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A preliminary study on the effect of intrauterine infusion of neem oil on innate immunity, endometrial inflammatory cytokines, recovery, and conception rate in repeat breeder dairy cows

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ABSTRACT

The present study evaluated the immuno-modulatory effect of intrauterine infusion of Neem oil (twice at 24 h interval) in repeat breeding cows (n=6) suffering with sub-clinical endometritis and compared with untreated control (n=6). There were no changes in the mean blood leucocyte count, lymphocytes (%), neutrophils (%) and monocyte (%) between and within groups. The phagocytic activity of blood neutrophils and monocyte was increased at next estrus compared to that at 0 h and 24 h of treatment in treated cows (NO), while, it did not differ in untreated control. The mean stimulation index (lymphocyte proliferation) was significantly higher at next estrus in NO treated cows compared to untreated control. The endometrial mRNA expression of IL-6 (0.18 fold), IL-1α (0.25 fold) and TNF-α (0.38 fold) were downregulated after the treatment in treated cows, while the mRNA transcript of IL-10 (3.28 fold) was upregulated after the treatment in group NO compared to control. All the cows showed negative endometrial cytology for PMNs after the treatment in NO group. The cervical mucus bacterial load reduced significantly at next estrus in treated cows compared to untreated control as well as after the treatment compared to before the treatment. The first service conception rate was 50% (3/6) in treated cows, while, the corresponding figure in untreated control was 16.67% (1/6). In conclusion, methanolic extract of neem oil may improve the recovery and conception rate in cows with sub-clinical endometritis through its bactericidal and immunomodulatory activity.

Keywords: Conception rate, Cow, Cytokines, Endometritis, Innate immunity, Neem oil

Endometritis is a common condition in dairy cows that negatively impacts reproductive efficiency and increases herd health cost (Sheldon *et al.* 2009). During the peripartum period, cows experience physiological changes that compromise their immune system and increase susceptibility to uterine infections (Varhidi *et al.* 2024). Neutrophils play a crucial role in protecting against invading microorganisms, and their decreased activity during the peripartum period is believed to influence the occurrence of uterine infections in cows. The cytokines system initiates signaling cascades that mobilize and activate immune cells, leading to the influx of PMNs and macrophages into the uterus and the secretion of antimicrobial factors by endometrial epithelial cells to eliminate potential pathogens (Herath *et al.* 2009).

Common treatment options for endometritis are prostaglandin analogues and antimicrobials (Sheldon *et al.* 2009, Ludbey *et al.* 2022). Antimicrobial use in the treatment of endometritis has come into question because of the growing prevalence of antimicrobial resistance (AMR) and probably residues in milk. Further, antibiotics destroy the phagocytic activity of polymorphonuclear leucocytes (Brodzki *et al.* 2017), inconsistent treatment success, higher cost and having milk residual effect. Use of the hormones requires sound reproductive knowledge. Hence, alternatives to antimicrobials are the need of the hour to treat endometritis.

Neem is the most versatile medicinal plant in India, thanks to its wide range of biological activity (Biswas *et al.* 2002). It is known for its excellent antibiotic and immunomodulatory properties (Upadhyay *et al.* 1992), making it a promising therapy for endometritis in repeat-breeding cows (Kumar *et al.* 2013c). However, its effect on uterine immunity and leucocyte functions is poorly known. Further, limited work has been carried out on the therapeutic effect of neem oil in cow with chronic endometritis, particularly during the late postpartum period. The present study evaluated the immunomodulatory effect of neem oil on recovery from sub-clinical endometritis in repeat breeding cows and the subsequent conception rate.

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MATERIALS AND METHODS

The present research work was conducted from March 2023 to August 2023 and approved by the Institutional Animal Ethics Committee (IAEC) vide no. VETCOLL/IAEC/2022/20/PROTOCOL-30.

