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Short communication

Antioxidant and anti-inflammatory properties of herbal medicine compound (KodiPur®) using *in vitro* assay

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Abstract

Kodipur®, a herbal medicine compound, is composed of five pharmaceutical herbs and has been used for treating common cold symptoms as well as modulating the immune function. However, the underlying mechanism has not been scientifically identified. The present study determined the efficacy of Kodipur® on antioxidant and anti-inflammatory properties using *in vitro* assay. Antioxidant activity was evaluated using oxygen radical absorbance capacity assay and 2,2-diphenylpicrylhydrazyl radical scavenging (DPPH) assay. Kodipur® had a radical absorbance capacity of 5,205.83 ± 0.04 μmol/g of sample dry weight with a half maximum concentration (IC_{50}) of 10.22 mg/mL and a % DPPH inhibition of 46.39 ± 0.2 with an IC_{50} of 10.29 mg/mL. Anti-inflammatory activity was determined using lipopolysaccharide (LPS)-activated mouse macrophage RAW264.7 cells. Pre-treatment with the herbal medicine formula at concentrations of 200–400 μg/mL for 1 hr prior to co-incubation with 5 ng/mL LPS for a further 24 hr, significantly inhibited the production of nitric oxide, interleukine-6, tumor necrosis factor-alpha and prostaglandin E2 in a dose-dependent manner as well as reducing reactive oxygen species.

These findings suggested that the herbal medicine compound has an efficacy to modulate antioxidant as well as anti-inflammatory properties. However, its potential use in animals or humans should be studied further.

Introduction

Inflammation is a physiological process to protect a host against either external or internal stimuli and macrophage is a dominant cellular agent which plays a role against viruses or bacterial infection, tissue injury or cell damage during inflammation (Medzhitov, 2008). Macrophage is activated by a lipopolysaccharide (LPS), the outer membrane of Gram-negative bacteria, to generate various pro-inflammatory mediators including nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), interleukine-6 (IL-6) and prostaglandin E2 (PGE_2) through NF-κB transcription factors, as well as enhancing the ability to phagocytose and kill the ingested microbes (Krishnamoorthy and Honn, 2006). However, if the protective inflammatory response is deregulated, macrophages will persistently accumulate and produce an excessive amount of several pro-inflammatory mediators leading to a systemic inflammatory response that has been associated with the onset of chronic inflammatory diseases and increased risk of morbidity and mortality (Krishnamoorthy and Honn, 2006).

LPS-stimulated macrophage induces NF-κB activation and phosphorylation of mitogen-activated protein kinases (MAPKs), resulting in the production of reactive oxygen species (ROS) such as $O_2^{\bullet-}$, H_2O_2 and •OH via activation of nicotinamide adenine dinucleotide

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phosphate (NADPH) oxidase, the enzyme of respiratory burst; however, without optimal antioxidant protection, cells and tissue surrounding the affected site would be exposed to excessive levels of ROS leading to damaged membrane, protein, and DNA which may contribute to the development of pathological conditions and diseases such as cancer, neurodegenerative diseases and diabetes (Guha and Mackman, 2001; Fang et al., 2002; Swindle and Metcalfe, 2007). Therefore, inhibiting pro-inflammatory mediators during a chronic state might be an effective approach to control inflammatory and severe subsequent complications.

A traditional herbal compound is an alternative medicine that has long been used for the treatment of various conditions and diseases in Asian countries for centuries (Huang, 1999). Each compound consists of different components and amounts of medicinal herbs specific to the symptoms or type of disease. Kodipur®, a herbal medicinal compound, is composed of five oriental medicinal herbs: *Aloe barbadensis* Miller, *Morus alba* L., *Gynostemma pentaphyllum, Ganoderma lucidum* and *Glycyrrhiza glabra* L. and it has been used specifically as a traditional medicine for the treatment of common cold symptoms as well as immune-modulation and a number of studies have investigated the various health properties of individual herbs such as anti-virus, anti-inflammation, anti-bacterial, and anti-cancer (Huang et al., 2013; Suarez-Arroyo et al., 2013; Yang et al., 2013; Chen and Zhang., 2014; Radha and Laxmipriya, 2015). However, there is still lack of scientific data regarding herbs in a compound formulation. Therefore, this study aimed to clarify the underlying properties of Kodipur® on antioxidation and anti-inflammation using *in vitro* assay.

