

Effects of *Taraxacum Mongolicum* Extract on Lipopolysaccharide-Induced Nitric Oxide and Cytokines Production by Bovine Mammary Epithelial Cells

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ABSTRACT

The objective of this study is to investigate *Taraxacum mongolicum* as a therapeutic alternative for preventing and treating bovine mastitis. The effect of the anti-inflammatory activity of *Taraxacum mongolicum* extract (TME) on lipopolysaccharide (LPS)-induced responses was studied in the bovine mammary epithelial cell line (MAC-T). The dried plant *Taraxacum mongolicum* was extracted with 10 volumes of distilled water to generate its water extract. MAC-T cells were pre-treated with various concentrations of TME

(0, 1, 10, 100, 1000 µg/ml) and subsequently incubated with LPS (10µg/ml). Cell viability was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) assay. The level of nitric oxide (NO) was determined by using Griess reagent assay. The mRNA expression levels of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, and granulocyte chemotactic protein (GCP)-2 were determined by using quantitative polymerase chain reaction (qPCR). The results showed no significantly cytotoxic effects on the MAC-T cells at various treated concentrations of TME. Treatment of TME (10, 100, 1000 µg/ml) significantly inhibited

NO production in LPS-stimulated MAC-T cells. TME (100 µg/ml) significantly inhibited LPS-stimulated IL-1β, IL-6, IL-8, TNF-α, and GCP-2 mRNA expression in MAC-T cells at a time-dependent manner. In this article, we reported for the first time that TME significantly inhibited production of NO and pro-inflammatory cytokines in LPS-stimulated MAC-T cells. This finding could be useful for clinical practice in preventing and treating bovine mastitis.

INTRODUCTION

Mastitis, defined as inflammation of the mammary gland, is usually caused by bacterial invasion into the udder.^{1,2} An inflammatory response is usually initiated when bacteria enter the mammary gland through the teat canal and multiply in the milk. Bacteria, leukocytes and mammary epithelial cells in the infected quarters release chemo-attractive products and inflammatory mediators for leukocytes. The mammary epithelial cells may be the first line of defense against mammary gland infection. Bacterial toxins, enzymes and cell-wall components have a direct effect on the function of the mammary epithelium to produce a variety of inflammatory mediators such as cytokines.^{3,4} Lipopolysaccharide (LPS) is a potent inducer of macrophages and mammary epithelial cells, which are key mediators of the innate immune response of mammary gland.⁵ In previous studies, mammary epithelial cells have the ability to rapidly produce pro-inflammatory cytokines, such as interleukin (IL)-1α, IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α, in response to *E. coli*-derived LPS.^{5,6,7,8} Yu et al.⁹ reported that the chemotactic effect of GCP-2 and IL-8 on bovine mammary epithelial cell line (MAC-T) were examined using an in vitro model.

Mastitis is one of the most common and costly infectious diseases in the dairy industry, which leads to huge economic losses mainly associated with decreased milk production and quality, therapeutic interventions, loss of antibiotic-contaminated milk and extra labor.¹⁰ The common practice of treating mastitis is intramammary infusion

of antibiotics. However, the efficacy is only moderate and the residue of antibiotics in milk has become a public concern.¹¹ Suriyasathaporn et al.¹² reported that the dairy farms with excessive use of antibiotics had a higher probability of antibiotic-resistant pattern than the farms with normal use. Therefore, using therapeutic agents other than antibiotics for preventing or treating bovine mastitis is an important research subject in the dairy industry.

Plant-based products constitute a major source of alternative therapies for a wide spectrum of diseases in human beings and animals. Herbs with anti-inflammatory properties that are used in traditional Chinese medicine may be potential candidates for a variety of treatments. In Taiwan, *Taraxacum mongolicum* (TM), is the famous traditional Chinese medicine, which was commonly used by Chinese local herbal physicians. The aqueous extract of TM is frequently used to treat hepatic and inflammatory disorders, certain diseases common to women associated with lactation, as diuretics and anti-inflammatory remedies in the Pharmacopoeia Chinensis. The amount of TM used on animals far exceeds that used on human beings.¹³ According to the TM specific anti-inflammatory properties, which suggested that TM may be a very potential alternative treatment (as intramammary infusion of TM extract) for preventing and treating bovine mastitis. Therefore, we were interested in examining the anti-inflammatory effects of TM extract (TME) on bovine MAC-T cells under LPS-stimulation.