Experimental animals: From a total of 25 repeat breeding crossbred cows, those evidencing subclinical endometritis based on endometrial cytology (n=12) (Gilbert *et al.* 2005) were treated at estrus either with 25 mL of methanolic extract of Neem oil (n=6) infused intrauterine for two days (NO) or with 25 mL of normal saline (n=6) for two days (C). At subsequent estrus, the endometrial cytology samples were obtained again and cows were inseminated with high fertility frozen semen and followed for pregnancy confirmation at 60 days post-insemination by trans-rectal examination.

Methanolic extract of neem oil (Unjha Ayurvedic Pharmacy, Unjha, Gujarat, India) was prepared according to Kumar *et al*. (2013b) with some modifications. In brief, Neem oil was processed for fractionation by mixing equal volume of methanol. This mixture was vigorously vortexed for 10 min and allowed to stand for another 10 min. The uppermost methanol miscible portion was collected and filtered with syringe filter. Further, filtered portion was kept in large sterile petri-dish at room temperature for complete evaporation of the methanol and stored at room temperature in a glass bottle.

Collection of blood samples: Blood samples were collected by jugular venipuncture aseptically using 21-G needle in sterile heparinized vacutainers before the treatment (0 h, at estrus), 24 h after the treatment and at subsequent estrus.

Collection of endometrial cytology samples: The endometrial cells were collected at estrus before treatment and on subsequent estrus after treatment using cytobrush (Care India Surgicals, Delhi, India) according to Ghasemi *et al*. (2012). Two separate cytobrush samples were collected using two different sterile cytobrush assembly. One cytobrush was used for endometrial cytology and another cytobrush sample was used for endometrial cytokines mRNA expression. Cytobrush was detached from the assembly and placed in sterile DEPC (0.1% Diethyl pyrocarbonate, HiMedia, India) treated microcentrifuge tube containing 1 mL Trizol reagent (Qiagen, Germany) transported in the thermocol box containing icepacks and stored at -20°C until further processing.

Innate immunity: The total leukocyte count (TLC) and lymphocytes (%), neutrophils (%) and monocyte (%) were estimated by automatic haematology analyzer (Exigo Hematology Analyzer, Boule Medical AB, Sweden) immediately after collection of blood samples. Phagocytic activity (PA) and Lymphocyte Proliferation Assay (LPA) were assessed according to Khatti *et al*. (2017).

mRNA expression of endometrial inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-10 and TNF): RNA was isolated using Trizol method according to Ghasemi *et al.* (2012). RNA quantity and purity was checked using the Agilent Biotek microplate reader (Agilent Technologies, USA) at wavelengths of 260 nm and 280 nm. The cDNA was synthesized from 1 µg of total RNA using commercially available Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions and were stored at -20°C till further use.

Published primers (Table 1) were synthesized commercially (Eurofins Genomics, Bengaluru, India). Primers were first tested using end-point PCR to optimize amplification conditions. The β-actin was used as an endogenous reference gene. All the Real-time PCR reactions in duplicate were carried out in a total volume of 20 µL with 1 µL cDNA (10 ng/µL), 10 µL SYBR Green master mix (Thermo Fisher Scientific), 0.6 µL forward, and 0.6 µL reverse primers (10 pmol of each) and 7.8 µL nuclease-free water. Dissociation curves were examined for the presence of a single PCR product. Real-time PCR was performed using an Applied Biosystem 7500 with the following cycling parameters: 95°C for 15 min and 40 cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 30 s,

Table 1. Oligonucleotides sequences used in qPCR

Gene		Primer Sequence (5'-3')	Amplicon size (bp)	Reference
IL-1 α	F	AGGTCCATACCTGACGGCTA	189	Dadarwal *et al.* (2019)
	R	TTGGGTGTCTCAGGCATCTC		
IL1- β	F	AAAAGCTTCAGGCAGGTGGT	155	
	R	AACTCGTCGGAGGACGTTTC		
IL-8	F	CGCTGGACAGCAGAGCTCACA	106	
	R	TGCCAAGAGAGCAACAGCCAGC		
IL-10	F	TGATGCCACAGGCTGAGAAC	135	
	R	CCGCCTTGCTCTTGTTTTCG		
TNF- α	F	CTCTTCTGCCTGCTGCACTTC	205	
	R	CCATGAGGGCATTGGCATACG		
IL-6	F	ATGACTTCTGCTTTCCCTACCC	180	Saut *et al*. (2014)
	R	GCTGCTTTCACACTCATCATTC		
β- actin	F	CTAGGCACCAGGGCGTAATG	177	Dadarwal *et al.* (2019)
	R	CCACACGGAGCTCGTTGTAG		