Materials and Methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin, *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) and lipopolysaccharide (LPS) (*E. coli* O11:B4) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH (Haidmannweg, Austria). Earle's minimal essential medium, penicillin, gentamycin and fungizone were purchased from GIBCO® (New York, NY, USA). L-glutamine and heat-inactivated fetal bovine serum (FBS) were purchased from Hyclone® (South Logan, UT, USA) and the WST-1 proliferation assay was purchased from Roche (Mannheim, Germany). TNF-α, IL-6 and the PGE_2 ELISA kit were purchased from Abcam (Cambridge, MA, USA). All chemical reagents were analytical grade. Kodipur® was prepared and supplied by Herb Plus Co., Ltd (Bangkok, Thailand).

Determination of phenolic contents using high performance liquid chromatography

The phenolic contents in Kodipur® were analyzed using high performance liquid chromatography (HPLC) with an Agilent Technologies 1100 series instrument coupled with a photodiode array detector (Agilent Technologies; Santa Clara, CA, USA.), with a Zorbax Eclipase XDB-C18 column (4.6 mm × 150 mm, 5 µm beads; Agilent Technologies; Santa Clara, CA, USA) protected with a cartridge guard column. The HPLC conditions were performed as previously described (Tuntipopipat et al., 2011). In brief, Kodipur® powder was dissolved in 2 mL of 62.5% methanol: 6M HCl (4:1) and shaken in a water bath at 70°C for 2 hr. The column temperature was controlled at 30°C. Phenolic compounds were eluted using a gradient program with 100% water containing 0.5% (weight per weight; w/w) trifluoroacetic acid (TFA; solvent A), 100% methanol containing 0.5% (w/w) TFA (solvent B) and 100% acetonitrile containing 0.5% (w/w) TFA (solvent C). Phenolic compounds were identified and quantified using comparisons of retention time, spectral profile and peak area with pure phenolic standards of: gallic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanilic acid, caffeic acid, syringic acid, p-courmaric acid, ferulic acid, sinapic acid, myricetin, quercetin, t-cinnamic acid, luteolin, naringenin, hesperitin, kaempferol, apigenin and isorhamnetin.

Determination of free radical scavenging activity.

The free radical scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined according to Barros et al. (2007). An amount of 100 mg of herbal medicine compound was dilated with 5 mL of 10% dimethyl sulfoxide. Then, 0.1 mL of diluted sample was added to 0.9 mL of 0.1 mM methanol DPPH solution. The reaction mixture was mixed and incubated at room temperature for 30 min. The absorbance was recorded at 515 nm using a microplate reader spectrophotometer and compared with the control consisting of 0.1 mL of 95% methanol and 0.9 mL of 0.1 mM DPPH solution. The data were expressed as the inhibition percentage of DPPH scavenging activity which was calculated using Equation. 1:

$$\% \text{ Inhibition of DPPH} = (\text{Abs control} - \text{Abs sample} / \text{Abs control}) \times 100 \quad (1)$$

where Abs is the absorbance measured at 515 nm.

Determination of radical absorbance capacity assay

The oxygen radical absorbance capacity (ORAC) assay was performed according to Huang et al. (2002). Fluorescence conditions were set with excitation at 485 nm and emission at 520 nm. The area under the curve was calculated following Wu et al. (2004). Data were expressed as micromoles of Trolox equivalent (TE) per gram of sample dry weight. The ORAC value was calculated by summing the H-ORAC and L-ORAC results.

Cell culture and treatment

RAW 264.7 murine macrophage cells were purchased from the American Type Culture Collection (Bethesda, MD, USA). Cells were cultured in DMEM, with 10% FBS, 15 mM HEPES, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere (95% air, 5% CO_2) and subcultured every 3 d and used for experiment between passages 12 and 25. In the experiment, RAW 264.7 cells were seeded at 0.75×10^6 cells/mL

in a 12-well plate for 24 hr before treating with the Kodipur® at concentrations of 100 µg/mL, 200 µg/mL or 400 µg/mL for 1 hr prior to inducing with 5 ng/mL LPS for a further 24 hr.

