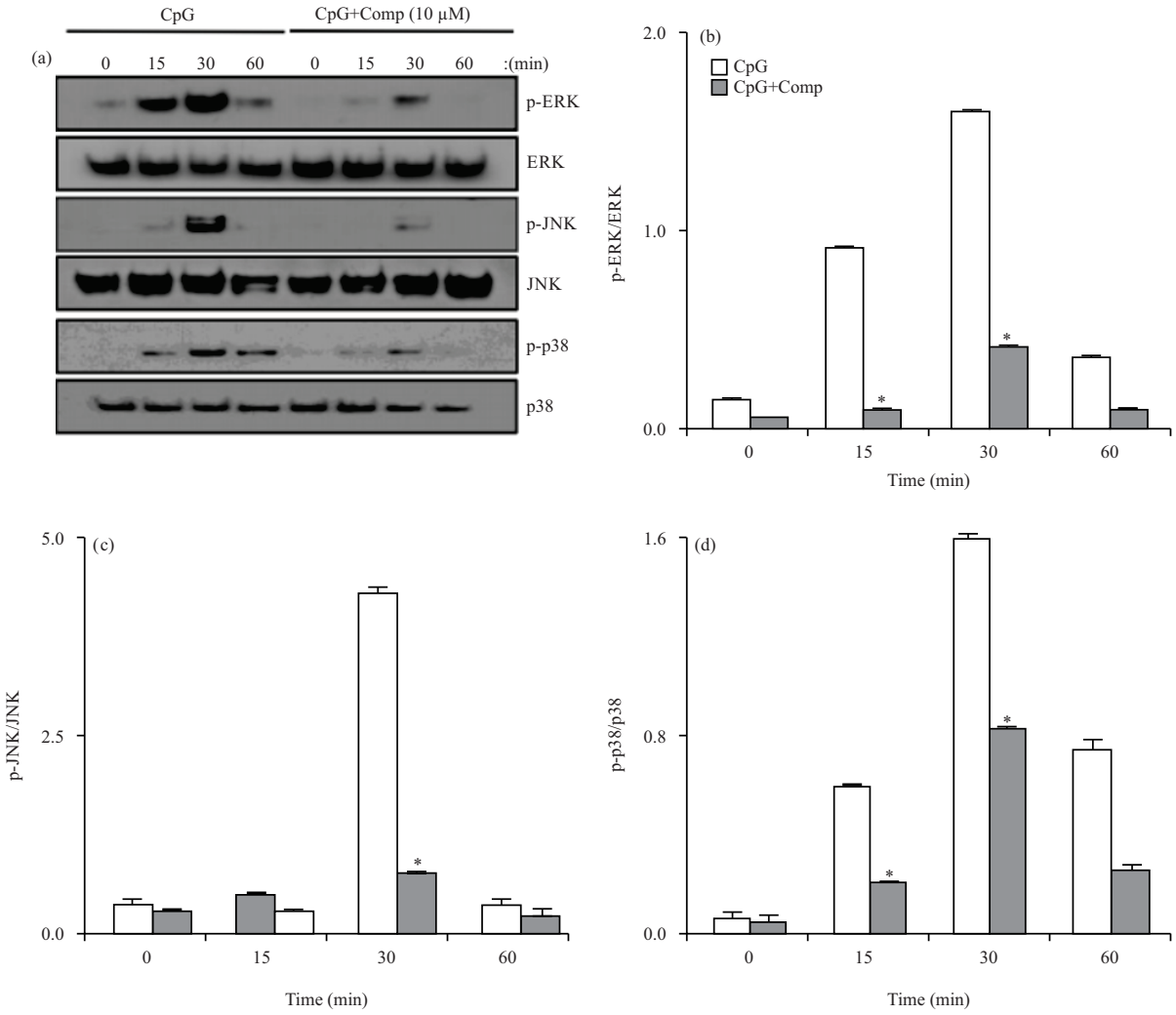

Fig. 3(a-d): Effect of Comp on MAPKs phosphorylation in TLR9-stimulated Dcs, (a) Western blot analysis of TLR9 with CpG-DNA, (b) Phosphorylation of Protein samples ERK1/2, at definite time differences, (c) Phosphorylation of Protein samples JNK1/2, at definite time differences and (d) Phosphorylation of Protein samples p38 at definite time differences

Western blot was done to observe MAPKs phosphorylation. Protein quantification was performed by scanning densitometry, *p<0.05 vs. Comp untreated and CpG-stimulated DCs

and scanning densitometry in Fig. 4b and c for relative phosphorylation and production of IκBα as shown in Fig. 4. It was observed that CpG-DNA can induce phosphorylation of IκBα (relative phosphorylation, 1.21±0.01 at 15 min, Fig. 4b) and Comp pretreatment can inhibit IκBα phosphorylation by 70% (relative phosphorylation, 0.32±0.02 at 15 min, Fig. 4b) compared to Compuntreated control (Fig. 4a, $p<0.05$, Fig. 4b). Also, CpG treatment resulted in degradation of IκBα (relative production, 0.08±0.01, Fig. 4c black bar) within 30 min of stimulation but Comp pretreatment inhibited its degradation by 80% (relative production, 0.42±0.01 at 30 min, Fig. 4c white bar) compared to Comp untreated control ($p<0.05$, Fig. 4c). So, these data indicate that Comp can inhibit IκBα phosphorylation and its degradation in TLR9-stimulated DCs (Fig. 4a-c).