MATERIALS AND METHODS

Preparation of *Taraxacum mongolicum* extract (TME)

The extract of *Taraxacum mongolicum* Hand.-Mazz. was obtained from Koda Pharmaceuticals Ltd (Taoyuan, Taiwan). Dried plant entire (100 g) was extracted twice by boiling for 1 h with 10 volumes of distilled water. The decoctions obtained were sieved through a 200-mesh (diameter 0.74 mm) screen and centrifuged at 10,000 rpm for 5 min to eliminate sediments. The total

crude plant extract (38.5 g) was collected by centrifugation twice at $8000 \times g$ for 15 min at 4°C . Filtration through a membrane filter (0.45 mm pore size, Nalgene, New York, USA) and collection of extract was done thrice. The resulting decoction was evaporated to dryness in vacuo at 37°C on Speedvac concentrator (Thermo Savant, Farmingdale, NY). Dry extract was weighted, dissolved in phosphate-buffered saline (stock 100 mg/ml) and stored in -20°C until further use.

Bovine mammary epithelial cell culture

The bovine mammary epithelial cell line (MAC-T) was maintained by serial passages in Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL) supplemented with 10% fetal bovine serum (FBS, Gibco/BRL) and 50 $\mu\text{g/ml}$ gentamicin (Gibco/BRL). Cells were cultured at 37°C a humidified atmosphere of 5% CO_2 until 90% confluent.

Viability of cells

The MAC-T cells were plated (at a density of 2.5×10^4 cells per well per 100 μl of medium) with the indicated concentrations of TME (0, 1, 10, 100, 1000 $\mu\text{g/ml}$) at 37°C under 5% CO_2 atmosphere in a 6-well plate for 24 h. Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylthiazolium bromide (MTT) assay¹⁴ and is expressed as a percentage of the control without TME. One-tenth volume (10 μl) of 5 mg/ml MTT (Sigma Aldrich, St. Louis, MO) was added to the culture medium. After 4 h incubation at 37°C , an equal volume of 0.04 N HCl in isopropanol was added to dissolve the MTT formazan and the absorbance was measured at 570 nm using an ELISA-Reader (Thermo Multiskan EX). The absorbance of the formazan formed by the untreated cells was defined as 100%.

Nitric oxide (NO) assay

The study used lipopolysaccharide (LPS) (Escherichia coli serotype O111:B4; Sigma, Cat. No. L4391). NO was measured with cell supernatant as nitrite and nitrate. The safe form of nitrite after being reduced to nitrate was measured using Griess reagent (Sigma Chemical Co., St. Louis, MO). MAC-T cells (2.5×10^4) were put into a 6

well plate and washed two times with PBS when the confluence was 90% and then cultured for at least 24 hours and TME samples were made into the final concentrations of 1, 10, 100, 1000 $\mu\text{g/ml}$ for the experiments. Four hours later, LPS (final concentration 10 $\mu\text{g/ml}$) was put into all wells except for the well for the control group to stimulate the cells. The amount of NO generated were measured with the supernatant 18 hours later at 540nm in an enzyme-linked immunosorbent assay (ELISA) microplate reader.¹⁵

In vitro LPS stimulation and TME treatment

Cultured MAC-T cells (90% confluence) were incubated with LPS (10 $\mu\text{g/ml}$) at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h, and cells were collected at 0, 2, 4, 8, 16, 24 h relative to the addition of LPS. In experiments to determine the effects of TME (indicated concentrations), was added for 4 hr before the addition of LPS. Thereafter, cells were trypsinised and centrifuged for RNA extraction.

RNA extraction and reverse transcription

Total RNA was extracted using Trizol (Invitrogen, Grand Island, NY, USA), according to the manufacturer's recommendations for cultured cells. The pellet of 5×10^6 MAC-T cells was lysed in 1 ml of Trizol and centrifuged after adding 0.2 ml chloroform. The RNA, retained in the aqueous phase, was precipitated by mixing with an equal volume of isopropanol and washed twice with 75% ethanol. Then, 10 μl of diethyl pyrocarbonate-treated water was added to dissolve the RNA and the concentration was determined by spectrophotometry at an optical density of 260 nm.