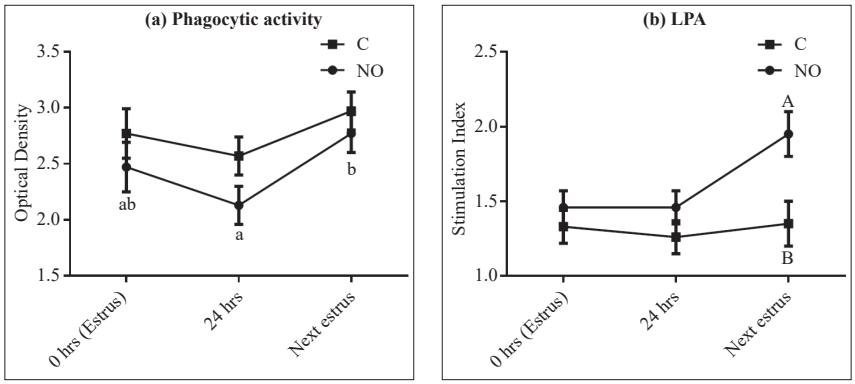

Fig. 1. Effect of intrauterine neem oil on (a) phagocytic activity and (b) lymphocyte proliferation assay (LPA) of peripheral blood mononuclear cells (PBMC) in subclinical endometritic cows [a:b = P<0.05 (Within group); A:B = P<0.005 (Between the groups). NO: Treated cows, C: Untreated control].

followed by amplicon dissociation (95°C for 30 s, 65°C for 30 s, 95°C for 30 s). Gene expression results were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Fold changes of cytokines mRNA transcripts in the treatment (group I) to the control (group II) and before relative to after treatment within groups were determined.

Recovery rate and conception rate: Recovery rate was assessed by endometrial cytology and bacterial load before treatment and at subsequent estrus after treatment. The first service conception rate was also calculated. The cervico-vaginal mucus was obtained using a sterilized plastic sheath and 20 mL disposable syringe, transferred into sterile plastic containers and transported in the thermocol box containing icepacks to the laboratory to count bacterial load within 1h of collection.

The bacterial load, Colony Forming Unit (CFU/ mL) in the CVM was determined by using serial dilutions of samples as per the standard technique according to Mali *et al.* (2020). The CFU/plate was calculated from a plate by manual counting of discrete bacterial colonies and then CFU/mL was derived mathematically by multiplying the dilution factor and number of colonies grew on NA.

Statistical analysis: The statistical difference in mean TLC, DLC PA and LPA between the groups and at the different time points were analysed using repeated measure ANOVA. The difference in fold change of cytokines (*IL-α, IL-1 β, IL-6, IL-8, IL-10* and *TNF-α*) mRNA expression between the groups as well as before and after the treatment were analysed using two-way ANOVA. The bacterial load variable was analysed by non-parametric Friedman test and data were presented as median (range). Data were presented as mean±S.E. Data were analysed using SPSS software (IBM® SPSS® statistics, version 20.0). Significance was set at 95%. The graphs were prepared using GraphPad Prism v. 6.

