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Vedula Vijaya Kameswari

Department of Veterinary
Microbiology, NTR College of
Veterinary Science,
Gannavaram, Sri Venkateswara
Veterinary University, Tirupati,
Andhra Pradesh, India

Ashok Kumar Kolli

Department of Veterinary
Microbiology, NTR College of
Veterinary Science,
Gannavaram, Sri Venkateswara
Veterinary University, Tirupati,
Andhra Pradesh, India

P Anand Kumar

Department of Veterinary
Microbiology, NTR College of
Veterinary Science,
Gannavaram, Sri Venkateswara
Veterinary University, Tirupati,
Andhra Pradesh, India

Corresponding Author:

Vedula Vijaya Kameswari

Department of Veterinary
Microbiology, NTR College of
Veterinary Science,
Gannavaram, Sri Venkateswara
Veterinary University, Tirupati,
Andhra Pradesh, India

Immunomodulatory activity of quercetin and thymol on bovine neutrophil functional activity and lymphocyte blastogenesis during *in vitro* *Escherichia* *coli* infection

Vedula Vijaya Kameswari, Ashok Kumar Kolli and P Anand Kumar

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Abstract

Escherichia coli is an important Gram-negative pathogen causing several infectious diseases in buffaloes. The present study evaluated the immunomodulatory potential of thymol and quercetin on innate and adaptive immune responses of buffaloes under *in vitro* conditions. Neutrophil phagocytic activity was assessed using the nitroblue tetrazolium (NBT) reduction assay, whereas lymphocyte proliferation was determined by the XTT assay using peripheral blood mononuclear cells (PBMCs). Treatment with thymol and quercetin significantly enhanced neutrophil phagocytic activity and lymphocyte proliferative responses compared with untreated controls. Increased immune responses were observed following stimulation with live *E. coli*, opsonized *E. coli*, zymosan, heat-inactivated *E. coli* antigen and Concanavalin A. The findings indicate that thymol and quercetin possess promising immunostimulatory properties and may serve as potential natural immunomodulators for improving host defence against bacterial infections in buffaloes.

Keywords: Buffalo, *Escherichia coli*, immunomodulation, neutrophils, peripheral blood mononuclear cells (PBMCs), quercetin, thymol

1. Introduction

Buffaloes (*Bubalus bubalis*) play a vital role in the livestock sector by contributing significantly to milk production, meat production and rural livelihoods in many developing countries, particularly India [22]. The productivity and profitability of buffalo farming are greatly influenced by animal health, and infectious diseases remain a major constraint to sustainable livestock production. Among the bacterial pathogens affecting buffaloes, *Escherichia coli* is an important opportunistic organism associated with enteritis, mastitis, septicaemia, neonatal infections and other clinical conditions, resulting in considerable economic losses to the dairy industry [20, 21].

The increasing emergence of antimicrobial-resistant bacteria has reduced the effectiveness of conventional antibiotics and has become a major concern in both veterinary and public health [4, 19]. Consequently, there is growing interest in alternative approaches that enhance the host immune system rather than relying solely on antimicrobial therapy. Natural bioactive compounds of plant origin have attracted considerable attention because of their antimicrobial, antioxidant and immunomodulatory properties [9, 15].

Thymol, a naturally occurring monoterpene phenol present in the essential oil of thyme (*Thymus vulgaris*), possesses antimicrobial, antioxidant, anti-inflammatory and immunomodulatory activities [10, 17]. Similarly, quercetin, a naturally occurring flavonoid widely distributed in fruits and vegetables, has been reported to modulate immune cell functions, reduce oxidative stress and regulate inflammatory responses [8, 9, 13]. These phytochemicals have emerged as promising alternatives for improving immune competence and disease resistance in livestock [6, 15].

Neutrophils constitute the first line of innate immune defence against invading pathogens through mechanisms such as phagocytosis and intracellular killing, whereas lymphocytes play a central role in adaptive immunity by initiating antigen-specific immune responses [1, 16, 24]. Therefore, evaluating neutrophil functional activity and lymphocyte proliferative responses provides valuable information regarding the immunomodulatory potential of therapeutic agents.