Effect of Comp on RAW 264.7 cells viability and NO production: Here, MTT assay was done to evaluate the influence of Comp on the viability of murine macrophage-like RAW 264.7 cells as shown in Fig. 5a. Cell viability (%) was expressed as the percentage of Comp non-treated viable cells. At the highest concentration of Comp (10 μM) cell viability decreased to 90.2±1.5%. Therefore, there is no or little effect (by 0-15% reduction) on RAW 264.7 cell viability on indicated

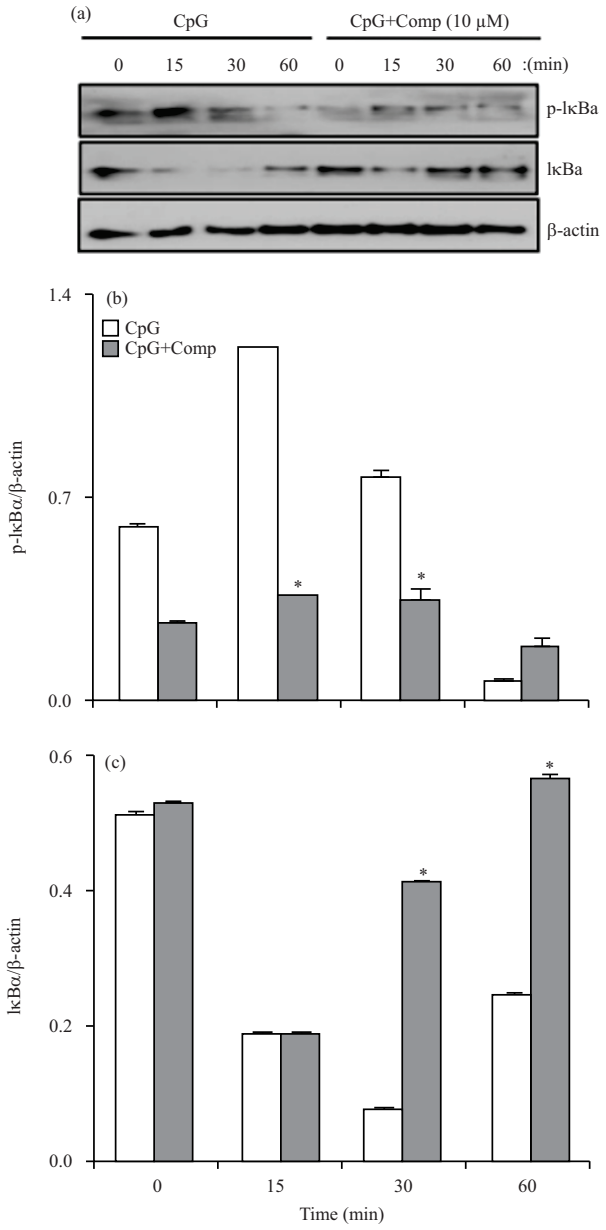

Fig. 4(a-c): Effect of Comp on the phosphorylation and degradation of lκBα in TLR9-stimulated Dcs, (a) Western blot analysis of TLR9 with CpG-DNA, (b) Phosphorylation of lκBα and β-actin and (c) Degradation of lκBa and β-actin

Protein samples were collected for specific time differences. Western blot was done to detect phosphorylation and degradation of I?Ba and ß-actin considered as a control. Scanning densitometry was used for the quantification of protein. *p<0.05 vs. Comp untreated and CpG-stimulated DCs

doses (0.1-10 μM) compared to Comp untreated control (Fig. 5a). Also, the effect of Comp treatment on NO production in TLR9-stimulated cells was assessed as shown in Fig. 5b.

