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TLR4/NFκB-Mediated Anti-Inflammatory and Antioxidative Effect of Hexanic and Ethanolic Extracts of *Curcuma longa* L. in Buffalo Mammary Epithelial Cells

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Abstract: Mastitis is the commonest disease of bovines imposing a great economic setback and a drastic public health concern worldwide. Antibiotic resistance is the preliminary con of excessive antibiotics use for disease treatment. Studies validate an alarming increase in the antibiotic resistance of both humans and animals. For ages, medicinal plants have been used to treat a number of ailments in humans and animals, including mastitis. *Curcuma longa*, or turmeric, is the prerequisite in Indian cooking and has been used in traditional medicine for its magical properties. The rhizome of *C. longa* possesses significant anti-inflammatory, antibacterial, antifungal, and antioxidative properties. In the current study, we evaluated the hexanic and ethanolic extracts of *C. longa* for their anti-inflammatory and antioxidative potential against LPS-induced inflammation in buffalo mammary epithelial cells (BuMECs). Pretreatment with the extracts downregulated the expression of proinflammatory cytokine TNFα via the TLR4/NFκB-mediated signaling pathway. However, IL-6 was downregulated in only the hexanic *C. longa*-pretreated group. A significant upregulation of NRF2 mRNA expression was seen in both hexanic- and ethanolic-treated groups. A GC-MS/MS study of the extracts revealed the presence of important sesqueterpenoids and phenolics as the main bioactive phytoconstituents in the extracts. Sesqueterpenoids, such as turmerone, ar-turmerone, curlone, and atlantone, and phenols, such as guaiacol (2-Hydroxyanisole phenol, 2-methoxy), and ethyl ferulate/ethyl 4′-hydroxy-3′methoxycinnamate, were found in *C. longa* extracts. The protective role of *C. longa* in BuMECs against LPS-induced inflammation and oxidant insult might be due to the presence of bioactive compounds, such as terpenoid and phenolic compounds. However, we further propose the isolation of these phytoconstituents and their analysis using HPLC and NMR studies.

Keywords: mastitis; BuMECs; ROS; TLR4; inflammation; LPS; oxidative stress; *Curcuma longa*; GC-MS/MS

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1. Introduction

The invasion of the parenchymatous tissue of the mammary gland by bacteria, a virus, or fungi leads to a highly complex disease in bovines known as mastitis [1]. It is the most prevalent disease of bovines, inflicting a huge economic burden on the dairy industry worldwide. Bovine mastitis is mostly caused due to common udder pathogens, such as *Staphylococci*, *Streptococci*, and *Coliform* species [2]. Bacteria, such as *Streptococcus uberis*,

Streptococcus dysagalactiae, *Klebsiella*, and *Escherichia coli*, can be isolated from both the animal's outer surface as well as its surroundings [3]. *E. coli* is responsible for causing a severe form of clinical mastitis. Lipopolysaccharides (LPS released from gram-negative bacteria are the main virulence factors that induce an inflammatory reaction after binding to the cell membrane receptors called Toll-Like Receptor-4 (TLR4) on mammary epithelial cells [4]. TLR4 stimulates the transcription factor, nuclear factor kappa B (NFκB), which activates downstream signaling proinflammatory molecules such as cytokines, which include interleukin (IL)-6, 8, 1α, 1β, and tumor necrosis factor (TNF)-α [5]. Leukocyte migration to the affected area is induced due to an increased chemokine secretion, eventually amplifying the inflammatory response. Serum amyloid A (SAA), haptoglobin (Hp), C-reactive protein (CRP), ceruloplasmin, α-1 antitrypsin, and fibrinogen are the major acute-phase proteins (APPs) which increase after inflammatory reaction [6]. The upregulation of all inflammatory mediators leads to bovine mastitis. Moreover, inflammation and generation of oxidative stress in the cells are quite interrelated phenomena and exhibit significant crosstalk within the diseased cell. The generation of reactive oxygen species (ROS) and the disruption of antioxidative depots are the predominant results of increased oxidative stress. The Nrf2 (nuclear factor erythroid 2-related factor 2) transcription factor is of high significance with regard to the antioxidative status of the cell. Significant downregulation of NRF2 expression has been observed in cells induced with LPS in many in vivo and in vitro studies [4].

Currently, the main line of treatment followed for anti-mastitis therapy in bovines is the administration of corticosteroids and antibiotics. Increased drug resistance to a number of frequently used drugs, such as tetracycline, penicillin, oxacillin, cefoxitin, etc., has developed as a result of the excessive use of antibiotics [7]. The widespread and unsystematic use of antibiotics contributes to the development of resistant strains of mastitis-causing pathogens, resulting in the emergence of drug resistance in animals. The withdrawal of these antibiotic residues in livestock products like meat and milk eventually leads to antibiotic resistance in humans [8]. This has led scientists to search for alternative anti-mastitic medications based on natural ingredients. Plant-based medications have been used to treat veterinary patients throughout the world and have delivered appreciable results, although the need for further technology drives studies [9]. For instance, Fuzvet-A (from pumpkin seeds), Vivatone (a decoction of different herbs and ammonia), Penosept (made from common nettle extract), and Mastig are some anti-mastitic drugs approved and marketed worldwide [10]. The effectiveness of plant-based ointments, namely, Pihtoin, synthesized from pine sapwood, was 16.7% effective in sub-clinical mastitis, while its combination with a trauma gel led to 100% recovery in cows diagnosed with mastitis. Many plants, such as *Ocimum tenuiflorum* (basil seeds), *Brassica juncea* (mustard oil), *Rheum emodi* (Indian rhubarb), *Curcuma longa* (turmeric), Nelumbo (lotus roots), *Syzygium aromaticum* (cloves), *Elletaria cardamonum* (cardamom), *Piper nigrum* (black pepper), *Terminalia* (Indian almond), *Trigonella* (fenugreek), etc., are known to be used by local pastoralists to treat udder infections in the state of Jammu and Kashmir [11]. Studies postulating the anti-inflammatory, antioxidative, or antibacterial effects of pure bioactive compounds, such as baicalin, curcumin, resvesterol, thymol, etc., in BALB/c mice, bovine mammary alveolar cell (MAC-T), and bovine mammary epithelial cell (bMEC) mastitis models have been carried out [12]. Moreover, studies elucidating the effect of natural compounds on bovine mastitis at transcriptomic, genomic, and proteomic levels are still very meagre and largely untapped.