Neutrophil function was assessed using the nitroblue tetrazolium (NBT) reduction assay, while lymphocyte proliferation was determined by the XTT assay using peripheral blood mononuclear cells (PBMCs). The study aimed to investigate whether these natural compounds could enhance both innate and adaptive immune responses and thereby serve as potential immunomodulators for improving host defence against *E. coli* infection in buffaloes.

2. Materials and methods

2.1 Blood sample collection

Blood samples were collected from apparently healthy she buffaloes (4–5 years of age) maintained under similar physiological conditions at the Livestock Farm Complex, NTR College of Veterinary Science, Gannavaram, Andhra Pradesh, during May–September 2019. Peripheral blood was collected aseptically into EDTA vacutainer tubes and used for the isolation of neutrophils and peripheral blood mononuclear cells (PBMCs).

2.2 Isolation and biochemical characterization of *Escherichia coli*

An *Escherichia coli* isolate obtained from a clinical case of bubaline mastitis was used throughout the study. The isolate was enriched in tryptic soy broth (TSB) at 37 °C for 18 h and identified based on Gram staining, growth on eosin methylene blue (EMB) agar, biochemical characterization, and PCR confirmation using *E. coli*-specific primers.

2.3 Preparation of bacterial DNA template and PCR confirmation

Genomic DNA was extracted from the bacterial culture by the high-salt extraction method with suitable modifications. PCR amplification was performed using *E. coli*-specific primers in a 25 µL reaction mixture under standardized cycling conditions. The amplified products were separated by electrophoresis on a 1.7% agarose gel stained with ethidium bromide and visualized using a gel documentation system.

2.4 Isolation of neutrophils

Neutrophils were isolated from peripheral blood according to previously described methods with slight modifications [12, 23]. Briefly, whole blood was centrifuged at 1200 × g for 20 min at room temperature. After removal of the plasma and buffy coat, the hematocrit fraction was mixed with an equal volume of 1.5% ammonium chloride solution for erythrocyte lysis. Following centrifugation, the cell pellet was washed with phosphate-buffered saline (PBS), layered over Histopaque-1077, and centrifuged to remove contaminating mononuclear cells. The purified neutrophil pellet was washed three times with PBS, resuspended in RPMI-1640 medium, and used for subsequent assays. Cell morphology was confirmed by Leishman's staining.

2.5 Neutrophil viability and counting

Neutrophil viability and cell count were determined by the trypan blue dye exclusion method using a Neubauer

haemocytometer. Only viable cells were used for subsequent functional assays.

2.6 Preparation of opsonized *E. coli*

Freshly grown 18 h *E. coli* culture was centrifuged, washed twice with sterile PBS, and adjusted to 0.5 McFarland turbidity. The bacterial suspension was mixed with an equal volume of normal bovine calf serum and incubated at 37 °C for 1 h to obtain opsonized bacteria.

2.7 Assessment of superoxide production by quantitative NBT reduction assay

Neutrophil suspension was adjusted to 5×10^6 viable cells/mL in RPMI-1640 medium supplemented with 10% fetal calf serum. Aliquots of the cell suspension were dispensed into 96-well tissue culture plates in triplicate and treated with quercetin (25 and 50 µM) or thymol (15 and 30 µg/mL). After incubation for 2 h at 37 °C in a humidified 5% CO₂ incubator, the cells were stimulated with live *E. coli*, opsonized *E. coli*, or zymosan. Nitro blue tetrazolium (NBT) solution was then added, and the plates were further incubated for 2.5 h [23]. Superoxide production was quantified by measuring the absorbance at 540 nm using a microplate reader. Appropriate blank, positive, and negative controls were included in each experiment.

2.8 Assessment of phagocytic activity

Phagocytic activity was determined by calculating the difference between the absorbance of stimulated neutrophil wells and the corresponding blank wells. All assays were performed in triplicate, and the mean absorbance values were used for analysis [23].

2.9 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by Histopaque-1077 density gradient centrifugation. Briefly, blood was diluted with an equal volume of PBS, layered over Histopaque-1077, and centrifuged at 400 × g for 40 min at room temperature. The mononuclear cell layer was collected, washed twice with PBS, and pelleted by centrifugation. Cell viability and recovery were determined by the trypan blue dye exclusion method before use in lymphocyte proliferation assays.

