# Effect of a natural plant ingredient — Pulsatilla saponin B4 on clinical mastitis and serum inflammatory indices in dairy cows

**Liuhong Shen** ( shenlh@sicau.edu.cn )
Sichuan Agricultural University College of Veterinary Medicine
**Bolin Qian**
Sichuan Agricultural University College of Veterinary Medicine
**Liuchao You**
Sichuan Agricultural University College of Veterinary Medicine
**Yue Zhang**
Sichuan Agricultural University College of Veterinary Medicine
**Yu Shen**
Sichuan Agricultural University College of Veterinary Medicine
**Shangkui Lv**
Sichuan Agricultural University College of Veterinary Medicine
**Jinbang Xiao**
Sichuan Agricultural University College of Veterinary Medicine
**Zhetong Su**
Guangxi Innovates Medical Technology Co., Ltd
**Ke Dong**
Sichuan Yuqiang Materia Medica Biotechnology Co., Ltd
**Xiaolan Zong**
Sichuan Agricultural University
**Shumin Yu**
Sichuan Agricultural University College of Veterinary Medicine
**Suizhong Cao**
Sichuan Agricultural University College of Veterinary Medicine
**Shilin Yang**
Jiangxi University of Traditional Chinese Medicine
**Yulin Feng**
Jiangxi University of Traditional Chinese Medicine

**Research article**

**Keywords:** Clinical mastitis, inflammatory factors, Pulsatilla saponin B4, somatic cell count

**Posted Date:** October 23rd, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-48799/v2

## Abstract

**Background:** Bovine mastitis is an inflammatory reaction of the breast caused by pathogenic bacteria infection or breast injury, which affect milk yield and quality and poses severe threat to the health of dairy cows. *Pulsatilla chinensis* has antibacterial, anti-inflammatory, and immune enhancing effects, etc. However, there was no research about the therapeutic effect of it towards the treatment of cow mastitis. In the study, twelve healthy cows were selected as the control group (group A), and 36 cows with mastitis were equally and randomly divided into group B, C and D. Group A didn't accept treatment, while group B, C and D were treated with 15, 30 and 60 mL *Pulsatilla* saponin B4 (a purified extract form *Pulsatilla chinensis*) injection by brachiocephalicus intramuscular injection once a day for 4-6 day and the optimal dose was selected from them. Then, we investigated the effect of this extract on the clearance rates of pathogenic bacteria, the regulation of somatic cell count (SCC) and inflammatory factors the in appropriate dose group.

**Results:** *Pulsatilla* saponin B4 was effective in all test groups and showed a dose-effect relationship. The extract had inhibitory effects on *Streptococcus agalactiae*, *Streptococcus dysenteriae*, *Klebsiella species*, *Mycoplasma bovis*, *Escherichia coli* and *Staphylococcus aureus*. Group C cured more cows than group B and D during the 6 days of treatment and was determined as the optimal dose group. The milk SCC, serum haptoglobin (HP), prostaglandin E2 (PGE2), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-8 (IL-8), interleukin-10 (IL-10) and tumour necrosis factor alpha (TNF-α), interleukin-4 (IL-4) and leukotriene B4 (LTB4) in Group C were significantly higher than those of healthy cows ($P < 0.05$) on the first day but had no significant difference with group A after treatment.

**Conclusion:** 30 mL *Pulsatilla* saponin B4 applied through intramuscular injection once a day for 4-6 days leaded to the significant amelioration of bacterial infection, SCC and levels of serum inflammatory factors in cows with clinical mastitis.


## Background

Mastitis in dairy cows is caused mainly by the inflammatory reaction induced by toxic products secreted by the infected bacteria [1]. With the incidence of clinical mastitis, the permeability of the mammary veins increased, which in turn enhanced the chemotaxis of leukocytes and elevated the somatic cell counts in the milk [2]. Meanwhile, inflammatory agents would lead to the synthesis and secretion of inflammatory factors, including leukotrienes B4 (LTB4), prostaglandin E2 (PGE2), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumour necrosis factor alpha (TNF-α) [3, 4] and induce hepatic secretion of C-reactive protein (CRP), serum amyloid A protein (SAA), and haptoglobin (HP) [5]. In response to this, the levels of anti-inflammatory factors such as interleukin-4 (IL-4) and interleukin-10 (IL-10) increased to inhibit the inflammation [6].

*Pulsatilla* saponin B4 is the main active ingredient of *Pulsatilla chinensis* (Bunge) Rege and plays a therapeutic role in the case of mastitis as an anti-inflammatory, anti-bacterial, and immune regulating agent [7, 8]. Recent studies showed that *Pulsatilla chinensis* has inhibitory effects on *Staphylococcus aureus*, *Streptococcus dysenteriae* and *Salmonella typhi* [9]. It inhibits the expressions of pro-inflammatory cytokines through NF-κB signaling pathway, which exhibited obvious inhibitory effect on LPS induced inflammation [10], and showd relatively high immunoregulatory activity and low cytotoxicity [11]. These pharmacological effects of *Pulsatilla* saponins closely coincided with the therapeutic needs of mastitis cows in clearing bacteria and alleviating inflammation. On the other hand, few attempts have been made to treat mastitis with *Pulsatilla* saponins.

Considered that *Pulsatilla* Saponin B4 has anti-bacterial and anti-inflammatory effects, it is hypothesized that this extract might be able to treat dairy cows with clinical mastitis. Accordingly, in this research, *Pulsatilla* saponin B4 were expected to potentially reduce infected bacteria and ameliorate the levels of inflammatory factors in cows so as to prevent and treat clinical mastitis. However, there was no vivo study of it towards treating the clinical mastitis cows. Thus, the objective of the present study was to investigate the effect of *Pulsatilla* saponin B4 on the clearance rates of bacterial infection, the regulation of somatic cell count and inflammatory factors in dairy cows with clinical mastitis.