Curcuma longa is an ancient Indian medicinal herb classically called Golden spice. It is a perennial plant, and the part recognized to have the most beneficial effects is its rhizome [13]. A variety of bioactive compounds, including terpenes, phenolic compounds, curcuminoids, fatty acids, and steroids, are present in the rhizome of turmeric [14]. *Curcuma longa* is known to inhibit the production of ROS by decreasing lipid peroxidation and increasing antioxidative enzyme concentrations [15]. Crude extracts of *C. longa* are known to decrease the essential markers of inflammation, such as cyclooxygenase (COX), thromboxanes (Txs),

prostaglandins (PGs), lipoxygenase (LOX), TNF, and interleukins [16]. Recently, curcumin-loaded nanoparticles (CUR-NP) have led to the amelioration of inflammation in mastitic mice by reducing oxidative markers and restoring histopathological aberrations [17]. Post-milking polyherbal teat dips such as Mastidip, having *Berberis lycium*, *Curcuma longa*, and *Eucalyptus* as its components, are known to decrease the occurrence of mastitis with the subsequent increase in milk yield [18]. *Curcuma longa* is also an essential component of Mastilep gel which has been used to alleviate the symptoms of subclinical mastitis in dairy cattle [19]. In buffalo cows, the hydroalcoholic *C. longa* extracts cause a reduction in the somatic cell count (SCC) and total bacterial count (TBC) with a concomitant increase in the levels of L-selectin, a leukocyte adhesion molecule (LAM) which is significantly decreased in mastitis and periparturient periods [20]. Curcumin treatment helps in attenuating LPS-induced mastitis in rats by targeting the TLR4/NFκB-mediated pathway, downregulating the activity of myeloperoxidase (MPO), and the expression of inflammatory mediators such as TNF-α, IL-6, and IL-1β in a dose-dependent manner [21]. Nanocurcumin alleviates inflammation and oxidative stress in LPS-induces mastitis in rats via the downregulation of the TLR4-mediated NF-κB signaling pathway and the activation of Nrf2 expression, respectively i [4]. A significant decrease in MDA levels and pro-inflammatory cytokines such as IL-1β and TNF-α is also observed in these rats. Very recently, matrix-transdermal patches with *C. longa* have been used in mastitic rat models wherein a significant alleviation of inflammation, a decrease in the pain score, and an increased mean threshold of pain due to an increased accumulation of curcuminoids and methoxyflavones were observed [22].

To date, no screens that have identified bioactive molecules from *C. longa* capable of explicitly and effectively producing the anti-inflammatory and antioxidative action in BuMECs have been published. Additionally, the research postulating the effect of pure extracts of *C. longa* in bovine mammary epithelial cell lines has not been undertaken largely. However, the activation of anti-inflammatory pathways and deactivation of prooxidative actions by the use of *C. longa* directly or its active compounds is postulated in many research studies, as stated above. In the current study, we used the hexanic and ethanolic extracts of *C. longa* in LPS-induced BuMECs to investigate their anti-inflammatory targeting TLR4/NFκB pathway and antioxidative potentials through the TLR4/NRF-2 pathway.

2. Material and Methods

2.1. Collection of Plant Material and Preparation of Extracts

The rhizome of *C. longa* was obtained from a traditional plant vendor (Zam Zam Ayurvedic and Unani Medicinal Store, Srinagar, Kashmir), cleaned with distilled water, and dried under a shade. The rhizomes were ground to form a fine powder and were stored in an air-tight jar. For the preparation of the extracts, 100 g (gm) of dried powder was subjected to cold extraction, wherein the powder was dissolved in the solvent and kept in a shaking incubator for 4 days at 25 °C. Two solvents, i.e., hexane and ethanol, were used in preference of their polarity indices. Extractions were carried out starting from less polar to more polar solvents, i.e., hexane, followed by ethanol. HECl represents the hexanic extract of *C. longa*, and EECl represents the ethanolic extract of *C. longa*. After 4 days, the extracts were taken out from the shaking incubator and filtered using Whatmann Filter paper No. 2. Then, the filtrates were kept in a hot air oven set at 25 °C in order to evaporate the solvents. The dried extracts were weighed and stored in eppendorf tubes at −20 °C for further experiments.

2.2. Chemicals and Cell Line

High glucose DMEM (Sigma) (Dulbecco's Modified Eagle Medium) with sodium pyruvate, sodium bicarbonate, and a HEPES buffer was used to make Complete DMEM with 10–15% FBS (Sigma, Victoria, Australia), 1% Penicillin-Streptomycin, EGF (10 ng/mL) (Sigma, Victoria, Australia), 1 µg/mL hydrocortisone (Sigma, Victoria, Australia), and 5 µg/mL bovine insulin (Sigma, Victoria, Australia).