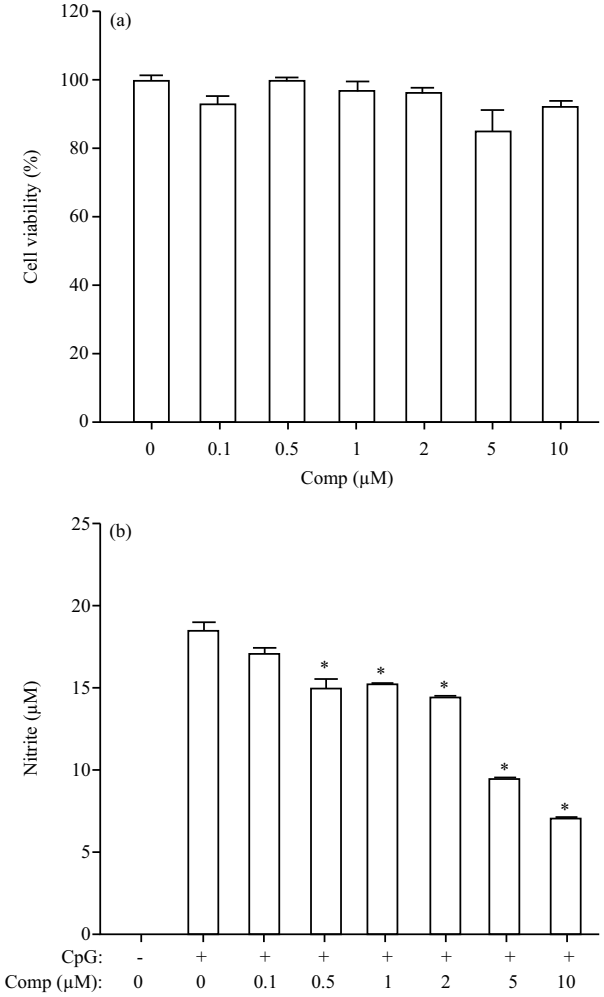

Fig. 5(a-b): Effect of Comp on the viability and nitrite production in RAW 264.7 cells, (a) Effect of Comp on the viability in RAW 264.7 cells and (b) Effect of Comp on the nitrite production in RAW 264.7 cells

Cells were pretreated with Comp at different concentrations for 18 hrs and cell viability was evaluated by MTT assay, nitrite concentration in the supernatants determined with a Griess reagent system, Comp (0-10 μM) pretreated cells were stimulated with CpG-DNA for 18 hrs, *p<0.05 vs. Comp untreated and CpG-stimulated cells

Nitrite production was measured with the Griess reagent system in culture supernatants as shown in Fig. 5b. Without the stimulation of CpG-DNA concentration of nitrite in the cell culture medium was 0.1±0.002. With CpG-DNA stimulation level of nitrite was 18.1±0.5 μM. Therefore, TLR9 stimulation in RAW 264.7 cells with CpG-DNA showed a 180-fold rise in nitrite production, which is a stable metabolite of NO (Fig. 5b). However, Comp pre-treatment with CpG stimulation showed inhibition of nitrite production

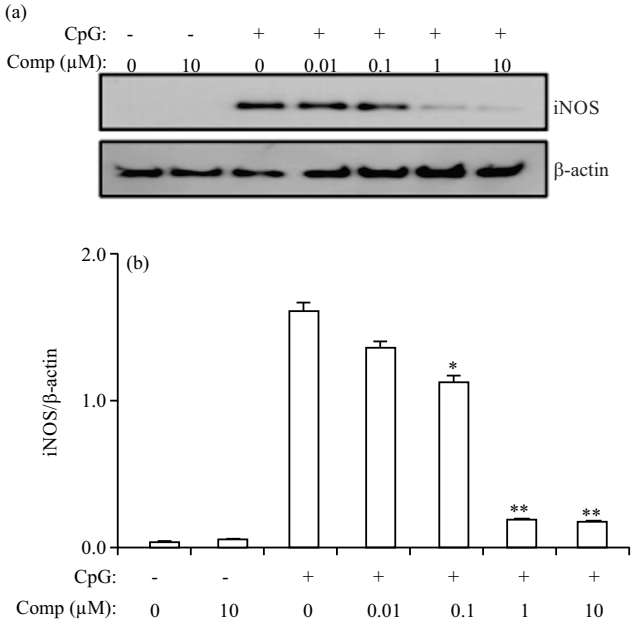

Fig. 6(a-b): (a) Western blot analysis and (b) Effect of Comp on iNOS expression in TLR9-induced RAW 264.7 cells

(Comp (0-10 μM) pretreated RAW 264.7 cells were stimulated with CpG-DNA and cell lysate was collected. Then iNOS expression was detected by western blot and β-actin was considered as a control. Scanning densitometry was utilized for the quantification of protein. *p<0.05, **p<0.01 vs. Comp untreated and CpG-stimulated cells

in a dose-dependent manner (0.5-10 μM) ($p<0.05$, Fig. 5b). Specifically, Comp at 10 μM strongly inhibited nitrite production (7.1±0.1 μM).