## Results

Effects of *Pulsatilla* saponin B4 on the cure rate and cure time of clinical mastitis in dairy cows

As shown in table 3, all three doses were effective in the 12-day experimental period and 9, 9 and 11 cows with clinical mastitis were cured in group B, C and D respectively. Within 6 days of usage of *Pulsatilla* saponin B4, there were 6, 9 and 7 mastitis cows were cured in group B, C and D, respectively. The total cure rates were 50.00, 75.00 and 58.30% and the average cure time were 5.67, 5.44 and 5.28 d respectively. After usage of extract, there was no cured cow in group C, but 3 and 4 cows in group B and D were cured.

Effects of *Pulsatilla* saponin B4 on the clearance of milk bacteria in the cows with clinical mastitis

As shown in figure 1, on the first day the *Streptococcus agalactiae* was detected in figure 1-1, 1-4, 1-7, 1-8 and 1-10, and it was suspected positive in figure 1-9. The Streptococcus dysgalactiae was detected in figure 1-3. The *Klebsiella species* was detected in figure 1-11 and it was suspected positive in figure 1-10. The *Mycoplasma bovis was* detected in figure 1-5 and 1-6. The *Escherichia coli* was detected in figure 1-4, 1-5, 1-9, 1-10 and 1-12 and it was suspected positive in figure 1-2, 1-6 and 1-11. The *Staphylococcus aureus* was detected in figure 1-2. After treatment with 30mL *Pulsatilla* saponin B4 injection, the results of bacteria detection on the 11th day was shown in Figure 2. No pathogen was detected in 9 cows (figure 1-1, 1-3, 1-4, 1-5, 1-7, 1-8, 1-9, 1-11). The *Streptococcus agalactiae* was detected in figure 1-10; the *Staphylococcus aureus* was detected in figure 1-2; the *Mycoplasma bovis was* detected in figure 1-5 yet.

Effects of *Pulsatilla* saponin B4 on the regulation of the milk SCC in the cows with clinical mastitis

As shown in table 4, milk SCC in group C was significantly higher than that in group A and showed a declining trend with the treatment. Milk SCC of cows with clinical mastitis dropped significantly from d 1 to d 5 ($P < 0.05$) and from d 7 to d 9 ($P < 0.05$) and showed no significant difference ($P > 0.05$) with group A on d 11 ($P > 0.05$).

Effects of *Pulsatilla* saponin B4 on the regulation of serum inflammatory indices in the cows with clinical mastitis

As shown in table 5, cows with clinical mastitis exhibited very significantly higher levels of serum SAA, HP, PGE2, IL-1α, IL-2, IL-6, IL-8, IL-10, and TNF-α than healthy cows ($P < 0.01$) and significantly higher serum LTB4, CRP, IL-1β, and IL-4 than those of healthy cows ($P < 0.05$). This showed a declining trend within the advancement of treatment except for serum PGE2, which was still significantly higher than healthy cows ($P < 0.05$), and the other indices showed no significant difference with the healthy cows ($P > 0.05$). Serum HP, IL-2, and IL-10 concentrations in cows with mastitis on d 3 was significantly lower than d 1 ($P < 0.05$). Serum CRP, SAA, HP, PGE2, IL-1α, IL-2, and TNF-α concentrations in cows with mastitis on d 5 were significantly lower than d 1 ($P < 0.05$). Moreover, on d 7, serum IL-6, IL-4 concentrations in cows with mastitis also became significantly lower than d 1 ($P < 0.05$).

## Discussion

*Pulsatilla* Decoction is famous formula prescription, and it is rich in triterpenoid saponins, such as *Pulsatilla* Saponin B4, *Pulsatilla* Saponin A3, and 23-hydroxybetulinic acid [12, 13]. As the major active component of *Pulsatilla chinensis*, *Pulsatilla* saponin B4 has also received attention as a pure compound for its therapeutic potential. In our study, different doses of *Pulsatilla* saponin B4 were effective for treating clinical mastitis in dairy cows and showed a dose-dependent effect. No side effects were observed in the cows or injection sites after usage of the extract. Within 6 days of treatment there were 6, 9 and 7 mastitis cows cured in group B, C and D, respectively. The total cure rates were 50.0, 75.0 and 58.3% and average cure time were 5.67, 5.44 and 5.28 d, respectively. There were more cured cows in group C in a similar time and it was regarded to be the optimal dose group. Therefore, milk and blood samples from group C were selected for next detection

SCC is the count of somatic cells in a millilitre of milk, and contains 75% of leukocytes and 25% of epithelial cells [14]. Mammary tissues released inflammatory factors like IL-1β, IL-6, and TNF-α when damaged or infected. This promoted the increase of vascular permeability and the detachment of epithelial cells, as well as, it stimulated the secretion of chemokines, attracting leukocytes to the infected area and causing the increase of somatic cell count in milk [15]. SCC reflectd the severity of mastitis, and was used in early diagnosis and treatment evaluation of mastitis in dairy cows [16]. Das et al. [17] found that the SCC value was significantly higher ($P < 0.05$) in clinical mastitis cows compared to subclinical mastitis and healthy cows. Our findings are consistent with them, indicating that the immune cells were accumulated abundantly in the damaged tissues through the non-specific and fluid immune response. In our study, SCC in cows with clinical mastitis decreased during the treatment and had no significant differences with the healthy cows finally ($P < 0.05$), indicating that *Pulsatilla* saponin B4 may relieve the auto-immune damage by decreasing the secretion of pro-inflammatory factors.