Cytotoxicity

The viability of treated cells was determined using a WST-1 proliferation assay kit (Roche; Mannheim, Germany). In the experiment, Kodipur® was diluted with serum-free medium to the desired concentrations at 100–400 µg/mL and passed through a 0.2 µm membrane sterile filter prior to addition into cell culture. After 1 hr of treatment, cells were induced using 5 ng/mL LPS for a further 24 hr. Then, the WST-1 reagents were added and incubation proceeded at 37°C for 1 hr before measuring the absorbance at 420 nm using a microplate reader, where the value of the control (non-treated) was defined as 100% viability.

Nitric oxide production

Production of nitric oxide was determined by quantification of its stable product as the nitrite concentration in culture medium by Griess reaction (Green et al., 1982). In brief, 100 µL of culture medium were collected to determine the nitrite concentration by adding 0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulfanilamide in 5% concentrated phosphoric acid. Absorbance at 530 nm was measured within 30 min and the nitrite concentration was calculated by comparison with a sodium nitrite standard.

Prostaglandin E2 production

Prostraglandin E2 production in culture medium was determined using a PGE_2 ELISA kit (ab133021; Abcam; Cambridge, MA, USA) following the manufacturer's instructions. In brief, culture medium, PGE_2 standard and PGE_2 alkaline phosphatase conjugate were added in a 96-well goat, anti-mouse, IgG-coated plate and incubated at room temperature on a plate shaker for 2 hr. Wells were washed with provided washing buffer and then PNPP substrate solution was added for 45 min. The intensity of the color production was measured at an absorbance of 405 nm. The amount of PGE_2 was calculated by comparison with standard curves.

IL-6 and TNF-α secretion

IL-6 and TNF-α levels in cell culture medium were measured using the IL-6 and TNF-α mouse ELISA kits (ab46100 and ab46105, respectively; Abcam; Cambridge, MA, USA) following the manufacturer's instructions. Antibody specific for mouse TNF-α or IL-6 alpha were coated on a 96-well plate. Standards and culture medium were added into the appropriate wells and bound to the wells by the immobilized antibody. Wells were washed three times and biotinylated anti-Mouse TNF-α or IL-6 alpha antibody was added. After washing three times, unbound biotinylated antibody, HRP-conjugated streptavidin, was pipetted into the wells. The wells were washed three times and TMB substrate solution was added to induce a colored reaction product. Absorbance was measured at 450 nm and the intensity was directly proportional to the concentration of TNF-α and IL-6 present in the culture medium which was calculated by comparison with TNF-α and IL-6 standards.

Intracellular reactive oxygen species generation

Intracellular ROS generation was determined by monitoring the fluorescent signal generated from oxidized 2′,7′-dichlorofluorescein diacetate (DCFH-DA) according to Wolfe and Liu (2007). In brief, RAW 264.7 cells were seeded at 0.5×10^5 cells/mL in a 96-black-well plate for 24 hr before treating with the herbal medicine formula at concentrations of 100 µg/mL, 200 µg/mL or 400 µg/mL for 1 hr prior to inducing with 5 ng/mL LPS for a further 24 hr. Cells were washed with warm basal medium and incubated with 5 µM DCFH-DA at 37°C for a further 30 min. The fluorescence signal in the intracellular layer was measured using a microplate reader with excitation at 485 nm and emission at 530 nm.

Statistical analysis

Statistical analysis was performed using the SPSS software (version 19; IBM Corp.; Armonk, NY, USA). Data were presented as mean ± SD from at least three separate experiments conducted on different days. Statistical differences were determined using one-way analysis of variance followed by Duncan's multiple range test for multiple comparison of group means. Significance was set at $p< 0.05$.