2.10 Assessment of lymphocyte blastogenesis by XTT assay

PBMCs were adjusted to 2×10^6 cells/mL in RPMI-1640 medium containing 10% fetal calf serum and seeded into 96-well tissue culture plates. Cells were treated with quercetin or thymol and incubated for 2 h, followed by stimulation with heat-inactivated *E. coli* or concanavalin A. After incubation for 72 h at 37 °C in a humidified 5% CO₂ incubator, freshly prepared XTT-PMS reagent was added to each well, and the plates were incubated for an additional 6 h. Cell proliferation was determined by measuring the absorbance at 450 nm using a microplate reader. The stimulation index (SI) was calculated as the ratio of absorbance of stimulated cells to that of unstimulated cells.

2.11 Statistical analysis

Statistical analysis was performed by analysis of variance using SPSS software (Version 25).

3. Results

3.1 Isolation and identification of *Escherichia coli*

The bacterial isolate produced characteristic greenish metallic

sheen colonies on eosin methylene blue (EMB) agar. Microscopic examination revealed Gram-negative coccobacilli. The isolate was positive for indole, methyl red and motility tests, and negative for Voges–Proskauer and citrate utilization tests. PCR amplification using *E. coli*-specific primers yielded the expected 662 bp amplicon, confirming the identity of the bacterial isolate (Figure 1).

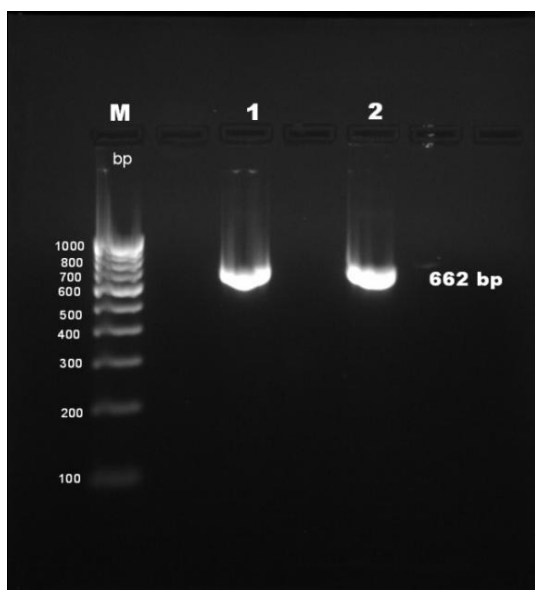


Fig 1: Polymerase Chain Reaction test for detecting *E. coli* [M = 100 bp DNA ladder; Lanes 1&2: *E. coli*.]

3.2 Effect of quercetin and thymol on neutrophil functional activity

Neutrophil functional activity was evaluated by the NBT reduction assay following stimulation with live *E. coli*, opsonized *E. coli* and zymosan. Approximately 5×10^6 neutrophils/ml were recovered from the peripheral blood of each buffalo and were confirmed by Leishman's staining (Figure 2). Superoxide production by neutrophils was indicated by the reduction of NBT. Baseline phagocytic activity of control neutrophils incubated with different stimulants was comparable among the experimental animals. The phagocytic activity of neutrophils treated with different concentrations of quercetin and thymol is presented in Tables 1–3.

3.3 Effect of quercetin and thymol on lymphocyte blastogenesis

Lymphocyte blastogenesis was assessed by the XTT assay using peripheral blood mononuclear cells (PBMCs). Lymphocyte proliferation was determined following

stimulation with Concanavalin A (Con A) and heat-inactivated *E. coli* antigen in the presence or absence of quercetin and thymol. The stimulation index of PBMCs under different treatment conditions is presented in Table 4.