Effect of comp on iNOS expression in CpG-induced RAW 264.7 cell: NO synthesis from its precursor L-arginine is regulated by the iNOS enzyme. This enzyme expression by immune cells depends on exposure to microbial products as well as inflammatory cytokines. This study evaluates the influence of Comp on iNOS expression by treating RAW 264.7 cells with particular concentrations of Comp before stimulation with CpG-DNA via western blotting analysis in Fig. 6a and scanning densitometry in Fig. 6b for relative production of iNOS as shown in Fig. 6. iNOS protein expression was measured by western blot (Fig. 6a). The relative level of iNOS was 1.81±0.01 in CpG-DNA stimulated RAW 264.7 cells but that reduced to 0.21±0.01 in Comp (10 μM) treated RAW 264.7 cells as shown in Fig. 6b. Therefore, results showed that Comp (1-10 μM) inhibits iNOS expression by 90% in RAW 264.7 cells, compared to comp untreated control ($p<0.01$, Fig. 6b).

DISCUSSION

In the pathogenesis of several human diseases (e.g., rheumatoid arthritis, bronchial asthma and psoriasis) inflammation plays a critical role. So, inflammation control is the key to avoid or limit such diseases progression[16,17]. Compound with anti-inflammatory properties can be used as a therapeutic agent to alleviate chronic inflammation. Here, an investigation was done to assess the anti-inflammatory potency of Comp, obtained from *Sanguisorba officinalis* L. There are not many detailed studies had done yet to evaluate the effect of Comp on inflammation control and its underlying mechanism.

IL-12, IL-6 and TNF-α production by activated DCs plays a pivotal role in host immune response. IL-12 is a unique heterodimeric cytokine originated from activated dendritic cells, B lymphocytes and macrophages[18]. IL-12 differentiates naïve T cells into $CD4^+$ helper T cells and potentiate the production of INF-γ from T lymphocytes and NK cells, which promote inflammation and Th1 cells mediate autoimmunity[19]. Therefore, suppression of IL-12 production is crucial for controlling inflammation and autoimmune diseases. Here, Comp pretreatment has shown significant suppression of IL-12 overproduction in CpG triggered DCs. IL-6 plays a crucial role in inflammation, hematopoiesis, cell growth and differentiation. In inflammation, IL-6 potentiates the differentiation of naïve $CD4^+$ T cells into helper T (Th17) cells with help of TGF-β[20,21]. It also influences $CD8^+$ T cells transformation into cytotoxic T cells and inhibits TGF-β mediated T_{reg} proliferation[21]. Therefore, IL-6 is crucial for inflammatory response and suppression of IL-6 production has significant value for inflammation control. TNF-α is a key inflammatory cytokine and is over expressed in acute and chronic inflammatory conditions like infection, trauma and rheumatoid arthritis[22]. So, the regulated production of TNF-α is crucial to improve inflammatory conditions. Here pretreatment with Comp significantly inhibit the expression of IL-12p40, IL-6 and TNF-α in CpG stimulated DCs, which demands further research regarding its potential use for the management of inflammatory diseases.

TLR9 stimulation by CpG DNA in immune cells results in activation of downstream MAPKs and NF-κB pathways and modulates pro-inflammatory cytokines gene expression[23]. Pretreatment with Comp inhibits MAPKs phosphorylation in TLR9-stimulated DCs, thereby suppress inflammatory cytokines gene expression. Comp pretreatment also inhibits IκBα phosphorylation and degradation as a result, inhibited activation of the NF-κB signalling pathway.

NO is a reactive oxygen molecule that plays a crucial role in inflammation, immune defences and acts as a neurotransmitter. In normal physiological conditions, basal NO production prevents inflammation by inhibiting cytokines and chemokine production, leukocytes adhesion and their transmigrations[24]. But during inflammatory reaction NO production increases by 1000 times by immune cells. This high concentration of NO is potentially toxic, pro-inflammatory and causes massive tissue damage[9]. NO production by immune cells from amino acid L-arginine catalyzed by enzyme iNOS[15]. Therefore, controlled production of NO and iNOS expression by immune cells is essential to check the inflammatory reaction. Here Comp inhibits NO production and also iNOS protein expression in TLR9-stimulated RAW264.7 cells, indicating its significant influence on inflammation control.

CONCLUSION

In conclusion, the present data indicate that Comp has a suppressive effect on cytokines production, which may be associated with blockage of MAPKs and NF-κB pathways activation. Also, it can down regulate inflammatory mediator nitric oxide production and iNOS expression in TLR9-stimulated immune cells. So, a detailed study regarding its mode of action and in vivo effect is required to prove its efficacy for the treatment of inflammatory diseases.

SIGNIFICANCE STATEMENT

This study discovered the anti-inflammatory activity of Comp via suppressing MAPK and NF-κB pathways that can be beneficial for the treatment of different inflammatory diseases. This study will help researchers to uncover critical areas and identify the importance of herbal Comp that could be used as a therapeutic agent for inflammation. However, further *in vivo* work needs to be validated.

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