Mastitis is an inflammatory process of the udder tissue caused mainly by *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus* [18]. Consistent with our study, Cheng et al. [19] reported tha that *Escherichia coli* was the commonest organism in mastitis cases, and Streptococcus uberis continued to be a prevalent pathogen closely followed by *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Streptococcus agalactiae*. *Pulsatilla chinensis* have inhibitory effects on Staphylococcus aureus, Streptococcus dysenteriae and Salmonella typhi [9]. Shafaghat [20] reported that *Pulsatilla* showed moderate inhibitory activity against some certain strains in Gram-positive and Gram-negative bacteria. In agreement with the foregoing studies, we found that most of the bacteria disappeared or decreased after treatment with *Pulsatilla* Saponin B4, indicating that *Pulsatilla chinensis* is potently inhibitory against the *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Klebsiella species* and *Escherichia coli*.

The infection-induced inflammatory response is the main cause of clinical mastitis in dairy cows, and it stimulates the cellular secretion of LTB4, PGE2, and histamine to enhance the immune response, promoting the production of inflammatory factors (interleukins, bradykinin and allergic toxins) and elevating the levels of acute-phase protein [21, 22]. Our study showed that CRP, SAA and HP of dairy cows with clinical

mastitis were significantly higher than those of healthy cows ($P < 0.05$), suggesting that the breasts of cows were damaged by inflammation. After treatment, the levels of CRP, SAA and HP were significantly decreased ($P < 0.05$), indicating that the inflammatory response rapidly subsided. It was speculated that *Pulsatilla* saponin B4 had a highly effective anti-inflammatory effect, which regulated the inflammatory response in the early stage of mastitis and reduce its damage to breast and body tissues.

In mastitis, activated Toll-like Receptors initiated the NF-κB/MAPKs pathways, which further triggered the gene expression of TNF-α, IL-1β and IL-6 [23]. Tomala et al. [24] showed that LPS, IL-1α and TNF-α modulated PGE2 and LTB4 secreted by bovine mammary gland in both vivo and vitro studies. In agreement with the foregoing studies, we found that the serum levels of PGE2, LTB4, IL-1, IL-2, IL-6, IL-8 and TNF-α in clinical mastitis cows were significantly higher than healthy cows. The incidence of mastitis stimulated body cells to synthesize and release inflammatory mediators, and induce the inflammatory stress in the dairy cows [25]. After treatment, the levels of serum inflammatory factors of clinical mastitis cows showed a declining trend except PGE2, which had was silimilar to that of the healthy group on 7th day ($P > 0.05$). *Pulsatilla* saponin B4 is the main active ingredient of the *Pulsatilla chinensis*, which has anti-bacterial, anti-inflammatory, vasodilatory, and immunoregulatory properties, and it regulated the expression of related inflammatory cytokines via the NF-κB signalling pathway, relieving the inflammatory response with high efficiency [26, 27]. Hu et al. [28] showed that *Pulsatilla* decoction and its active ingredients could inhibit the secretion of NO, ET-1, TNF-α, and IL-1α in LPS-induced rat intestinal microvascular endothelial cells. *Pulsatilla* saponin B4 significantly decreased the expression of NO, TNF-α, IL-1β and IL-6, improve lung tissue damage and down-regulate the protein expression of NF-κB in mice [29]. In the present research, *Pulsatilla* saponin B4 significantly reduced serum pro-inflammatory factors levels. Therefore, this extract was proposed for inhibiting the secretion of inflammatory mediators during the process of mastitis in dairy cows.

Bochniarz et al. [6] found that the levels of serum anti-inflammatory factors (IL-4 and IL-10) in cows with sub-clinical mastitis were significantly lower than healthy cows, but our study showed that the serum levels of IL-4 and IL-10 were significantly and very significantly higher than healthy cows respectively. The reason may be that the inflammatory symptoms are more obvious in cows with clinical mastitis, and the elevation of acute pro-inflammatory factors damaged the mammary tissues and even the entire body. As a result, the serum levels of IL-4 and IL-10 increased rapidly to antagonize the inflammation. Unlike the current researches, we found that serum levels of anti-inflammatory factors sexhibited a declining trend during the treatment, it may be that the secretion of pro-inflammatory factors was more related to the cellular secretion and immune response. In the acute phase of mastitis, levels of pro-inflammatory factors elevated because of the severe inflammation, and the concentrations of anti-inflammatory factors also increased as the consequence of the body's defense mechanism. When the inflammation controlled, the pro-inflammatory factors and anti-inflammatory factors decreased sequentially. However, the reason of the changes in the levels of anti-inflammatory factors still should be distinguished.

## Conclusion

30 mL *Pulsatilla* saponin B4 injection by brachiocephalics intramuscular injection once a day for 4-6 days was the most appropriate usage for the treatment of clinical mastitis. Treatment of *Pulsatilla* saponin B4 can significantly decrease the somatic cell count, eliminate pathogenic bacteria, and down-regulate the levels of serum CRP, SAA, HP, IL-1α, IL-1β, IL-2, IL-6, IL-8, TNF-α of cows with clinical mastitis.