Buffalo mammary epithelial cells (BuMECs) were obtained from the Animal Biotechnology Centre, National Dairy Research Institute, Karnal, India. The cells were cryopreserved in the NDRI lab itself and transported to our lab in a liquid nitrogen (LN_2) can. This cell line exhibited epithelial cell characteristics by immunostaining positively with cytokeratin 18 and negatively with vimentin, as already performed at the NDRI lab [23]. The cell line was revived and cultured in a T-25 flask kept in a CO_2 incubator set at 37 °C with 5% CO_2. The cells were grown to 70% confluency and trypsinised for further passages.

2.3. Free Radical Scavenging Activity (DPPH Assay)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to assess the free radical scavenging activity (RSA%) of the extracts by UV spectroscopy as per Padmapriya et al. [24]. A total of 20 µL of the different concentrations of each extract was pipetted out in a 96-well plate, and subsequently, 180 µL of the DPPH reagent was added to make a final volume of 200 µL. Ascorbic acid was also added to the same plate and served as the positive control. All the experiments were performed in a triplicate manner. The plate was incubated at 37 °C for 1 h following absorbance, which was measured at 517 nm using the ELISA plate reader. The RAS% of the extracts were expressed as the mean of IC 50 ± S.D.

2.4. MTT Assay

The MTT assay is a colorimetric assay used to measure cellular activity by detecting changes in color. The MTT assay was first carried out by Mosman et al., 1983 [25]. Cells were seeded in 96 well plates at a cell density of 1×10^4 cells per well and allowed to attach for 24 h in a CO_2 incubator. The next day cell attachment was checked under a microscope in 100× and the media was slowly taken out using a multichannel pipette, and fresh media was added. The cells were then treated with various concentrations of the extracts per mL of the media and were kept in an incubator at 37 °C for 24 h. Each concentration was repeated in a triplicate manner. After overnight incubation, the media containing the extracts was discarded from each well, and fresh media was added to each well. Subsequently, 20 µL of the MTT working solution was added from a stock MTT of 5 mg/mL and incubated for 4 h at 37 °C in a CO_2 incubator. Then, the media solution with MTT was discarded, and DMSO was added; the plates were wrapped in aluminum foil and incubated for 15 min. The absorbance was then measured for each well against a blank column containing 100 µL of DMSO per well using an ELISA reader at 595 nm. The cell viability was determined by comparing the absorbance values of the treated cells to the absorbance values of the control cells. Data were expressed as a ratio of the percentage reduction in the cell viability relative to that of the control cells and were expressed as the mean ± standard error of the mean (SEM).

2.5. Preparation of LPS

Lyophilised LPS from *E. coli*, strain O111:B4, was ordered from Sigma. A total of 1 mg LPS was dissolved in 1 mL sterile DPBS; it was dispensed in aliquots and kept at −20 °C till further use. Do not filter the LPS.

2.6. Protocol for Treatment with Different Extracts

Both the extracts were dissolved in DMEM and heated in the water bath set at temperatures varying between 30 and 35 °C to make a homogenous solution. The extracts were syringe filtered through 0.23 µm nylon filters (Himedia). BuMECs were seeded in 6 well plates at a seeding density of 3×10^5, and 1 mL of complete DMEM was added to each well. The plates were kept in a CO_2 incubator, and the media was changed every three days with fresh complete DMEM until the cells became 60–70% confluent. Then, the media was discarded, new media was added, and the cell groups were subjected to different treatments. Group I served as the control where BuMECs were grown in complete DMEM without any treatment; Group II served as the infected group in which LPS treatment was given @5 µg/mL of complete DMEM for 6 h. In Group III, BuMECs were treated with

HECl @ 50 µg/mL for 24 h followed by LPS @ 5 µg/mL for 6 h, and in Group IV, BuMECs were treated with EECl @ 50 µg/mL for 24 h followed by LPS @ 5 µg/mL for 6 h.

2.7. Total RNA Extraction

RNA from each group was extracted manually using the TRIzol method, as per Sambrook and Russel [26]. The quality of RNA was checked by running the samples on 1.5% Agarose to check for three bands of mRNA. The quantity of RNA was checked by using the spectrophotometer at an optical density (OD) of 260 nm and 280 nm. The samples showing an OD 260/280 ratio between 1.9 and 2 were used for cDNA synthesis and qRTPCR.

2.7.1. cDNA Synthesis

cDNA was synthesized using a cDNA Synthesis kit (Promega) as per the kit guidelines. To validate cDNA synthesis and primer Tm, the gradient polymerase chain reaction (PCR) was carried out using all committed primers (Table 1). The PCR products were run on 2% Agarose gel electrophoresis to check for the desired band.

Table 1. Melting temperature and amplicon size of the primers used.

S. No.	Primer Name	Primer Sequence	Amplicon Size	Tm (°C)
1.	β-Actin/ ACTB	Fwd: CCCTGGAGAAGAGCTACGAG Rev: GTAGTTTCGTGAATGCCGCAG	160 bp	60
2.	TLR4	Fwd: TCCCCGACAACATCCCCATA Rev: GGCCCTGAAATGTGTCGTCT	159 bp	60
3.	NFκB	Fwd: CAGCCTGGTGGGAAAACACT Rev: CAGGCATCTGTCATTCGTGC	150 bp	65
4.	IL-6	Fwd: GCTGAATCTTCCAAAAATGGAGG Rev: GCTTCAGGATCTGGATCAGTG	200 bp	65
5.	TNFα	Fwd: CCACGTTGTAGCCGACATC Rev: CCCTGAAGAGGACCTGTGAG	155 bp	65
6.	NRF2	Fwd: CATGGCATCACCAGACCACT Rev: CGGTGTTTTGGGACCCTTCT	130 bp	63