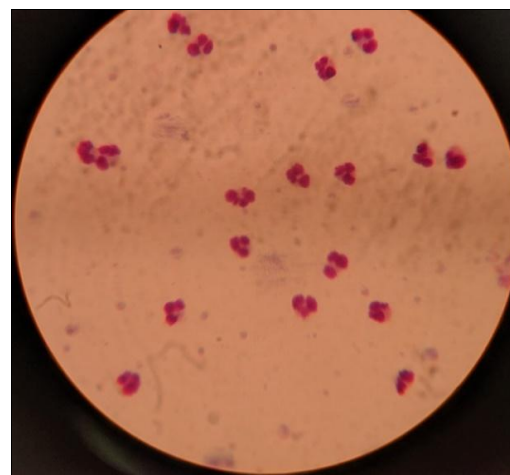


Fig 2: Neutrophils observed in Leishman staining

Table 1: Effect of quercetin and thymol on neutrophil phagocytic activity following stimulation with live *Escherichia coli*

Treatment	Concentration	Mean $\pm$ SE (OD)
Control (1% DMSO)	—	0.015 $\pm$ 0.0003
Quercetin	25 μ M	0.055 $\pm$ 0.009
Quercetin	50 μ M	0.052 $\pm$ 0.010
Thymol	15 μ g/ml	0.058 $\pm$ 0.011
Thymol	30 μ g/ml	0.052 $\pm$ 0.009

Table 2: Effect of quercetin and thymol on neutrophil phagocytic activity following stimulation with opsonized *Escherichia coli*

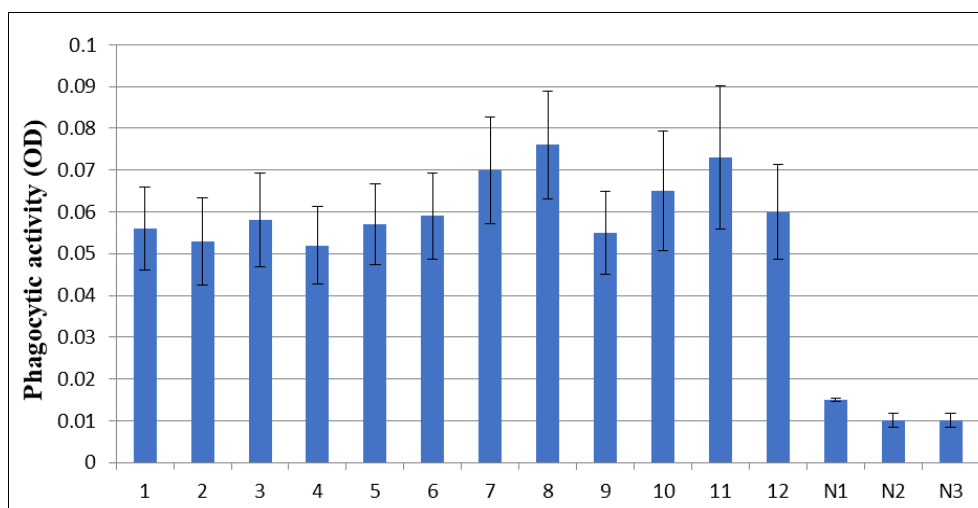
Treatment	Concentration	Mean $\pm$ SE (OD)
Control (1% DMSO)	—	0.010 $\pm$ 0.0002
Quercetin	25 μ M	0.057 $\pm$ 0.009
Quercetin	50 μ M	0.059 $\pm$ 0.010
Thymol	15 μ g/ml	0.074 $\pm$ 0.012
Thymol	30 μ g/ml	0.076 $\pm$ 0.001

Table 3: Effect of quercetin and thymol on neutrophil phagocytic activity following stimulation with zymosan

Treatment	Concentration	Mean $\pm$ SE (OD)
Control (1% DMSO)	—	0.010 $\pm$ 0.0002
Quercetin	25 μ M	0.055 $\pm$ 0.010
Quercetin	50 μ M	0.065 $\pm$ 0.014
Thymol	15 μ g/ml	0.073 $\pm$ 0.017
Thymol	30 μ g/ml	0.060 $\pm$ 0.011