## Methods

Experiment animals and treatment

The Chinese Holstein cows were obtained from Sichuan Ninggang Animal Husbandry Co., Ltd. Twelve healthy cows and 36 cows with first-class clinical mastitis with similar date of parturition and milk production were selected form 50 healthy and 50 cows with mastitis (with weight 612 ± 47 kg, 3-4 years of age, and 2-3 parity) in a semi-closed unified dairy farm. Twelve healthy cows were placed into control group (group A), the other 36 cows with clinical mastitis were divided into 3 groups (group B, C, and D) equally and randomly by simple randomizaton. In group B, C and D, the cows were applied with *Pulsatilla* saponin B4 Injaction through brachiocephalicus intramuscular injection with 15, 30, and 60 mL respectively, and the first day of the experiment was recorded as day 1, then administrated the same doses continuously for 4-6 days until the recovery of cows. The control group didn't receive any administration. Our research was a field trial. All cows were diagnosed as clinical mastitis for the first time and didn't receive antibiotic treatment for 14 days before diagnosis. All the groups were under observation for 12 days. All experimental cows had the same feeding and management procedure without other diseases occurred and antibiotic treatment during the experimental period. We selected a small sample size because the *Pulsatilla* saponin B4 was evaluated for the first time in the present study, the initial intention was to gather basic evidence regarding the usage of this drug in the further reseaches.

Sample collection

10 mL of tail venous blood of experimental cows were collected before feeding at 8 am. The sampling was done on day 1, 3, 5, and 7 in group A, B, C, and D. The blood was loaded in a centrifuge tube without anticoagulant and centrifuged with a centrifuge (Sigma, Germany) at 3 000

rpm for 10 min to separate serum after 1 hour deposition at room temperature (20-25 ℃), and the upper serum was transferred to EP tube, stored at -70 ℃ in refrigerator (Haier, China). Washed the udder by warm water, and sterilized by 75% ethanol. Discarded several streams of milk, then collected 5 mL milk and measured the SCC on day 1, 3, 5, 7, 9, and 11 in group A and group C. All sampling process were administrated before treatment.

Animals treatment after experimentation

Cows in group A and cured cows in group B, C, and D were under the normal breeding management again after the study, and the rest were treated by other medicine until cured. All cows are raised according to animal welfare principles.

Reagents

*Pulsatilla* saponin B4 injection (100 mL/bottle, 66% *Pulsatilla* saponin B4 , ethanol solution), PubChem CID: 11636713 (https://pubchem.ncbi.nlm.nih.gov/compound/71307558), donated by Sichuan Innovate Medical Technology Co., Ltd, Chengdu, China, identified by National Pharmaceutical Engineering Center for Solid Preparation in Chinese Herbal Medicine, Jiangxi University of Traditional Chinese Medicine .

Criteria of clinical mastitis

All cows with clinical mastitis were diagnosed by the same veterinarian of the dairy farm. Detailed criteria included red and swollen mammary gland with sensitive tenderness, infected quarters heated, decreased milk production, milk with yellow or red color, SCC higher than 500 000/mL, and other abnormal traits [30].

Clinical cure and bacteriological cure

A cow was regarded as clinically cured if both milk and mammary gland had a normal appearance in the clinical examination approximately 24 hours after the last injection. A cow was considered bacteriologically cured if a microorganism was identifiedin the milk sample on d 1, and the same species was not isolated in any of milk samples collected post-treatment (d 1 or 11). If the same pathogen isolated on d 1 was identified in any of the post-treatment samples, the infected quarter was considered non-cured. Only milk samles with positive results were included in this evaluation. In addition, samples with negative culture (no growth) on d 1 were not included in the analysis of bacteriological cure.

PCR tests and indices measurement

A commercial eight combined bovine mastitis pathogen nucleic acid detection reagents kit (bioinfee Biotechnology Co., Ltd, Shenzhen, China) was employed to measure pathogenic bacteria on the milk sample on day 1 and day 11 in the optimal dose group according to the manufacturer's instructions with the help of real-time fluorescent quantitative PCR instrument (Stratagene Mx3005P, U.S.A). Kit item number: YRMBP7045-2; lot number: 20021001.

Serum levels of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, LTB4, PGE2, SAA and HP in group A and the optimal dose group was measured by bovine ELISA kits (Shanghai Enzyme Biotechnology Co., Ltd, Shanghai, China) according to the manufacturer's instructions with the help of full wavelength microplate reader (Thermo Scientific, U.S.A). The intra and inter-assay coefficient of variance was less than 10% and 15%, respectively. The Detection range and minimum detection dose of ELISA Kits was shown in table 1..

SCC measurement and cure rate calculation

Detected the SCC of milk in group A and the optimal dose group by milk somatic cell detector (De Laval, Sweden), observed the clinical symptoms of the cows with mastitis every day during the experiment period, and then calculated the cure time, effective rate, and cure rate.

Criteria of PCR results

The criteria of PCR results were shown in table 2.

Data Analysis

Regarded the average of the data in group A as the control and compared it with the optimal dose group. The data was normally distributed and distinguished the differences and correlations between groups by independent sample t-test and Person relation analysis by SPSS 19.0 (IBM SPSS statistics for Windows, version 21.0). All data was recorded as`X ± SD.

For each cow, three different investigators were involved as follows: a first investigator administered the treatment and collected samples based on the randomization table. This investigator was the only person aware of the treatment group allocation. A second investigator was responsible for the measurement of somatic cell count, inflammatory factors, and qPCR and the calculation of cure rate. Finally, a third investigator (also unaware of treatment) analyzed the above results.

## Abbreviations

IM: intramuscular injection; qPCR: quantitative real-time polymerase chain reaction; SCC: somatic cell count; d: day; LTB4: leukotrienes B4; PGE2: prostaglandin E2; IL-1α: interleukin-1 alpha; IL-1β: interleukin-1 beta; Il-2: interleukin-2; IL-4: interleukin-4; IL-6: interleukin-6; IL-8: interleukin-8; IL-10: interleukin-10; TNF-α: tumour necrosis factor alpha; CRP: C-reactive protein ; SAA: serum amyloid A protein; HP: haptoglobin

## Declarations


### Acknowledgements

The authors are grateful to the farmers for allowing the collection of samples.