Results

Phenolic content and antioxidant activity

The Kodipur® powder contained 752.07 ± 12.23 µg/g caffeic acid, 329.5 ± 9.8 µg/g chlorogenic acid and 91.04 ± 1.30 µg/g vanillic acid (all as dry weight). Antioxidant activity was evaluated using ORAC and DPPH assays. Kodipur® showed radical absorbance capacity at 5,205.83 ± 0.04 µmol TE/g dry weight with a half maximum concentration (IC_{50}) at 10.22 mg/mL and % DPPH inhibition at 46.39 ± 0.2 with IC_{50} at 10.29 mg/mL.

Kodipur® inhibition of lipopolysaccharide-induced NO production

Exposure of macrophages to LPS activated NO generation from L-arginine through iNOS activity (Kim et al., 2006). Treated RAW264.7 cells with 200 µg/mL and 400 µg/mL of Kodipur® for 1 hr before stimulating with LPS for 24 hr significantly inhibited nitrite generation in a dose-dependent manner without affecting the cell viability (Fig. 1). The viability of treated cells with Kodipur® at concentrations 100–400 µg/mL was not significantly different in cell numbers compared with the control (data not shown).

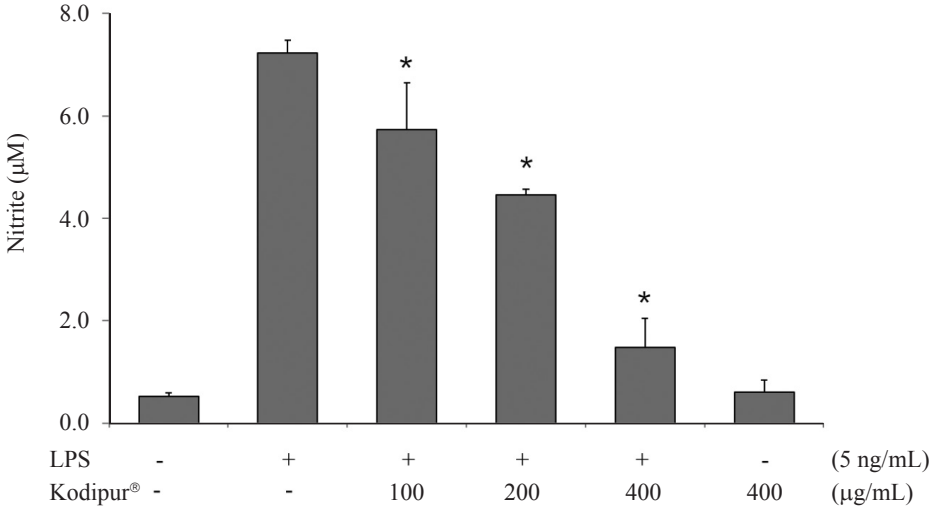

Fig. 1 Kodipur® inhibits LPS-induced NO production in RAW 264.7 cells, where cells were treated with indicated amounts of extract for 24 hr prior to nitrite determination using Griess reaction. * = $p < 0.05$ versus treatment with LPS alone.

Kodipur® suppression of lipopolysaccharide-activated TNF-α and IL-6 cytokine secretion

TNF-α and IL-6 cytokine are induced by various stimuli including LPS through activation of NF-κB and MAPK (Mekhora et al., 2012). As expected, challenging RAW264.7 cells with LPS significantly resulted in secretion of TNF-α and IL-6. Kodipur® at 100–400 μg/mL and significantly attenuated LPS-induced TNF-α and IL-6 cytokine secretions in a dose dependent manner (Figs. 2A and 2B).

Kodipur® suppression of prostraglandin E2 secretion.

Prostraglandin E2 is a mediator of inflammation induced by various stimuli including LPS and after exposure to LPS, cells significantly induced PGE_2 secretion. Pretreatment of cells with Kodipur® at 100–400 μg/mL effectively inhibited PGE_2 secretion in a dose dependent manner (Fig. 3).

Kodipur® inhibition of intracellular reactive oxygen species

Reactive oxygen intermediates are produced by activated macrophages via activation of NADPH oxidase activity to eliminate any invading microbes (Choi et al., 2007). Pre-treatment for 1 hr of RAW 264.7 cells with Kodipur® at 200–400 μg/mL significantly decreased in a dose-dependent manner the intracellular ROS induced by exposure to LPS for 24 hr. (Fig. 4).