Reverse transcription of mRNA into cDNA

Total RNA was reverse transcribed into cDNA according to the manufacturer's directions (iScript, BioRad). Briefly, a 20 μl reaction mixture consisting of 1 μg of total RNA, 4 μl of $5 \times$ iScript Reaction Mix, 1 μl of iScript Reverse Transcriptase, and a known amount of nuclease-free water and placed into the Mastercycler Autorisierter

Table 1. Sequences of primers for bovine cytokines and β -actin in qPCR

Target gene	Primer	Sequences (5'-3')	Predicted Size (bp)	Gene bank accession number
β -actin	Forward	CCTTTTACAACGAGCTGCGTGTG	391	AH00130
	Reverse	ACGTAGCAGAGCTTCTCCTTGATG		
IL-1 β	Forward	AAATGAACCGAGAAGTGGTGT	185	M35589
	Reverse	TTCCATATTCCTCTTGGGCTAGA		
IL-6	Forward	CTGGGTCAATCAGGCGAT	205	X57317
	Reverse	CAGCAGGTCAGTGTTTGTGG		
IL-8	Forward	CACTGTGAAAATTCAGAAATCATTGTTA	105	NM173925
	Reverse	CTTCACAAATACCTGCACAACCTTC		
TNF- α	Forward	CTGGTTCAGACACTCAGGTCCT	183	AF011926
	Reverse	GAGGTAAGCCCGTCAGCA		
GCP-2	Forward	CTGGCCCACTGAAGACAA	169	AF149249
	Reverse	AAATACGGAGCAGGAAATAATA		

thermocycler (Eppendorf, Hamburg, Germany) with the following temperature/time protocol: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. All isolated RNA and cDNA samples were stored at -80°C until further analysis.

Primer design

The primer sequences used for all experiments were designed to bind specifically to bovine cDNA according to published sequences. All sequences were purchased from Integrated DNA Technologies, Inc. (Coralville, IA, USA; XXIDT). The housekeeping gene, β -actin, was used as an internal standard. The GenBank accession numbers of the targets as well as the primer sequences are shown in Table 1.

Quantitative real-time PCR

The relative quantities of the gene transcripts were measured after LPS stimulation by quantitative real-time polymerase chain reaction (qPCR). The measurements were performed on a MyiQ iCycler Single Color RT-PCR detection system (Bio-Rad) using IQ EvaGreen PCR Supermix (Bio-Rad), according to the manufacturer's guidelines. Briefly, each well contained a 20- μ l reaction mixture that contained 10 μ l of the master

mix, 1 μ l each of the forward and reverse primers, 6 μ l of water, and 2 μ l of cDNA samples. The EvaGreen dye was measured at 530 nm during the extension phase. PCR was conducted in 96-well optical reaction plates. The iCycler was programmed in four steps: (1) enzyme activation at 95°C for 30 sec; (2) denaturation at 95°C for 5 sec; (3) 40 cycles of denaturation at 95°C for 5 sec, annealing and extension at 55°C for 10 sec; and (4) melting curve analysis followed by cooling at 65°C and then increasing the temperature to 95°C at 0.5°C/10 sec. The resulting PCR cycle time (Ct) values were collected by using the software provided for the MyiQ system, and the data were then analyzed.