### Authors' contributions

LHS and YLF provided the concept on this study. LHS, BLQ and LCY designed the study. LCY, YZ, JBX, SKL and YS acquired the samples. SMY, SZC, BLQ and YZ interpreted the data. ZTS, SLY, YLF and KD developed and provided the experimental *Pulsatilla* saponin B4. BLQ and LHS drafted the work. LHS revised the manuscript. All authors read and approved the manuscript.


### Funding

This study has been supported by the National Key R&D Program of China (2018YFD0500900) and the Sichuan Department of Science and Technology Support Project (2019YJ0650).The funders had no role in the design of the study, collection, analysis and interpretation of data, and in writing the manuscript.


### Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

All procedures were approved by Insti-tutional Animal Care and Use Committee of Sichuan Agricultural University (approval number: DYY-13309).

### Consent for publication

All authors to have approved the submitted version (and any substantially modified version that involves the author's contribution to the study).

### Competing interests

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

## References


1. Guilherme GF, Maurício D, André KG, Tatiana SP, Marcia M, Juliana RP, Francisco LFF. Influence of mastitis on electrophoretic fractions of colostrum from Holstein cows. Pesqui Vet Bras. 2018; doi:10.1590/1678-5150-pvb-4919.
2. Jadhav PV, Das DN, Suresh KP, Shome BR. Threshold somatic cell count for delineation of subclinical mastitis cases. Vet World. 2018; doi:10.14202/vetworld.2018.789-793.
3. Borin T, Angara K, Rashid M, Achyut B, Arbab A. Arachidonic acid metabolite as a novel therapeutic target in breast cancer metastasis. Int J Mol Sci. 2017; doi:10.3390/ijms18122661.
4. Blum SE, Heller ED, Jacoby S, Krifucks O, Leitner G. Comparison of the immune responses associated with experimental bovine mastitis caused by different strains of *Escherichia coli*. J Dairy Res. 2017; doi:10.1017/S0022029917000206.

5. Faty A, Ferre P, Commans S. The acute phase protein Serum Amyloid A induces lipolysis and inflammation in human adipocytes through distinct pathways. PLoS One. 2012; doi:10.1371/journal.pone.0034031.
6. Bochniarz M, Zdzisińska B, Wawron W, Szczubiał M, Dąbrowski R. Milk and serum IL-4, IL-6, IL-10, and amyloid A concentrations in cows with subclinical mastitis caused by coagulase-negative staphylococci. J Dairy Sci. 2017; doi:10.3168/jds.2017-13552.
7. Liu T, Ye L, Guan X. Immunopontentiating and antitumor activities of a polysaccharide from *Pulsatilla* chinensis (Bunge) Regel. Int J Biol Macromol. 2013; doi:10.1016/j.ijbiomac.2012.12.012
8. Lian S, Jiang Y, Xue R. Research progress on chemical constituents and pharmacological effects of Pulsatilla chinensis (Bunge) Rege. Asia-Pac Trad Med. 2016; doi:10.11954/ytctyy.201602015.
9. Fong SC, Mulyana Y, Girawan D. Antibacterial effect of Pulsatilla chinensis towards Staphylococcus aureus, Shigella dysenteriae, and Salmonella typhi. Alt Med J. 2016; doi:10.15850/amj.v3n2.467.
10. Kang N, Shen W, Zhang Y, Su Z, Yang S, Liu Y, Xu Q. Anti-inflammatory and immune-modulatory properties of anemoside B4 isolated from *Pulsatilla* chinensis in vivo. Phytomedicine. 2019; doi:10.1016/j.phymed.2019.152934.
11. He L, Zhang Y, Kang N, Zhang Z, Zha Z, Yang S, Xu Q, Liu Y. Anemoside B4 attenuates nephrotoxicity of cisplatin without reducing anti-tumor activity of cisplatin. Phytomedicine 2019; doi:10.1016/j.phymed.2018.10.035.
12. Wang R, Wang X. Analysis on pharmacology and clinical application of *Pulsatilla* Acta Chi Med.2010; doi:10.16368/j.issn.1674-8999.2010.02.068.
13. Li Y, Zuo M, Han Q, Deng L, Richard M. Therapeutic potential of triterpenoid saponin anemoside B4 from *Pulsatilla* Pharmacol Res. 2020; doi:10.1016/j.phrs.2020.105079.
14. Nyman AK, Emanuelson U, Holtenius K, Ingvartsen KL, Larsen T, Waller KP. Metabolites and Immune Variables Associated with Somatic Cell Counts of Primiparous Dairy Cows. J Dairy Sci. 2008; doi:10.3168/jds.2007-0969.
15. Zhang W, Li X, Xu T, Ma M, Zhang Y, Gao M. Inflammatory responses of stromal fibroblasts to inflammatory epithelial cells are involved in the pathogenesis of bovine mastitis. Exp Cell Res. 2016; doi:10.1016/j.yexcr.2016.09.016.
16. Hegde R, Isloor S, Prabhu KN, Shome BR, Rathnamma D, Suryanarayana VVS, Yatiraj S, Prasad CR, Krishnaveni N, Sundareshan S, Akhila DS, Gomes AR, Hegde NR. Incidence of Subclinical Mastitis and Prevalence of Major Mastitis Pathogens in Organized Farms and Unorganized Sectors. Indian J Microbiol. 2013; doi:10.1007/s12088-012-0336-1.
17. Das D, Panda SK, Jena B, Sahoo AK. Somatic Cell Count: A Biomarker for Early Diagnosis and Therapeutic Evaluation in Bovine Mastitis. Int J Curr Microbiol Appl Sci. 2018; doi:10.20546/ijcmas.2018.703.174.
18. Cheng W, Han S. Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments - A review. Asian-Australas J Anim Sci. 2020; doi:10.5713/ajas.20.0156.
19. Cheng D, Zhu S, Yin Z, Ding W, Mu Z, Su ZR, Sun, HC. Prevalence of bacterial infection responsible for bovine mastitis. African Afr J Microbiol Res. 2010; doi:10.1186/1471-2164-11-359.
20. Shafaghat A. Antimicrobial activity and volatile constituents of the essential oil of Pulsatilla albana from Iran. Nat Prod Commun. 2010; doi:10.1002/mnfr.200900106.
21. Gronlund U, Hulten C, Eckersall PD, Hogarth C, Persson WK. Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced Staphylococcus aureus mastitis. J Dairy Res. 2003; doi:10.1017/S0022029903006484..
22. Turk, R. The role of oxidative stress and inflammatory response in the pathogenesis of mastitis in dairy cows. Mljekarstvo. 2017; doi:10.15567/mljekarstvo.2017.0201.
23. Akhtar M, Guo S, Guo Y, Zahoor A, Shaukat A, Chen Y, Umar T, Deng G, Guo M. Upregulated-gene expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) via TLRs following NF-κB and MAPKs in bovine mastitis. Acta Trop. 2020; doi:10.1016/j.actatropica.2020.105458.
24. Piotrowska TKK, Bah MM, Jankowska K, Lukasik K, Warmowski P, Galvao AN, Skarzynski DJ. Lipopolysaccharides, cytokines, and nitric oxide affect secretion of prostaglandins and leukotrienes by bovine mammary gland during experimentally induced mastitis in vivo and in vitro. Domest Anim Endocrinol. 2015; doi:10.1016/j.domaniend. 2015.03.001.
25. Turk R, Koledić M, Maćešić N, Benić M, Dobranić V, Đuričić D, Cvetnić L, Samardžija, M. The role of oxidative stress and inflammatory response in the pathogenesis of mastitis in dairy cows. Mljekarstvo. 2017; doi:10.15567/mljekarstvo.2017.0201.
26. Gong Q, He L, Wang M, Ouyang H, Gao H, Feng Y, Yang S, Du L, Li J, Luo Y. Anemoside B4 Protects Rat Kidney from Adenine-Induced Injury by Attenuating Inflammation and Fibrosis and Enhancing Podocin and Nephrin Expression. Evid Based Complement Alternat Med. 2019; doi:10.1155/2019/8031039.
27. Hu Y, Mao A, Tan Y, Zhao Y, He K. Role of 5 saponins in secretion of cytokines by PRRSV-induced endothelial cells. Drug Res. 2016; doi:10.1055/s-0042-106577.