Discussion

The herbal medicinal compound showed inhibition of pro-inflammatory mediators including NO, TNF-α, PGE_2 and IL-6 as well as intracellular ROS produced by LPS-activated murine RAW 264.7 macrophages. Phenolic components present in the compound included caffeic acid, chlorogenic acid and vanillic acid, which have been reported to have a role regarding their antioxidant and anti-inflammatory properties through inhibiting cell signaling pathway leading to suppress downstream inflammatory mediator such as NO, TNF-α, IL-6 and PGE_2 (Chao et al., 2009; Kang et al., 2009; Kim et al., 2011; Shan et al., 2009).

Caffeic acid, a metabolite product of chlorogenic acid, is a phenolic acid found in various plants including *Morus alba* L. leaf, *Ganoderma lucidum* and *Glycyrrhiza glabra* L. (Dey et al., 2009; Ramadan et al., 2017; Sánchez, 2017). Caffeic acid was present in the herbal medicinal compound at 752.07 ± 12.23 μg/g. It is known as an anti-inflammatory, anti-oxidant and anti-carcinogenic compound (Shin et al., 2004; Kang et al., 2009) and it has been demonstrated to down-regulate effectively COX-2 expression and PGE_2 production by modulation through the suppression of Fyn kinase and MAPKs, consequently causing inhibition of AP-1 and NF-κB activity in UVB-induced in JB6 P+ mouse skin epidermal cells (Kang et al., 2009). Caffeic acid methyl ester, a derivative form of caffeic acid was reported to possess potent anti-inflammatory and anti-nociceptive effects in carrageenan-induced edema and acetic acid-induced abdominal constriction in rats and mice, respectively (Shin et al., 2004). Chao et al. (2009) reported that intake of caffeic acid significantly lowered cardiac levels of malondialdehyde, an oxidized lipid product resulting from oxidative stress, IL-1β, IL-6, TNF-α and monocyte chemoattractant protein-1 (MCP-1). The same study also showed that caffeic acid significantly up-regulated cardiac mRNA expression of glutathione peroxidase (GPX1), superoxide dismutase (SOD) and catalase in cardiac tissue of diabetic mice. Caffeic acid in the present study partially contributed to suppression of COX-2, IL-6 and TNF-α production and decreased ROS accumulation in LPS-activated murine RAW 264.7 macrophages.