Table 4: Effect of quercetin and thymol on PBMC lymphocyte proliferation

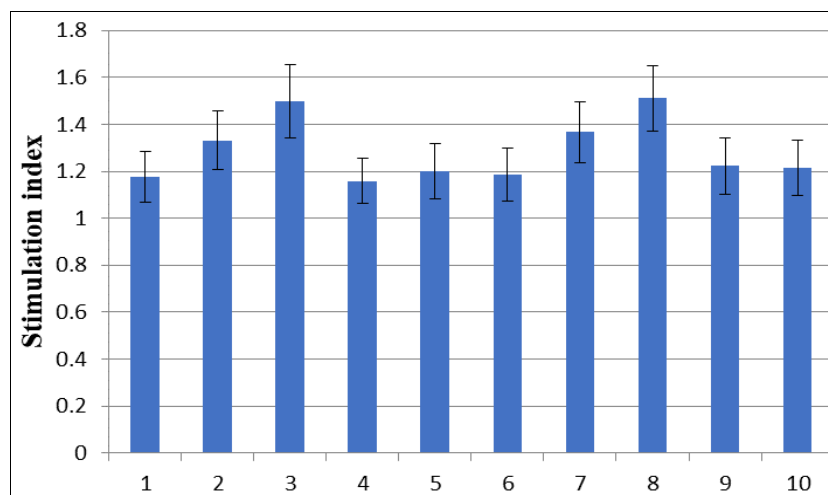
Stimulant	Treatment	Concentration	Mean $\pm$ SE (Stimulation Index)
Concanavalin A	Control	—	1.175 $\pm$ 0.107
Concanavalin A	Quercetin	25 μ M	1.332 $\pm$ 0.126
Concanavalin A	Quercetin	50 μ M	1.496 $\pm$ 0.156
Concanavalin A	Thymol	15 μ g/ml	1.159 $\pm$ 0.097
Concanavalin A	Thymol	30 μ g/ml	1.198 $\pm$ 0.117
Heat-inactivated <i>E. coli</i>	Control	—	1.185 $\pm$ 0.112
Heat-inactivated <i>E. coli</i>	Quercetin	25 μ M	1.367 $\pm$ 0.130
Heat-inactivated <i>E. coli</i>	Quercetin	50 μ M	1.510 $\pm$ 0.140
Heat-inactivated <i>E. coli</i>	Thymol	15 μ g/ml	1.224 $\pm$ 0.120
Heat-inactivated <i>E. coli</i>	Thymol	30 μ g/ml	1.216 $\pm$ 0.117



Treatment groups

Figure 3. Effect of quercetin and thymol on neutrophil phagocytic activity. Whereas, 1 = Live *E. coli* + Quercetin 25 μ M; 2 = Live *E. coli* + Quercetin 50 μ M; 3 = Live *E. coli* + Thymol 15 μ g/ ml; 4 = Live *E. coli* + Thymol 30 μ g/ ml; 5 = Opsonized *E. coli* + Quercetin 25 μ M; 6 = Opsonized *E. coli* + Quercetin 50 μ M; 7 = Opsonized *E. coli* + Thymol 15 μ g/

ml; 8 = Opsonized *E. coli* + Thymol 30 μ g/ ml, 9 = Zymosan + Quercetin 25 μ M; 10 = Zymosan + Quercetin 50 μ M; 11 = Zymosan + Thymol 15 μ g/ ml; 12 = Zymosan + Thymol 30 μ g/ ml; N1 = PMN + Solvent + Live *E. coli*; N2 = PMN + Solvent + Opsonized *E. coli*; N3 = PMN + Solvent + Zymosan.



Treatment groups

Figure 4. Effect of quercetin and thymol on PBMC lymphocyte proliferation. 1 = Concanavalin A; 2 = Quercetin 25 μ M + Concanavalin A; 3 = Quercetin 50 μ M + Concanavalin A; 4 = Thymol 15 μ g/ ml + Concanavalin A; 5 = Thymol 30 μ g/ ml + Concanavalin A; 6 = Heat inactivated *E. coli*; 7 = Quercetin 25 μ M + heat inactivated *E. coli*; 8 = Quercetin 50 μ M + heat inactivated *E. coli*; 9 = Thymol 15 μ g/ ml + heat inactivated *E. coli*; 10 = Thymol 30 μ g/ ml + heat inactivated *E. coli*.