28. Hu Y, Chen X, Duan H, Hu Y, Mu X. Pulsatilla decoction and its active ingredients inhibit secretion of NO, ET-1, TNF-α, and IL-1α in LPS-induced rat intestinal microvascular endothelial cells. Cell Biochem Funct. 2009; doi:10.1002/cbf.1570.
29. Zhou M, Chen L, Hu H, Luo Y, Fang C et al. Protective effect of Pulsatilla saponin B4 on lipopolysaccharide-induced acute lung injury. Traditional Chinese Drug Research and Clinical Pharmacology 2019; 30 (6): 664-670. doi: 10.19378/j.issn.1003-9783.2019.06.005.
30. Pinzón SC, Cabrera VE, Ruegg PL. Decision tree analysis of treatment strategies for mild and moderate cases of clinical mastitis occurring in early lactation. J Dairy Sci. 2011; doi:10.3168/jds.2010-3930.

# Tables

**Table 1. The Detection range and minimum detection dose of ELISA Kits**

| Index | Detection range | Minimum detection dose |
|---|---|---|
| C-reactive protein (mg/mL) | 0.375-12 | < 0.1 |
| Serum amyloid A (μg/mL) | 0.375-12 | < 0.1 |
| Haptoglobin (ng/mL) | 12.5-400 | < 1.0 |
| Leukotrienes B4 (ng/mL) | 0.625-20 | < 0.1 |
| Prostaglandin E2 (pg/mL) | 20-640 | < 1.0 |
| Interleukin-1α (pg/mL) | 20-640 | < 1.0 |
| Interleukin-1β (pg/mL) | 20-640 | < 1.0 |
| Interleukin-2 (pg/mL) | 37.5-1200 | < 1.0 |
| Interleukin-6 (pg/mL) | 6.25-200 | < 1.0 |
| Interleukin-8 (pg/mL) | 7.5-240 | < 1.0 |
| Tumor necrosis factor-α (pg/mL) | 6.25-200 | < 1.0 |
| Interleukin -4 (pg/mL) | 2-64 | < 0.1 |
| Interleukin-10 (pg/mL) | 5-160 | < 1.0 |

**Table 2. The criteria of PCR results**

| Target genes | Strong positive | Positive | Weak positive | Suspected positive | Negative |
|---|---|---|---|---|---|
| *β-lactam resistance* | ≤ 20 | (20-28] | (28-33] | (33-36] | (36-40] |
| *Streptococcus agalactiae* | ≤ 25 | (25-33] | (33-37] | (37-38.5] | (38.5-40] |
| *Pseudomonas aeruginosa* | ≤ 22 | (22-30] | (30-36] | (36-38] | (38-40] |
| *Klebsiella species* | ≤ 20 | (20-28] | (28-36] | (36-38] | (38-40] |
| *Streptococcus dysgalactiae* | ≤ 25 | (25.33] | (33-37] | (37-38.5] | (38.5-40] |
| *Staphylococcus aureus* | ≤ 25 | (25-33] | (33-37] | (37-38.5] | (38.5-40] |
| *Escherichia coli* | ≤ 20 | (20-28] | (28-35] | (35-37] | (37-40] |
| *Mycoplasma bovis* | ≤ 25 | (25-33] | (33-37] | (37-38.5] | (38.5-40] |