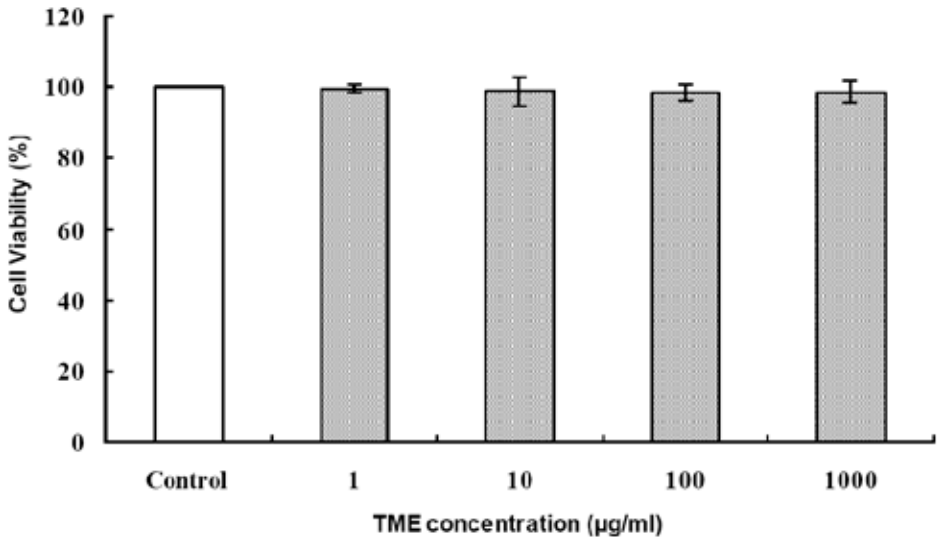
Data analysis of qPCR

The relative mRNA amount in each sample was calculated based on the Ct in comparison with the Ct of the housekeeping genes β -actin and the mRNA was ascribed a fold induction of 1. The results are presented as $2^{-(Ct \text{ of the target gene} - Ct \text{ of the housekeeping gene})}$ in arbitrary units.

Statistical analysis

Statistical analysis of the data was performed in Excel and GraphPad Prism

Figure 1. The MAC-T cells were treated with TME for 24h. The culture medium was replaced with medium containing various concentrations of TME or medium alone. Cell viability in each culture dish was determined by MTT assay. Values represent the average of duplicate wells from a representative of three independent experiments. The cell number is expressed as 2.5×10^4 cells/well. The results were presented as cell viability relative to control (relative value = 100). Cell viability = (absorbance of treated sample) / (absorbance of control). Values are mean \pm SD of three independent experiments.



software package version 5.0. All results were expressed as the mean \pm SD and were analyzed using a two-tailed Student's t test. Differences were considered statistically significant at $P < 0.05$ and highly significant at $P < 0.01$.

RESULTS

Viability of cells

The MAC-T cells were treated with various concentrations of TME (0, 1, 10, 100, 1000 µg/ml) for 24 h. The cell viability was determined by MTT assay. Figure 1 showed TME did not inhibit cell growth and the highest concentration (1000 µg/ml) did not reduce the cell viability. The results showed no significantly cytotoxic effects on the MAC-T cells at 1-1000µg/ml of TME.

Inhibition of NO production by TME

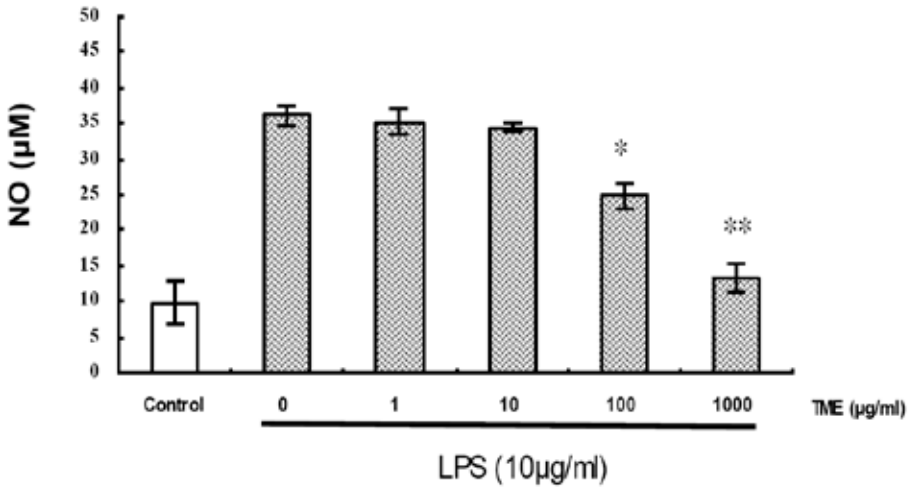
NO has been considered as a marker of inflammatory response that come with bovine mastitis.¹⁶ The effects of TME on NO production in LPS-stimulated MAC-T

cells were investigated. The MAC-T cells were treated with LPS alone or with various concentrations of TME for 24 h. NO production was measured from its stable metabolite, nitrite in the medium. None of the LPS or TME treatments caused toxicity to cells. When the MAC-T cells were treated with 100, 1000 µg/ml TME, NO production induced by LPS was significantly suppressed in 25.0 ± 2.2 and 13.2 ± 1.8 µM, respectively, in compared with the LPS-treated alone (36.1 ± 1.3 µM) (Fig. 2).