4. Discussion

Escherichia coli is one of the most important opportunistic bacterial pathogens affecting buffaloes and is associated with mastitis, enteritis, septicaemia and other economically significant diseases [20, 21]. Owing to the increasing prevalence of antimicrobial resistance, there is considerable interest in identifying natural compounds capable of enhancing host immune responses [4, 19]. In the present study, the immunomodulatory effects of quercetin and thymol on innate and adaptive immune responses of buffaloes were evaluated under *in vitro* conditions. The findings demonstrated that both phytochemicals enhanced neutrophil functional activity and

lymphocyte proliferative responses, suggesting their potential role as natural immunomodulators. The bacterial isolate was identified as *E. coli* based on characteristic cultural, morphological and biochemical properties, and its identity was further confirmed by PCR amplification of the expected 662 bp product. Accurate identification of the pathogen ensured the reliability of the subsequent immunological assays performed in this study.

Neutrophils constitute the first line of defence against invading microorganisms through phagocytosis and intracellular killing mediated by reactive oxygen species. The nitroblue tetrazolium (NBT) reduction assay is a well-established method for assessing the respiratory burst activity of neutrophils [18]. In the present investigation, treatment with quercetin and thymol enhanced neutrophil phagocytic activity following stimulation with live *E. coli*, opsonized *E. coli* and zymosan. The increase in NBT reduction observed in treated cells indicates enhanced production of superoxide radicals, reflecting improved functional activity of neutrophils. The enhanced neutrophil activity observed with quercetin may be attributed to its antioxidant and immunomodulatory properties. Quercetin has been reported to regulate intracellular signalling pathways involved in activation of

immune cells, improve phagocytic function and protect cells against oxidative damage during inflammatory responses [8, 9, 13]. Likewise, thymol possesses antimicrobial, antioxidant and anti-inflammatory activities and has been shown to improve innate immune responses by enhancing phagocytic efficiency while maintaining cellular integrity [10, 17]. The present findings are therefore consistent with previous reports demonstrating the immunostimulatory effects of these naturally occurring phytochemicals.

Lymphocyte proliferation is an important indicator of adaptive immune competence [1, 14, 24]. In the present study, PBMCs treated with quercetin and thymol exhibited enhanced proliferative responses following stimulation with Concanavalin A and heat-inactivated *E. coli* antigen, indicating improved lymphocyte activation. These findings suggest that both compounds are capable of modulating cell-mediated immune responses in addition to enhancing innate immunity. The greater stimulation index observed in treated PBMCs may be associated with the ability of quercetin and thymol to regulate cytokine production, promote lymphocyte activation and reduce oxidative stress, thereby improving cellular responsiveness. Previous studies have demonstrated that phytochemicals can influence immune cell proliferation and function through modulation of intracellular signalling pathways and inflammatory mediators [6, 15]. The present observations support these findings and indicate that natural plant-derived compounds may serve as effective immunomodulators in livestock.

Overall, the present investigation demonstrates that both quercetin and thymol positively influenced innate and adaptive immune responses of buffaloes under *in vitro* conditions. Enhancement of neutrophil phagocytic activity together with increased lymphocyte proliferation suggests that these compounds may improve host defence mechanisms against bacterial infections. Considering the growing concern regarding antimicrobial resistance, natural immunomodulators such as quercetin and thymol may represent promising adjuncts for improving disease resistance and reducing dependence on conventional antimicrobial therapy. Nevertheless, further *in vivo* investigations are required to establish their efficacy, optimal dosage and practical application under field conditions.

5. Conclusion

The present study demonstrated that thymol and quercetin enhanced both innate and adaptive immune responses of buffaloes under *in vitro* conditions. Treatment with these phytochemicals improved neutrophil phagocytic activity, as evidenced by increased NBT reduction following stimulation with live *Escherichia coli*, opsonized *E. coli* and zymosan, and also promoted lymphocyte proliferation in PBMCs stimulated with Concanavalin A and heat-inactivated *E. coli* antigen. These findings indicate that thymol and quercetin possess promising immunomodulatory properties and may serve as potential natural alternatives for improving host defence against bacterial infections. Considering the increasing concern over antimicrobial resistance, the use of such plant-derived immunomodulators could contribute to sustainable disease management strategies in livestock. However, further *in vivo* studies are required to validate their efficacy, safety and practical application under field conditions.

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Conflict of Interest

Not available

Financial Support

Not available

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