**Table 3. The influence of different dosage *Pulsatilla* saponin B4 on cure time and cure rate of clinical mastitis in dairy cows**

| Time | | Group B (15 mL, *n*=12 ) | Group C (30 mL, *n*=12) | Group D (60 mL, *n*=12) |
|---|---|---|---|---|
| Used *Pulsatilla* saponin B4 | 4$^{th}$ day | 0 | 1 | 1 |
| | 5$^{th}$ day | 2 | 3 | 3 |
| | 6$^{th}$ day | 4 | 5 | 3 |
| | Total cured cows | 6 | 9 | 7 |
| | Total cure rate(%) | 50.00 | 75.00 | 58.33 |
| | Average cure time(d) | 5.67±0.52a | 5.44±0.73a | 5.28±0.76a |
| Stopped use *Pulsatilla* saponin B4 | 7-12$^{th}$ day | 3 | 0 | 4 |
| | Total cured cows | 9 | 9 | 11 |

Note: The same letters show that there is no significant difference (P > 0.05), and the different letters show that there is a significant difference (P < 0.05), independent of case, only capital letters indicate a significant difference (P  0.01), the same as below.

**Tabel 4. The effect of 30 mL *Pulsatilla* saponin B4 on somatic cell counts of clinical mastitis in the milk of dairy cows**

| Somatic cell counts (10000/mL) | Group A (*n*=12) | Group C (30mL, *n*=12) | | | | | |
|---|---|---|---|---|---|---|---|
| | Average | 1$^{st}$ day | 3$^{rd}$ day | 5$^{th}$ day | 7$^{th}$ day | 9$^{th}$ day | 11$^{th}$ day |
| | 27.71±7.26e | 294.41±61.64a | 235.88±51.90b | 150.63±58.10c | 130.99±34.36c | 68.16±14.93d | 43.35±10.36de |

**Table 5. The effect of 30 mL *Pulsatilla* saponin B4 on the serum inflammatory factor index of clinical mastitis in dairy cows**

| Index | Goup A (*n*=12) | Group C (30mL, *n*=12) | | | |
|---|---|---|---|---|---|
| | Average | 1$^{st}$ day | 3$^{rd}$ day | 5$^{th}$ day | 7$^{th}$ day |
| C-reactive protein (mg/mL) | 6.55±1.68$^{b}$ | 8.25±2.50$^{a}$ | 7.02±2.08$^{ab}$ | 6.66±1.05$^{b}$ | 6.47±1.93$^{b}$ |
| Serum amyloid A (μg/mL) | 6.95±1.01$^{C}$ | 10.86±3.20$^{A}$ | 9.40±2.63$^{Ab}$ | 8.85±2.31$^{bc}$ | 8.71±2.60$^{bc}$ |
| Haptoglobin (ng/mL) | 167.37±36.40$^{B}$ | 236.34±55.99$^{A}$ | 178.46±45.14$^{B}$ | 168.68±35.05$^{B}$ | 160.01±52.80$^{B}$ |
| Leukotrienes B4 (ng/mL) | 11.15±2.69$^{b}$ | 14.71±3.82$^{a}$ | 14.49±4.77$^{a}$ | 14.27±4.68$^{a}$ | 14.13±4.78$^{a}$ |
| Prostaglandin E2 (pg/mL) | 253.70±52.08$^{C}$ | 351.80±56.39$^{A}$ | 331.47±82.63$^{AB}$ | 307.83±66.35$^{b}$ | 299.62±51.36$^{b}$ |
| Interleukin-1α (pg/mL) | 175.90±47.49b$^{C}$ | 228.47±69.62$^{A}$ | 210.98±63.04$^{Ab}$ | 181.65±60.07$^{bC}$ | 163.23±47.55$^{C}$ |
| Interleukin-1β (pg/mL) | 276.71±81.61$^{b}$ | 358.78±113.73$^{a}$ | 331.88±99.60$^{ab}$ | 325.92±98.89$^{ab}$ | 295.64±98.06$^{ab}$ |
| Interleukin-2 (pg/mL) | 449.65±95.09$^{B}$ | 606.38±130.78$^{A}$ | 500.38±151.23$^{b}$ | 470.21±145.64$^{b}$ | 415.76±121.10$^{B}$ |
| Interleukin-6 (pg/mL) | 97.67±22.63$^{C}$ | 155.00±39.96$^{A}$ | 134.52±38.21$^{AB}$ | 124.65±42.53$^{b}$ | 117.07±36.88$^{bC}$ |
| Interleukin-8 (pg/mL) | 108.61±38.62$^{B}$ | 165.45±53.22$^{A}$ | 153.94±50.83$^{a}$ | 152.60±54.40$^{a}$ | 139.76±47.86$^{AB}$ |
| Tumor necrosis factor-α (pg/mL) | 122.65±33.50$^{C}$ | 174.65±49.07$^{A}$ | 151.22±41.09$^{Ab}$ | 143.77±35.69$^{bc}$ | 133.20±46.61$^{BC}$ |
| Interleukin-4 (pg/mL) | 13.42±4.36b | 16.56±3.71a | 14.28±4.87ab | 13.78±4.98ab | 12.48±4.60b |
| Interleukin-10 (pg/mL) | 109.41±24.04B | 146.54±31.43A | 122.99±26.85b | 119.47±26.07b | 119.84±30.35b |