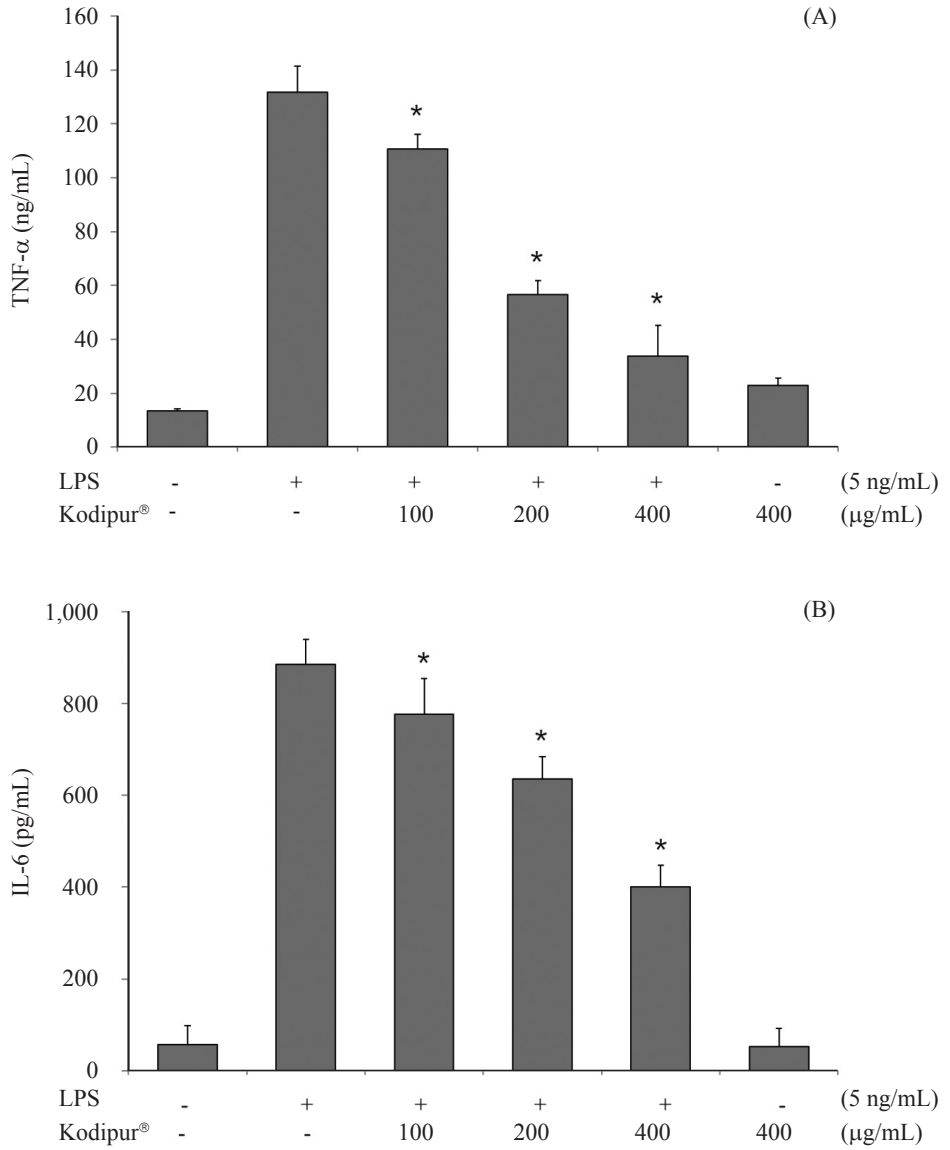

Fig. 2 Kodipur® attenuates LPS induced TNF-α and IL-6 cytokine secretion in RAW 264.7 cells, where cells were treated with indicated amounts of extract for 24 hr prior to determination of TNF-α (A) and IL-6 (B) cytokines using ELISA. * = $p < 0.05$ versus treatment with LPS alone.

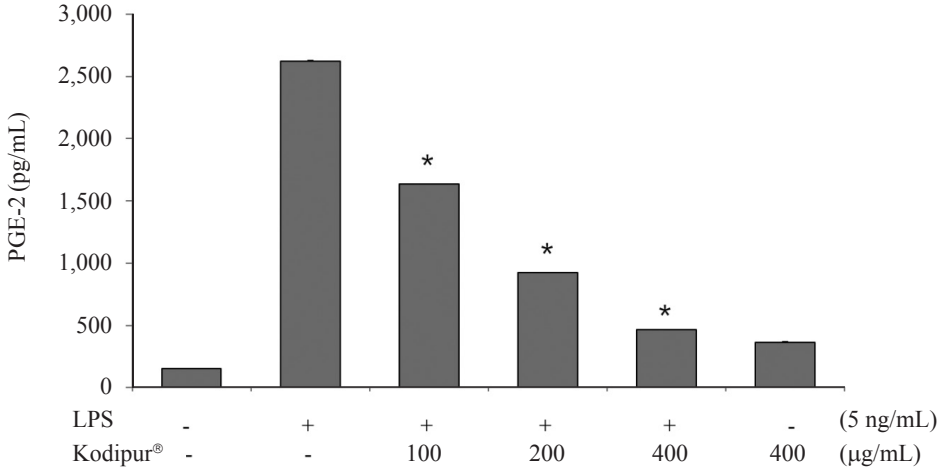

Fig. 3 Kodipur® suppresses LPS-induced PGE_2 secretion in RAW 264.7 cells, where cells were treated with indicated amounts of extract for 24 hr prior to determination of PGE_2 using ELISA. * = $p < 0.05$ versus treatment with LPS alone.