RESULTS AND DISCUSSION

The mean circulatory TLC, % lymphocytes, neutrophils and monocytes did not change after the treatment between and within the groups (Table 2). There was no significant (P=0.768) interaction of group and time on the phagocytic activity of neutrophils and monocytes (Fig. 1). The mean O.D. of formazan crystal did not differ significantly (P=0.135) between the groups at any time point. However, it was significantly (P<0.05) increased at the next estrus than the 0 h and 24 h of treatment in group NO, while it did not differ in group C. The LPA, indicated by the stimulation index was significantly higher at the next estrus

Table 2. Effect of intrauterine infusion of neem oil on circulatory Mean (± SE) total leucocyte count (TLC ×10³/µL), mean (%) of lymphocyte, neutrophils and monocytes in cows with sub-clinical endometritis

Group	0 h (Estrus)	24 h	Next estrus	P-value		
		TLC (×10³/µL)		G	T	G×T
NO	7.4 ± 0.91^{ab}	7.9 ± 0.74^{a}	5.92 ± 0.95^{b}	0.572	0.02	0.167
C	8.4 ± 0.91^{a}	7.52 ± 0.74^{a}	7.25 ± 0.95^{a}			
		Lymphocytes (%)				
NO	75.16 ± 0.11	70.63 ± 0.48	70.72 ± 0.46	0.628	0.234	0.234
C	74.29 ± 0.11	68.51 ± 0.49	64.74 ± 0.46			
		Neutrophils (%)				
NO	17.00 ± 0.12	22.07 ± 0.50	20.59 ± 0.49	0.832	0.324	0.972
C	17.23 ± 0.12	23.24 ± 0.50	22.73 ± 0.49			
		Monocytes (%)				
NO	6.91 ± 0.03	7.69 ± 0.05	8.34 ± 0.02	0.394	0.701	0.414
C	7.37 ± 0.03	6.27 ± 0.05	6.91 ± 0.02			

Means bearing different superscripts (a,b) differ significantly within row (P<0.05). NO, Treated cows; C, Untreated control.