2.7.2. qRT PCR

The Go Taq qPCR master mix from Promega Biotech India (A6001) was used to study the expression of various genes in the open system qPCR machine by Qiagen (Rotor-Gene Q). The experiment was run as per the kit guidelines. The amplification parameters were as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, the annealing temperature according to Tm for each primer and extension at 72 °C for 20 s, and a melting curve from 60 to 95 °C, increasing in increments of 0.5 °C every 5 s. Normalization was performed using the housekeeping gene, β-Actin. The ΔΔCt/Comparative Ct method (Livack and Schmittgen, 2001) was used to calculate the fold expression (given by $2^{-\Delta\Delta Ct}$) in each group to achieve the relative quantification. The threshold cycle (Ct) is the cycle number at which fluorescence is detected by the machine.

$$\Delta\Delta Ct = \Delta Ct \text{ (Treatment)} - \Delta Ct \text{ (Control)}$$

where, ΔCt = {Ct (Target gene) − Ct (Housekeeping gene)}.

2.8. Gas Chromatography-Mass Spectrometry

Both the samples were analyzed using a gas chromatograph (GC-7890B, Agilient Technologies, Wilmington, NC, USA) and a mass spectrometer detector (MS-7000D, Agilient Technologies, Wilmington, DE, USA) at the Centre for Residue and Quality Analysis (RCRQA), SKUAST-Kashmir. Ethanolic and hexanic extracts of *C. longa* were dissolved in

ethanol and hexane, respectively, to a concentration of 5 ppm. The samples were filtered using syringe filters and then analyzed using GC-MS. The operating conditions were set as the injector volume 1 µL, injector and detector temperatures 280 °C, inlet flow 1 mL/min, and the oven temperature was programmed at an initial temperature of 60 °C, rising to 310 °C. The run time was 40.5 min, and helium was used as a carrier gas. Compound identification in the extracts was based on GC retention times and computer matching with the library. The relative percentage (Area Sum %) of the constituents was calculated from the GC peak areas.

2.9. Statistics

The data generated were subjected to appropriate statistical analysis by using appropriate software (Mini Tab 21.1.0, Pennysylvia University, Philadelphia, PA, USA). The results were analyzed using one-way ANOVA followed by Tukey's multiple comparisons test.

3. Results

3.1. Cytotoxicity/MTT Assay

Both the extracts were tested for cytotoxicity using different doses of 10, 30, 50, and 100 µg/mL overnight. The extracts had no cytotoxic effects up to a dose of 50 µg/mL compared to the control (Figure 1). Therefore, the dose of 50 µg/mL was used in the subsequent experiments.

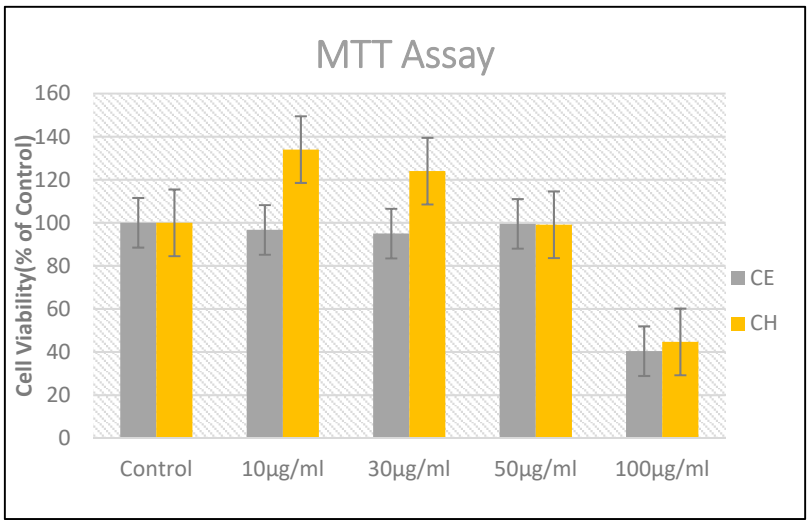

Figure 1. Cytotoxicity assay using different doses (10, 30, 50, and 100 µg/mL) of Hexanic (CH) and Ethanolic (CE) extracts of *C. longa* in BuMECs.

3.2. DPPH Assay of Extracts

Both the hexanic and ethanolic extracts showed the highest DPPH scavenging activity at the doses of 50 and 100 µg/mL with IC50 values of 8–10 µg/mL. The positive standard ascorbic acid/vitamin C showed the highest antioxidative or RAS scavenging potential with IC50 of 7.81 µg/mL. Here, we found a dose-dependent increase in the antioxidative potential of the extracts by decreasing the IC50 values from lower to higher extract concentrations (Figure 2).

3.3. Effect of HECl and EECl on TLR4 Expression

There was a significant downregulation in the mRNA expression of the TLR4 gene in both HECl and EECl-treated groups (group III and IV) as compared to the LPS-treated group (group II), wherein a significantly upregulated expression of TLR4 was seen when compared to the control group (group I) (Figure 3A).