Kinetics of cytokine mRNA expression in MAC-T cells stimulated with LPS

To analyze the anti-inflammatory mechanism of TME, the effects of LPS on inducing inflammatory cytokine mRNA expression of TNF- α , IL-1 β , IL-6, IL-8 and GCP-2 in MAC-T cells were measured first. Release of the pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-8 and GCP-2) are important mechanisms by which immune cells regulate inflammatory responses and con-

Figure 2. Effect of TME on LPS-induced NO in MAC-T cells. MAC-T were pretreated with the indicated concentrations of TME for 4 h and treated with LPS (10 µg/ml). After 24 h, the amounts of NO in the culture supernatants were measured using the Griess reaction assays. Data are mean ± SD values of three individual experiments. * $P < 0.05$, ** $P < 0.01$, significant differences from the LPS control group (TME 0 µg/ml).



tribute to various inflammatory disorders. The relative expression of TNF- α , IL-1 β , IL-6, IL-8 and GCP-2 genes in MAC-T cells after LPS stimulation for 0, 2, 4, 8, 16, and 24 h. As shown in Figure 3, stimulation of MAC-T cells with LPS led to significantly increase production of the pro-inflammatory cytokines including TNF- α , IL-1 β , IL-6, IL-8 and GCP-2. The mRNA expression levels of TNF- α , IL-1 β , and IL-8 increased rapidly within 4 h and remained relatively constant over the following 20 h. The peaks of expression for all three genes were 100 - 3000 fold greater than the unstimulated cells. IL-6 and GCP-2, compared to the other genes, showed slower time courses of up-regulation. IL-6 and GCP-2 showed increasing expression within the 24 h time period. These results indicated that LPS could induce a strong inflammatory response on MAC-T cells.

Effect of TME on LPS-induced mRNA expression of inflammatory cytokines in MAC-T cells

The mRNA expression levels of inflammatory cytokines in MAC-T cells cultured with LPS in the presence and absence of

TME (100 µg/ml) were determined by using qRT-PCR. As shown in Figure 4, the mRNA expression level of TNF- α , IL-1 β , IL-6, IL-8 and GCP-2 in LPS-induced MAC-T cells was markedly decreased by treating TME. TNF- α , IL-1 β , IL-6, IL-8, and GCP-2 level of treatment group (MAC-T+LPS+TME) was significantly decreased as compared to control group (MAC-T+LPS) in 2, 4, 8, 16, and 24h, respectively. These results suggested that TME could inhibit LPS-induced inflammatory response effectively in MAC-T cells.

DISCUSSION

Stimulation of macrophages and mammary epithelial cells by LPS leads to a cascade of intracellular signaling events that ultimately results in production of secretion of cytokines and other inflammatory mediators that constitute the pro-inflammatory response.¹⁷ Huynh et al.¹⁸ indicated that the MAC-T cells was an in vitro model for bovine lactation research. Therefore, we investigated the anti-inflammatory effects of TME in LPS-stimulated MAC-T cells. In the present study, we found that production

of inflammatory mediators in mammary epithelia cells, such as LPS-induced NO and inflammatory cytokines (TNF- α , IL-1 β , IL-6, IL-8 and GCP-2), were significantly inhibited by TME. In addition, the TME did not show any significant toxicity to MAC-T cells.

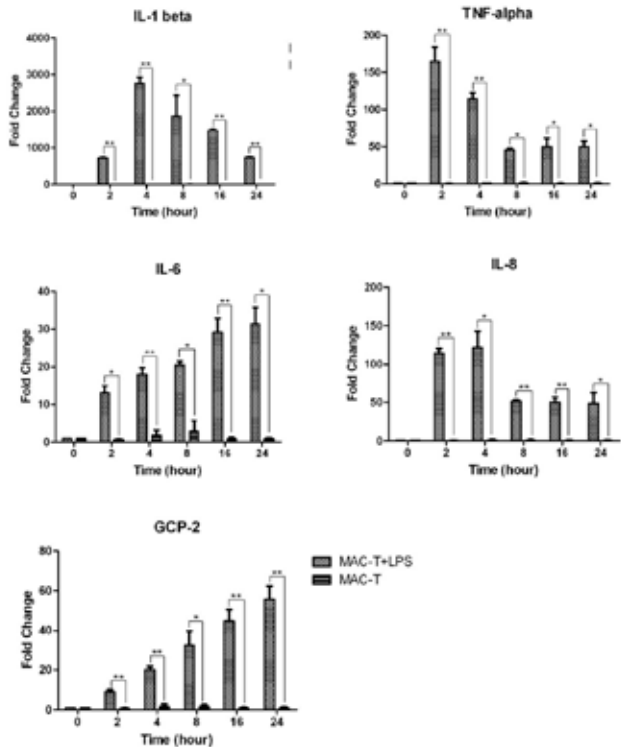
Excessive NO production in the mammary gland is believed to contribute to inflammatory processes.^{6,19} Therefore, TME that inhibits NO production may have therapeutic potential against inflammation.^{20,21,22}