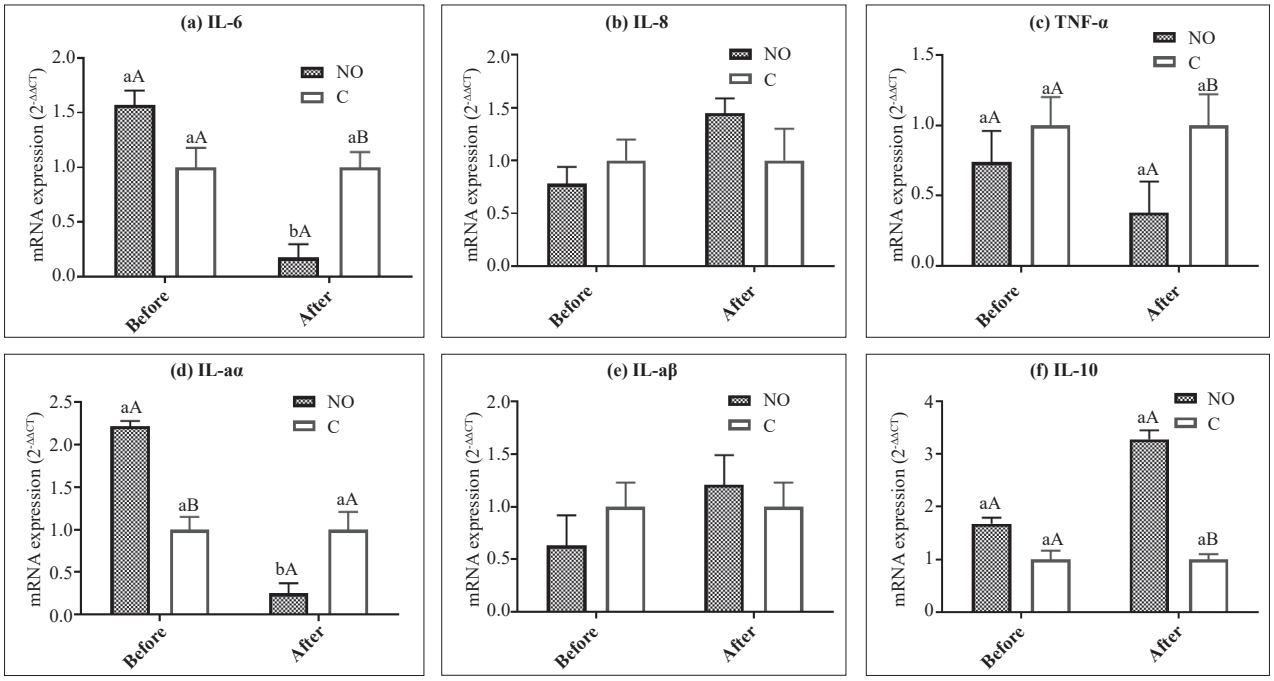

Fig. 2. Effect of intrauterine infusion of neem oil on mRNA expression ($2^{-\Delta\Delta CT}$) of cytokines in endometritic cows [a:b, P<0.05 (Within group); A:B, P<0.005 (Between the groups); IL, Interleukins; TNF, Tumor necrosis factor; NO, Treated cows; C, Untreated control].

in neem oil-treated cows (group I) than in the control group (Fig. 1). Apparently increased mean TLC at 24 h and higher neutrophils (%) counts after the treatment is in accordance with earlier report (Kumar *et al.* 2013a, Krishnan *et al.* 2015). The significant difference between the groups was not observed in the present study, can be attributed to low statistical power or spontaneous self-cure (Noakes *et al.* 2019) from subclinical uterine infection in control group. Further, subclinical infection has little effect on the general health of cows. Decreased neutrophilic respiratory burst and phagocytic activity around calving leads to postpartum diseases, such as endometritis, mastitis, metritis and retained placenta (Khatti *et al.* 2017). The neutrophils' phagocytic activity is one of the essential defence line against the establishment of uterine infections (LeBlanc 2008). Talpur and Ikhwanuddin (2013) proved that neem (*A. indica*) leaf extract in the diet could significantly increase the phagocytic activity of WBCs and affect the haematological and immunological parameters and survival rate of fish (*Asian seabass*) against viral infection. No literature is available on the impact of neem on functional activity of leucocyte in domestic animals. The research suggests that neem has immunomodulatory properties that stimulate the immune system through macrophage activation and leucocyte sub-population enhancement (Upadhyay *et al.* 1992, Talwar *et al.* 1997). The immunomodulatory activity of neem seed extract may be due to the presence of bioactive molecules such as triterpene, carbohydrate, and phenol (De and Mukherjee 2009). Further, they have also revealed that intramammary infusion of neem seed powder extract increased the *IL-2* cytokine and interferon-gamma expression in milk cells of cows with mastitis. Improved PA and LPA after the intrauterine neem oil infusion in treated Group (NO) cows may be due to selective activation of the cell-mediated immunity by stimulating lymphocytes and, especially, macrophages for phagocytosis by enhancing their antigen-presenting ability (Sarkar *et al.* 2021).

The mRNA expression of *IL*-6 was significantly (P<0.05) reduced after the treatment in treated cows (NO) (Fig. 2). Further, the mRNA transcript of *IL*-6 (0.18 fold-change) and *TNF-α* (0.38) fold change) were significantly downregulated, and, L-*10* was significantly (P<0.05) upregulated (3.28 fold) after the treatment (NO) than untreated control (C) (Fig. 2). The mRNA expression of *IL*-8 and *IL*-1*β* was not moderated. Ruslie and Darmadi (2020) reported that reduced IL-6 and TNF-*α* expression and increased IL-10 genes in colitis-induced rats fed with neem leaf extract. Kaur *et al.* (2004) observed a significant reduction of IL1 concentration in *in vitro* macrophage culture media in male Wistar rats fed with nimbidin (the active component of neem) for three consecutive days. The significant downregulated *IL-6*, *TNF-α* and *IL-1* α mRNA transcript in the present study may be due to the anti-inflammatory activity of neem (Sarkar *et al.* 2021) and suggest the recovery from the inflammation.