# Figures

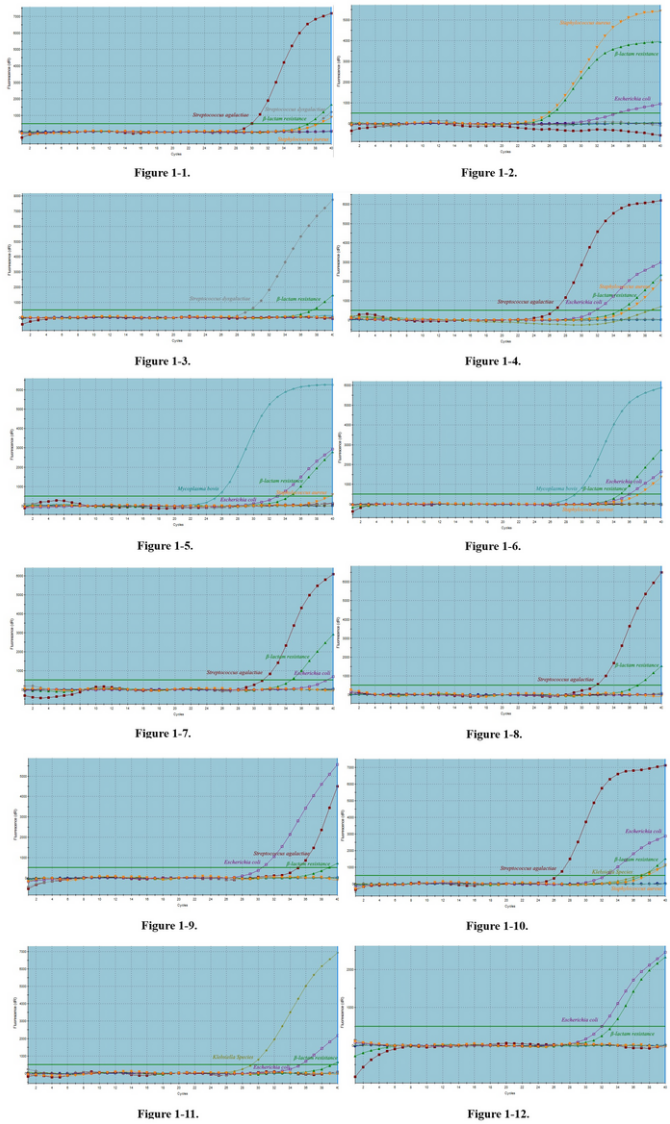


**Figure 1**

Results of pathogenic bacteria in the milk of clinical mastitis cows by qRT-PCR before treatment with 30 mL Pulsatilla saponin B4. (1-12) Pathogenic bacteria detection results of No.1-12 clinical mastitis cows.

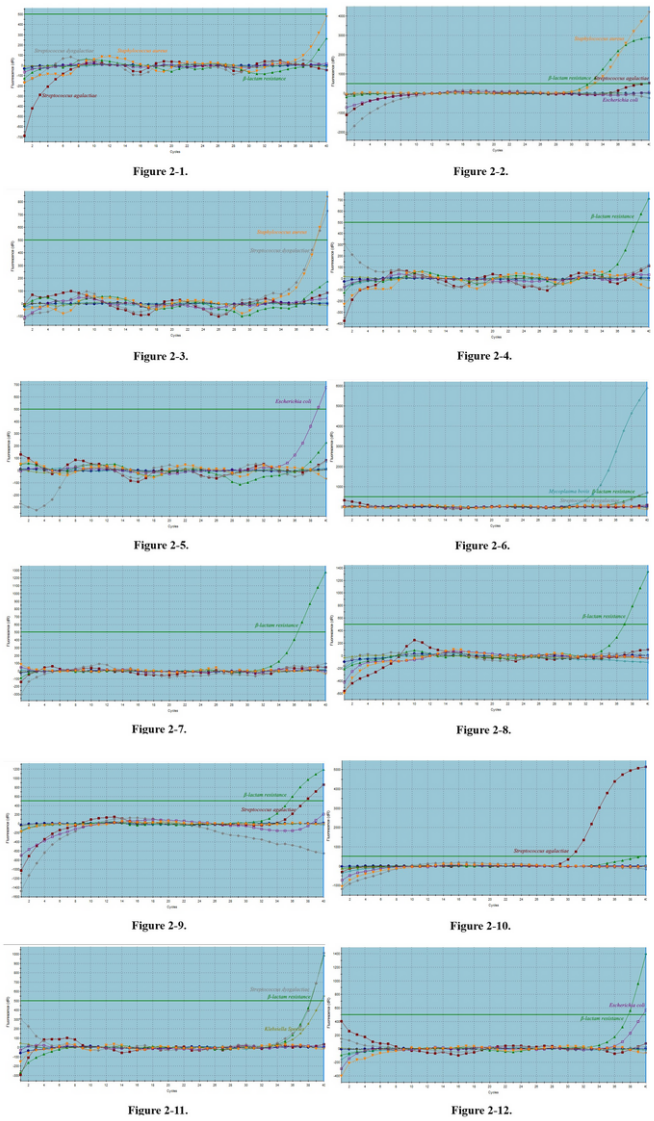


**Figure 2**

Results of pathogenic bacteria in the milk of clinical mastitis cows by qRT-PCR after treatment with 30 mL Pulsatilla saponin B4 for 4-6 days. (1-12) Pathogenic bacteria detection results of No.1-12 clinical mastitis cows.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ARRIVEEssential10.docx