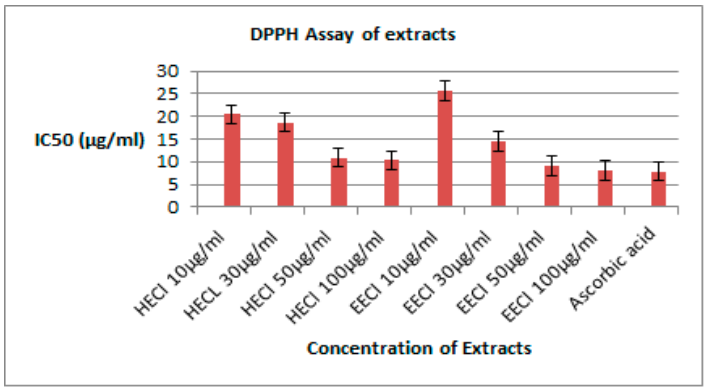

Figure 2. DPPH free radical scavenging of HECl and EECl at different doses.

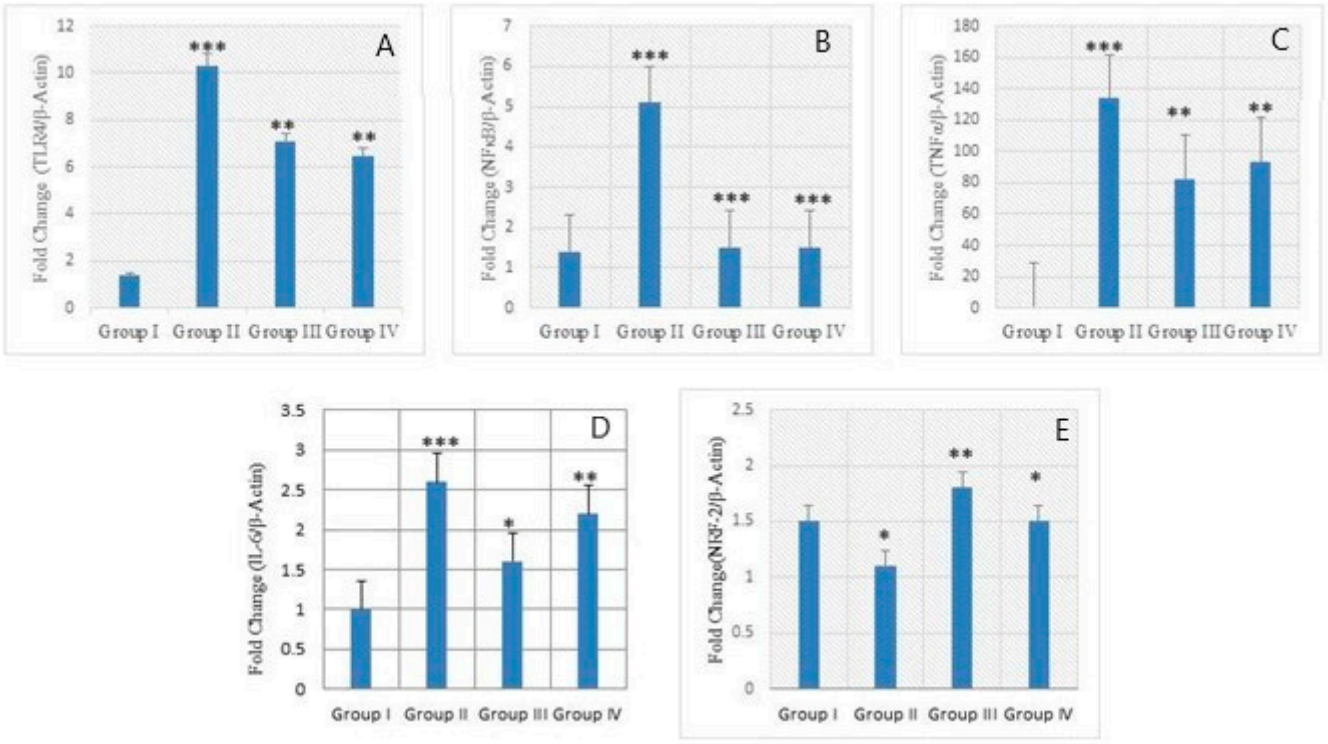

Figure 3. Relative expression of various genes in different groups of BuMECs normalized to housekeeping gene, β-Actin, using the qRTPCR technique. (**A**) TLR4 (**B**) NFκB (**C**)TNFα (**D**) IL-6 (**E**) NRF-2. Values represent mean ± SEM. Bars with ***, **, * represent $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively. Different superscript letters denote significant differences ($p < 0.05$). Comparison of Group II with Group I and Group III, IV with Group II has been conducted.

3.4. Effect of HECl and EECl on Inflammatory Gene Expression

Inflammatory cytokines viz TNFα and IL-6 were significantly downregulated in the HECl-treated group compared to the LPS-treated group (group II) (Figure 3C,D). NFκB expression was also downregulated in this group (group III) compared to group II (Figure 3B).

TNFα expression was downregulated in the EECL-treated group (group IV) (Figure 3C) compared to group II while a non-significant downregulation in the IL-6 expression was seen in this group compared to the LPS-treated group (group II) (Figure 3D). NFκB expression showed significant downregulation in group IV compared to the LPS-treated group (group II) (Figure 3B).

3.5. Effect of HECl and EECl NRF2 Expression

NRF2 mRNA expression was significantly upregulated in both groups III and IV compared to the LPS-treated group (group II) (Figure 3E).

3.6. GC-MS/MS Chromatogram Analysis

Tables 2 and 3 represent the compounds in HECl and EECl, respectively, with their area sum (%), molecular weights, RT, compound class, and common plants in which these compounds are present as observed in GC-MS/MS. Figures 4 and 5 represent the GC-MS/MS chromatograms of HECl and EECl, respectively.

Table 2. Important bioactive compounds in HECl as analyzed by GC-MS/MS.