All the endometrial cytology-positive treated cows (NO) became cytology-negative for PMNs at the subsequent estrus, while only two were cytology-negative in untreated control (χ^2_{dfl}= 12.00; P=0.001). The cervical bacterial load ($\times 10^3$ cfu/mL) was significantly (P<0.05) reduced after than before the treatment in treated cows. Further, the bacterial load was also significantly (P<0.05) reduced after the treatment in treated cows (2.47×10^3 cfu/mL) than compared to untreated control (48×10^3 cfu/mL). The bacterial load did not differ significantly in untreated control cows (Fig. 3). A total of 3/6 (50%) treated cows conceived at first service

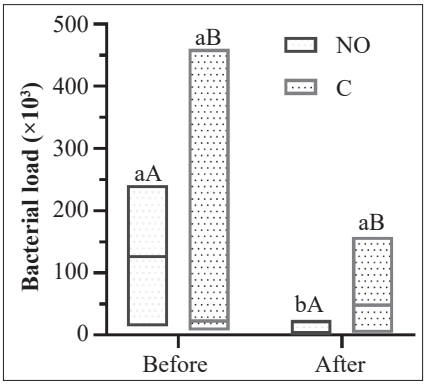

Fig. 3. Box blot showed the effect of intrauterine infusion of neem oil on bacterial load [(×10³ cfu/mL; Median (range)] in cervico-vaginal mucus (CVM) in endometritic cows. [a:b, P<0.05 (Within group); A:B, P<0.005 (Between the groups); NO, Treated cows; C, Untreated control].

while only 1/6 (16.6750%) control cows conceived. The chi-square test revealed a non-significant (*P*=0.221) difference in conception between the groups. However, cows with sub-clinical endometritis treated with neem oil were five times more likely to become pregnant compared to the untreated control [Odd Ratio (confidence interval=5 (0.46-75.79)]. Recovery rate in the present study was in accordance with previous studies (Kumar *et al.* 2013b, Nikhade *et al.* 2019). The reduction in the bacterial load after the infusion of methanolic fraction of neem oil in the current study may be due to enhancement of phagocytic activity and intracellular killing of bacteria (Williamson 2002). The results of the conception rate in the present study are in accordance with Kumar *et al.* (2013a) and Nikhade *et al.* (2019). Apparently higher conception rate in the current study may be due to enhanced phagocytic activity, proliferation of lymphocytes, stimulating anti-inflammatory cytokine, and reduced uterine bacterial load. Despite the smaller sample size, results of this study indicated significant differences between the treatment and control groups. However, the results must be interpreted as preliminary findings until further confirmation in randomized, and blind studies involving a larger number of animals is conducted.

The preliminary results suggest that methanolic extract of Neem oil (25 mL, intrauterine o.d. for two days) can improve the recovery rate and conception rate in cows with subclinical endometritis by stimulating phagocytic activity, lymphocyte proliferation, and modulating inflammatory cytokines.

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REFERENCES

Biswas K, Chattopadhyay I, Banerjee R K and Bandyopadhyay U. 2002. Biological activities and medicinal properties of neem (*Azadirachta indica*). *Current science* **10**: 1336–45.

Brodzki P, Lisiecka U, Brodzki A, Pyz-Łukasik R and Krakowski L. 2018. Phagocytic and oxidative burst activity of phagocytic cells in peripheral blood and uterine washings in cows with clinical endometritis before and after intrauterine use of cephapirin and methisoprinol. *Animal Science Journal* **89**(10): 1389–97.

De U K and Mukherjee R. 2009. Expression of cytokines and respiratory burst activity of milk cells in response to *Azadirachta indica* during bovine mastitis. *Tropical Animal Health and Production* **41**: 189–97.

Ghasemi F, Gonzalez-Cano P, Griebel P J and Palmer C. 2012. Proinflammatory cytokine gene expression in endometrial cytobrush samples harvested from cows with and without subclinical endometritis. *Theriogenology* **78**(7): 1538–47.

Gilbert R O, Shin S T, Guard C L, Erb H N and Frajblat M. 2005. Prevalence of endometritis and its effects on reproductive performance of dairy cows. *Theriogenology* **64**(9): 1879–88.

Kaur G, Sarwar Alam M and Athar M. 2004. Nimbidin suppresses functions of macrophages and neutrophils: Relevance to its antiinflammatory mechanisms. *Phytotherapy Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives* **18**(5): 419–24.