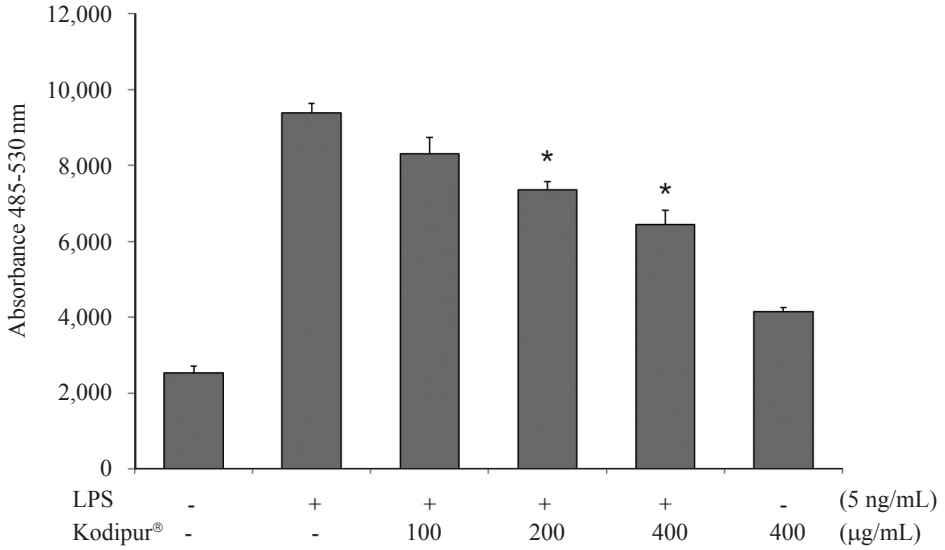

Fig. 4 Kodipur® reduces LPS-induced ROS accumulation in RAW 264.7 cells, where cells were treated with indicated amounts of extract for 24 hr prior to measuring the fluorescent signal of DCFH-DA. * = $p < 0.05$ versus treatment with LPS alone.

Chlorogenic acid (CGA) is an esterified form of caffeic and quinic acid and was dominant in the herbal medicinal compound at 329.5 ± 9.8 µg/g. CGA *plays* therapeutic roles due to its *anti-inflammatory activity, antioxidant,* antiviral and anti-microbial properties (Naveed et al., 2018). It *has been found in various plants including Morus alba* L. leaf (Ramadan et al., 2017) Previous study demonstrated that CGA at 37.5 µg/mL inhibited LPS-induced COX-2 expression via activation of NF-κB and JNK/AP-1 pathways in a murine macrophage cell line (Shan et al., 2009). It also reduced edema and pain in carrageenan-induced rats by inhibiting synthesis and release of inflammatory mediators (Shan et al., 2009). Zhang et al. (2010) reported that CGA had anti-inflammatory and analgesic activities in LPS-induced acute lung injury by decreasing the activity of iNOS and preventing NO release in mice. Therefore, the anti-inflammatory activity was partly in response to the chlorogenic acid found in the herbal medicine compound.

Vanillic acid, an oxidized form of vanillin, is a phenolic compound found in substantial parts of plants and fruits. The concentration of vanillic acid in the herbal medicinal compound was 91.04 ± 1.30 µg/g. Vanillic acid has been reported the anti-inflammatory mechanism in the carrageenan-induced paw edema model by inhibiting oxidative stress, suppressing pro-inflammatory cytokine production and NF-κB activation as well. (Calixto-Campos et al., 2015). Previous study showed that vanillic acid inhibited inflammatory mediators

including NO, COX-2, IL-6 and TNF-α, by blocking NF-κB and caspase-1 activation in LPS-induced d inflammatory responses in mouse peritoneal macrophages (Kim et al., 2011). The presence of vanillic acid in the herbal medicinal compound might have been responsible for some of the antioxidant and anti-inflammatory activities.

In summary, the herbal medicinal compound (Kodipur®) has the potential to alleviate LPS-induced pro-inflammatory actions by suppressing nitric oxide, PGE_2, IL-6 and TNF-α and by decreasing ROS accumulation in LPS-activated murine RAW 264.7 macrophages. These effects might be partially caused by the phenolic compounds present which have been reported to possess anti-inflammatory and antioxidant properties. However, efficacy studies in populations suffering from inflammatory-associated diseases need to be conducted to confirm such activities.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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