S. No.	Compound Name	Molecular Formula	Molecular Weight (g/mol)	RT	Area Sum (%)	Compound Subclass	Common Plants
1.	Atlantone	$C_{15}H_{22}O$	218.33	11.4	0.37	Sesqueterpenoid	*Cedrus deodara, Curcuma*
2.	dihydro-ar-Turmerone	$C_{15}H_{20}O$	218.339	12.45	0.77	Sesqueterpenoid	*C. longa, Peltophorum dasyrachis*
2.	Ar-Turmerone	$C_{15}H_{20}O$	218.339	13.33	24.58	Sesqueterpenoid	*C. longa, Peltophorum dasyrachis*
3.	Tumerone	$C_{15}H_{22}O$	218.33	13.3	7.20	Sesqueterpenoid	*C. longa*
4.	beta-Turmerone) (4-methylidenecyclohex-2-en-1-yl) hept-2-en-4-one	$C_{15}H_{22}O$	218.33	14.03	11.7	Sesqueterpenoid	*Gundelia tournefortii, Turmeric*
5.	Atlantone	$C_{15}H_{22}O$	218.33	15.51	2.92	Sesqueterpenoid	*Cedrus deodara, Curcuma*

Table 3. Important bioactive compounds in EECl as analyzed by GC-MS/MS.

S. No.	Compound Name	Molecular Formula	Molecular Weight (g/mol)	RT	Area Sum (%)	Compound Subclass	Common Plants
1.	Guaiacol (2-Hydroxyanisole Phenol, 2-methoxy)	$C_7H_8O_2$	124.137	4.336	2.58	Phenol	*Solanum torvum, Guaiacum officinale*
2.	dihydro-ar-Turmerone	$C_{15}H_{20}O$	218.339	13.2	26.84	Sesqueterpenoid	*C. longa, Peltophorum dasyrachis*
3.	Tumerone	$C_{15}H_{22}O$	218.33	13.3	7.39	Sesqueterpenoid	*C. longa*
4.	Curlone	$C_{15}H_{22}O$	218.33	13.9	12.67	Sesqueterpenoid	*C. longa, Karungkuravai" rice medicinal variety*
5.	Atlantone	$C_{15}H_{22}O$	218.33	15.36	2.22	Sesqueterpenoid	*Cedrus deodara, Curcuma*
6.	Ethyl ferulate/Ethyl 4′-hydroxy-3′-methoxycinnamate	$C_{12}H_{14}O_4$	222.24	18.18	0.99	Polyphenol	*Stemona tuberosa*

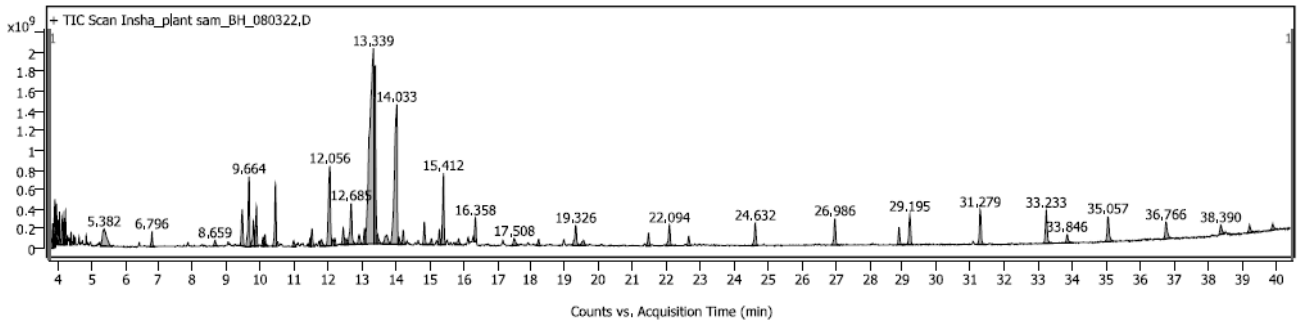

Figure 4. GC-MS/MS chromatogram of HECl.

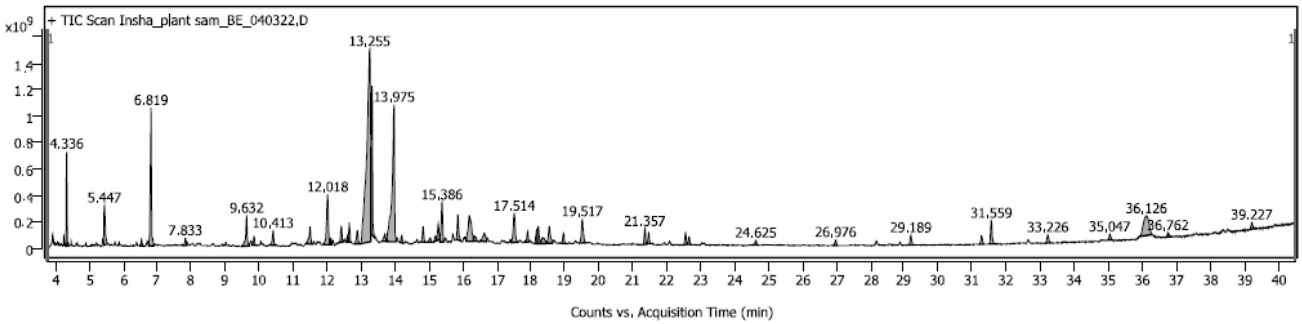

Figure 5. GC-MS/MS chromatogram of EECl.