Khatti A, Mehrotra S, Patel P K, Singh G, Maurya V P, Mahla A S, Chaudhari R K, Das G K, Singh M, Sarkar M, Kumar H and Krishnaswamy N. 2017. Supplementation of vitamin E, selenium and increased energy allowance mitigates the transition stress and improves postpartum reproductive performance in the crossbred cow. *Theriogenology* **104**: 142–48.

Krishnan B B, Kumar H, Mehrotra S, Singh S K, Goswami T K, Khan F A, Patra M K and Islam R. 2015. Effect of leukotriene B4 and oyster glycogen in resolving subclinical endometritis in repeat breeding crossbred cows. *Indian Journal of Animal Research* **49**(2): 218–22.

Kumar A, Gupta H P and Prasad S. 2013a. Studies on the immunomodulatory and therapeutic efficacy of neem (*Azadirachta indca*) on endometritis in repeat breeding crossbred cows. *The Indian Journal of Animal Reproduction* **34**(2): 1–5.

Kumar H, Singh, B, Goswami T K and Rawat M. 2013b. Use of neem preparations for the treatment of endometritis in cows. *Advances in Animal and Veterinary Sciences* 10–27.

LeBlanc S J. 2008. Postpartum uterine disease and dairy herd reproductive performance: A review. *The Veterinary Journal* **176**(1): 102–14.

Livak K J and Schmittgen T D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**(4): 402–08.

Mali, S S, Rangnekar M N, Amle M B, Khillare K P, Mali A B and Mhase P P. 2020. Efficacy of intrauterine ozone therapy in repeat breeder cows with subclinical uterine infection. *Haryana Veterinarian* **59**(SI): 83–86.

Nikhade C T, Deshmukh S G, Kuralkar S V, Ratnaparkhi A R, Chepte S D and Bankar P S. 2019. Comparative efficacy of different herbal extract on subclinical endometritis in postpartum cows. *Journal of Entomology and Zoology Studies* **7**: 1422–26.

Ruslie R H and Darmadi D. 2020. Administration of neem

(*Azadirachta indica* A. Juss) leaf extract decreases TNF-α and IL-6 expressions in dextran sodium sulfate-induced colitis in rats. *Journal of Advanced Veterinary and Animal Research* **7**(4): 744.

Sarkar S, Singh R P and Bhattacharya G. 2021. Exploring the role of *Azadirachta indica* (neem) and its active compounds in the regulation of biological pathways: An update on molecular approach. *3 Biotech* **11**(4): 178.

Sheldon I M, Price S B, Cronin J, Gilbert R O and Gadsby J E. 2009. Mechanisms of infertility associated with clinical and subclinical endometritis in high producing dairy cattle. *Reproduction in Domestic Animals* **44**: 1–9.

Takeuchi O and Akira S. 2010. Pattern recognition receptors and inflammation. *Cell* **140**(6): 805–20.

Talpur A D and Ikhwanuddin M. 2013. *Azadirachta indica* (neem) leaf dietary effects on the immunity response and disease resistance of Asian seabass, Lates calcarifer challenged with *Vibrio harveyi*. *Fish and Shellfish Immunology* **34**(1): 254–64.

Talwar G P, Vanshi P R, Misra R, Mukherjee S and Shah S. 1997. Plant immunomodulators for termination of unwanted pregnancy and for contraception and reproductive health. *Immunology and Cell Biology* **75**(2): 190–92.

Upadhyay S N, Dhawan S, Garg S and Talwar G P. 1992. Immunomodulatory effects of neem (*Azadirachta indica*) oil. *International Journal of Immunopharmacology* **14**(7): 1187–93.

Várhidi Z, Csikó G, Bajcsy Á C and Jurkovich V. 2024. Uterine disease in dairy cows: A comprehensive review highlighting new research areas. *Veterinary Sciences* **11**(2): 66.

Williamson E M. 2002. *Major Herbs of Ayurveda*. 361 p, Churchill Livingstone, Edimburgh: Elsevier Science Limited.