TME, at concentrations of 100-1000 $\mu\text{g/ml}$, inhibited 30–63% of LPS-stimulated NO production in MAC-T cells. This is in agreement with in a previous study indicating that eudesmanolides from *Taraxacum mongolicum* was able to inhibit the production of NO in vitro.²³

Furthermore, TME (100 $\mu\text{g/ml}$) also significantly inhibited the mRNA expression of IL-1 β , IL-6, IL-8, TNF- α , and GCP-2 in LPS-stimulated MAC-T cells.

Clinical severity of bovine mastitis has been positively correlated with these cytokines.²⁴ The presence of IL-6 and TNF- α have been associated with severe clinical coliform mastitis, in the milk whereas they might play a role in generating inflammation of the mammary gland as suggested by the high level of milk IL-6, even in the cows that died of coliform mastitis.²⁵ IL-8 is produced by bovine mammary epithelial cells,^{3,5} and plays an important role in neutrophil migration across the blood/milk barrier during the course of mastitis.²⁶ The concentration of IL-8 is dramatically increased in mammary secretions dur-

Figure 3. Effect of TNF- α , IL-1 β , IL-6, IL-8 and GCP-2 mRNA expression in MAC-T cells stimulated with LPS. The MAC-T cells were pretreated with LPS (0 or 10 $\mu\text{g/ml}$) at 37°C in a 96-well for 24 hours. The relative mRNA expression levels of TNF- α , IL-1 β , IL-6, IL-8 and GCP-2 in MAC-T cells after LPS stimulation of 0, 2, 4, 8, 16, and 24 h were measured using qPCR. Data are expressed as mean \pm SD of three individual experiments. * $P < 0.05$, ** $P < 0.01$, significantly different from that of the group without LPS (0 $\mu\text{g/ml}$) stimulation.



ing the course of coliform mastitis. GCP-2 is structurally and functionally similar to IL-8 in chemotactic effect on polymorphonuclear leucocytes and angiogenesis and enhanced the expression by IL-6.²⁷ In our study, we found that MAC-T extinguishes the production of cytokines (e.g., IL-1, TNF- α , and IL-8) within a few hours following the LPS stimulus. Whereas LPS-induced expression of IL-6 and GCP-2 were relatively later in compared with the expression of IL-1, TNF- α , and IL-8. This may indicate that these cytokines are relevant to various acute and chronic inflammations, so reduction of

these cytokines may retard inflammatory response.²⁸ Through down-regulation of TNF- α , IL-1 β , and IL-6, local inflammation response could be relieved by TME. As well as IL-8 and GCP-2, it was indicated that TME significantly decreased their expression, which resulted in preventing the excessive influx of polymorphonuclear leucocytes for the further injuries.

Several studies have demonstrated the plants of the genus *Taraxacum*, known as dandelion, have the diuretic, anti-inflammatory and anti-oxidative activities, for medicinal purposes.²⁹ The aqueous extract of TM was elucidated to interact with a fluoroquinolone-type antibiotic, ciprofloxacin, which modifies its bioavailability and disposition.³⁰ Additionally, other species of the genus *Taraxacum* were elucidated to contain various pharmacological activities. *Taraxacum officinale* (TO) was assessed to contain acute anti-inflammatory activity by showing its protective effect against cholecystokinin-induced acute pancreatitis in rats.³¹ On the other hand, Koh et al.³² indicated that the anti-inflammatory effects of TO leaves are probably due to down-regulation of NO, TNF- α and IL-1 β and reduced expression of iNOS via inactivation of the MAP kinase signal pathway.

In conclusion, the TME significantly inhibited production of NO and pro-inflammatory cytokines in LPS-stimulated MAC-T cells for the first time. This finding provided an alternative treatment for clinical practice in preventing and treating bovine mastitis. Further studies are needed to evaluate the anti-inflammatory activities by TM through several inflammatory parameters and their underlying molecular mechanisms.

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