4. Discussion

Research on plant-based medicine is gaining importance among the scientific community all over the world. The comparatively low/no side effects arising as a result of herb-based drugs compared to allopathic medicines create a high level of interest in elucidating the diseases and alleviating the properties of these plants [27]. Ethnoveterinary medicine (EVM) has been used for ages past to treat a number of animal diseases, mostly by pastoralists and rural farmers [28]. The dairy industry faces the challenge of bovine mastitis, which incurs a huge economic loss each year the world over [29].

LPS has been well studied to induce a strong inflammatory response in many cells, including Bovine Mammary Epithelial Cell lines (BMECs) [30]. TLR4 is crucial for eliciting the LPS-mediated inflammatory cascade. LPS is known to induce the mRNA expression of several proinflammatory cytokines (IL-8, IL-1β, IL-6, and TNFα) via the NFκB transcription factor activation in BMECs, which is in concomitance with our results [31]. In another study, LPS-induced oxidative stress led to the downregulation of the NRF2 gene with the simultaneous increase in superoxides and ROS levels [32]. Similar results were obtained in our study wherein LPS caused a significant upregulation of pro-inflammatory genes viz TLR4, NFκB, TNF- α, and IL-6 with a simultaneous downregulation of the NRF-2 transcription factor, which suggests that LPS has manifested a prominent inflammatory and oxidative stress cycle in the BuMECs.

4.1. Anti-Inflammatory Effects of C. longa Extracts

Turmeric is an important ingredient in our cuisines and has been used for ages past as an herbal remedy for various diseases. Many species of Curcuma have been used in EVM for treating mastitis [33,34]. Many post-milking teat dips and teat ointments, such as Mastidip and Mastilep, respectively, contain Curcuma as an active ingredient leading to the inhibition of the growth of mastitis and bacteria [19]. These topicals have shown remarkable results in mastitis by restoring the pH and SCC of milk, decreasing the microbial load on teat surfaces, and the process of inflammation in subclinical mastitic animals, along with the increase in milk yield. Very recently, Sedky and co-workers found that the ethanolic extract of *C. longa* shows antioxidative action through the scavenging free radicals and antibacterial

activity by diminishing growth of a wide range of mastitic milk isolated bacteria such as *E. coli*, *S. aureus*, *Strep. Agalactiae*, *Bacillus subtilis,* and *Pseudomonas* [35]. Many in vivo and in vitro studies also validate the essential biological roles, such as the anti-inflammatory, antibacterial, and antioxidative role of the bioactive compounds isolated from *C. longa*. For instance, Ghadiri et al., 2019 found that quercetin (5 µM) isolated from *C. longa* potentiated the detoxifying system and decreased aflatoxin-mediated cytotoxicity in Bovine MECs [36].

C. longa extracts are known to cause a significant antioxidative and anti-inflammatory effect in LPS-induced RAW 264, a macrophage cell line, by downregulating iNOS and TLR4, respectively [37]. So far, no study is available which has proposed the anti-inflammatory effect of the raw extract of *C. longa* on LPS-induced BMECs or in mastitic rat models using expressional analysis. The administration of curcumin, as an important constituent of *C. longa,* leads to the downregulated expression of classical inflammatory genes such as TLR4, NF-κB, IL-6, IL-1β, and TNFα in the LPS-induced mastitis of mice [21]. In the current study, both the extracts of *C. longa* viz HECl and EECl led to a significant downregulation of TLR4, NF-κB, and TNFα mRNA gene expression compared to the LPS group (Group II). However, IL-6 mRNA levels were downregulated significantly in HECl and non-significantly in EECl treated cells. Similarly, the downregulation of proinflammatory cellular molecules using bioactive compounds of *C. longa* has been seen in dental pulp stem cells (hDPSCs) induced with 2-hydroxyethyl methacrylate (HEMA) [38]. This invitro study proposed that nanotechnology-based liposomal curcumin formulation downregulates the NF-κB/ERK transcription factor mediating inhibition of the Monocyte Chemoattractant Protein-1 (MCP1) and Interferon-gamma (IFNγ) along with IL-6 and 8. Furthermore, nanocurcumin administration in animal models significantly decreased the protein expression of TLR4, NF-κB, and TNFα, thereby validating the anti-inflammatory effect of nanocurcumin on cerulein-induced acute pancreatitis [39]. Our study is in corroboration with a very recent study conducted by Li et al., 2021, which proposes that treatment with curcumin causes a downregulation in NF-κB along with proinflammatory cytokines such as IL-6, IL-8, IL-1β, and TNFα in the LPS-induced inflammation of MAC-T: an immortalized bovine mammary epithelial cell line [5]. A non-significant effect of EECl on IL-6 expression might be possible in our study, and here, some other interleukins might be involved in the inflammatory cycle, which needs to be studied in the future.

4.2. Anti-Oxidative Effects of C. longa Extracts

4.2.1. RAS Activity of the Extracts

The analysis of the antioxidative potential of the extracts was performed by evaluating the potential extracts to quench DPPH radicals through their hydrogen-donating ability. The conversion of DPPH to 1-1diphenyl-2-picryl hydrazine, a stable diamagenetic molecule, is observed through a change in color from purple to yellow. A more intense yellow color indicates the increasing RAS activity of the extract. In the present study, all the concentrations of both HECl and EECl demonstrated remarkable DPPH scavenging abilities with IC50 values of <20 µg/mL, explaining the potent antioxidative potential of the *C. longa* extracts. According to Souri et al., 2008, it has been postulated that plant extracts with IC50 < 20 µg/mL are known to have significant antioxidation properties [40]. Similarly, in our study, all the extracts showed significant free radical degradation abilities, which increased in a dose-dependent manner as observed by decreasing IC50 values. Our results are in corroboration with the findings of Kodjio et al., 2016 who also found the highest DPPH scavenging activity of *C. longa* extracts at a dose of 200 µg/mL [41].

Here, we found a more significant free radical scavenging property in ethanolic extracts of *C. longa* at doses of 50 and 100 µg/mL compared to hexanic extracts at the same doses, with IC_{50} values almost similar to ascorbic acid indicating EECl to be a potent antioxidative candidate. Studies have shown that ethanolic extracts of *C. longa* possess a higher antioxidant activity compared to aqueous extracts, as found by their lower IC_{50} concentration of 1.08 µg/mL [42]. The higher antioxidative properties of *C. longa* extracts may be attributed to the presence of different bioactive constituents responsible for increasing the antioxida-

tive depot. Phenolics, terpenoids, and flavonoids are largely involved in maintaining the antioxidative properties of *C. longa* as they have strong reducing capacities.

4.2.2. Effect on NRF-2 Gene Action

The antioxidative properties of *C. longa* are indispensable and highly significant as far as the alleviation of ROS levels and other oxidative parameters are concerned. A number of studies postulate that curcumin from *C. longa* causes the activation of oxidative stress related to the transcription factor, i.e., NRF2 along with KEAP (Kelch-like ECH-associated protein 1), which highly potentiates cellular protection against oxidant insult [43]. Recently, the antioxidative effect of curcumin in LPS-induced oxidative injury in MAC-T cells through the activation of NRF2, along with a decrease in ROS levels, was studied and is in corroboration with our result. In our study, both the extracts of *C. longa* showed a significant upregulation in the mRNA expression of the NRF2 gene compared to group II. Several other studies validate the crosstalk between NRF2 and NF-κB signaling. For example, curcumin, by inhibiting NF-κB and activating the Nrf2/HO-1 axis, leads to the alleviation of quinocetone-induced apoptosis injury in human leukocytic cells [44] and muscle damage in vivo animal models [45].

Another important bioactive compound of *C. longa*, i.e., ar-turmerone (aromatic turmerone/ART), also displays many essential activities such as anti-inflammatory, antimicrobial, antibacterial, and antioxidative qualities. Turmerone is structurally similar to curcumin and 6-shogaol. The attenuation of LPS-induced neuroinflammation in mice using turmerone extracted from *C. longa* essential oil was studied by Chen et al., 2018 [46]. A significant downregulation in the TLR4-mediated inflammatory cascade with the deceased protein expression of inflammatory markers such as TNFα, IL-1β, and NFκB was observed in turmerone-treated mice. The anti-neuroinflammatory and antioxidative effects of ART are explained in the study of Park et al., 2012 where the downregulation of both proinflammatory and oxidative stress genes was validated [47]. The NFκB dependent downregulation of inflammatory mediators such as MCP-1, IL-1β, IL-6, and TNF-α with a concomitant decrease in ROS and iNOS levels was seen in the affected microglial cell.

4.3. Phytoconstituents in C. longa Extracts

Here, in our study, the GC-MS/MS of both HECl and EECl showed the highest presence of ART. Sesqueterpenoids and phenols were the major active compound classes analyzed in the *C. longa*. HECl was rich in sesqueterpenoids viz ART (24.58%), tumerone (7.20%), Beta-turmerone (11.7%), dihydro ar-turmerone (0.77%), and atlantone (0.37%). On the other hand, EECl was rich in sesqueterpenoids viz dihydro ar-turmerone (26.84%), curlone (12.67%), tumerone (7.39%), and atlantone (2.2%), phenols viz Guaiacol (2-Hydroxyanisole Phenol, 2-methoxy) (2.58%), and ethyl ferulate/ethyl 4′-hydroxy-3′-methoxycinnamate (0.99%). Previous GC-MS/MS studies have also confirmed the presence of these sesqueterpenoids in the essential oil from *C. longa* rhizome and their importance for exhibiting a wide range of pharmacological properties [48].

Phenolic compounds are secondary metabolites of plant metabolism, and these compounds have received considerable attention because of the exhibition of potential bioactivity such as antioxidant, antimicrobial, antipyretic, antibacterial, antihypercholestremic, etc. [49]. The prominent metal ion chelating and free radical scavenging activity are quite fundamental to phenolic compounds, and therefore, these compounds can be candidate drugs for disease prevention and cure [50]. After careful analysis of the published articles, we found that two phenolic compounds, i.e., Guaiacol and ethyl ferulate, which were found in the current study, have not been reported so far in *C. longa.*

5. Conclusions

HECl downregulated the inflammation in LPS-induced BuMECs through TLR4/IL-6/TNFα/NFκB signaling pathways. On the contrary, EECl downregulated the LPS-induced inflammation via TLR4/TNFα/NFκB. Both extracts were able to decrease the LPS-induced

oxidative stress by upregulating the NRF2 gene expression. The high DPPH free radical scavenging activities of the *C. longa* extracts were confirmed by their corresponding low IC50 values and also indicates the high antioxidative potentials of *C. longa* extracts. The anti-inflammatory and antioxidative action of the *C. longa* extracts might be due to the presence of phytoconstituents such as sesque-terpenoids, phenolic compounds, and certain essential oils, which need to be further validated. Furthermore, studies are warranted to determine the precise mechanism at the protein level and elucidate the regulation of other downstream genes which might be involved in the protective effects of *C. longa* against LPS-induced inflammation in BuMECs. The effect of the tandem mass spectroscopy detected phytoconstituent on the LPS-induced inflammation in BuMECs needs to be studied vividly using high throughput techniques.

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