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BAICALIN DECREASES SOMATIC CELL COUNT IN MASTITIS OF DAIRY COWS*

Artur Burmańczuk[1♦], Beata Wojciechowska[2], Małgorzata Gbylik-Sikorska[3], Anna Gajda[3], Włodzimierz Markiewicz[4], Ewa Sosin[5], Tomasz Grabowski[6]

[1]Sub-Department of Pharmacology, Toxicology and Environmental Protection, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-033 Lublin, Poland
[2]Labkonsulting, Duńska 9, 54-427 Wrocław, Poland
[3]Department of Pharmacology and Toxicology, National Veterinary Research Institute, Aleja Partyzantów 57, 24-100 Puławy, Poland
[4]Department of Pharmacology and Toxicology, University of Warmia and Mazury, Oczapowskiego 13 bl. A, 10-719 Olsztyn, Poland
[5]Department of Animal Nutrition and Feed Sciences, National Research Institute of Animal Production, 32-083 Balice n. Kraków, Poland
[6]Polpharma Biologics SA, Trzy Lipy 3 bl. A, 80-172 Gdańsk, Poland
♦Corresponding author: artur.burmanczuk@up.lublin.pl

Abstract

Baicalin is a flavonoid that has an influence on molecular processes. It possesses anticancer, anti-inflammatory, antiviral, antioxidative, and antithrombotic properties. It was found that baicalein treatment attenuated the damage of the mammary gland induced by LPS, suppressed the activity of myeloperoxidase, TNFα, and IL-1β in mice with mastitis. The aim of the study was a pilot analysis of baicalin tolerability after intramammary (IMM) administration and its impact on somatic cell count (SCC) after multiple IMM treatment on dairy cows with clinical mastitis. Moreover, the determination of baicalin in milk was performed by the sensitive ultra-high performance liquid chromatography with tandem mass spectrometry. The pharmacokinetic analyses were performed using Phoenix® WinNonlin® 6.4 and ThothPro v 4.1 software. Twelve dairy cows with clinical mastitis were selected for this study. The pharmacodynamic endpoint was SCC level and the clinical investigation was also carried out. Baseline SCC analysis was performed every 24 h among all cows three days before the first dose (B1–B3). After the baseline monitoring, 8 days of treatment (T1–T8) was performed and 8 days within recovery period SCC level was observed (R1–R8). Starting from T1 to T8, a decrease of SCC in relation to baseline was characterized by a declining trend. The presented results confirm the effect of baicalin on the reduction of SCC in mastitis in dairy cows after this therapy. The current study has shown that baicalin accumulation was not confirmed.

Key words: mastitis, baicalin, flavonoids, immunomodulator, inflammation

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Baicalin is a lipophilic flavonoid occurring in the roots of *Scutellaria baicalensis*. This flavonoid affects molecular processes and consequently possesses anticancer, anti-inflammatory, antiviral, antioxidative, antithrombotic properties and modulates bacterial virulence (Ma et al., 2005; Li-Weber, 2009; Li et al., 2011; He et al., 2015; Yang et al., 2016; Chen et al., 2018; Wu et al., 2018; Zhao et al., 2018; Oo et al., 2019). Baicalin, and other *Scutellaria* spp. flavonoids inhibit cancer cells proliferation in concentrations of 20–200 μM and induce apoptosis (Li-Weber, 2009). The cytostatic activity of baicalin was found against myeloma cancer cells, leukemia, bladder, and prostate cancer cells (Chen et al., 2001; Ma et al., 2005; Shieh et al., 2006). The anti-inflammatory effect of baicalin has also been experimentally demonstrated by inhibiting prostaglandin E2 (PGE2) and leukotriene B4/C4 (LTB4/LTC4) biosynthesis and had such activities by affecting the enzymes cyclooxygenase (COX) and lipoxygenase (Schapoval et al., 1998). Baicalin blocks the biological functions of chemokines but also inhibits the activation of leukocytes as a result of antioxidant activity (Shen et al., 2003). Baicalein, baicalin, and quercetin, by decreasing Th1 lymphocytes differentiation, decrease the levels of numerous cytokines and factors like IL-1β, IL-4, IL-6, IL-10, IL-12, IL-25, IL-33, MCP-1, NF-κB, VEGF-A, COX-2, 5-LOX, iNOS, NO, CRP, TNFα, TNFγ, IgE and reduce the expression of VCAM-1, ICAM-1, MIP-2 as was shown in different models, including the model of mammary tissue inflammation in mice (Muthian and Bright, 2004; Bhaskar et al., 2016; Caglayan Sozmen et al., 2016; Chen et al., 2016; Meng et al., 2016; Maurya and Vinayak, 2017). Studies on the effects and molecular mechanism of baicalein on lipopolysaccharide (LPS) induced mastitis in mice were performed (He et al., 2015). As a result of this study, it was found that baicalein treatment attenuated the damage of the mammary gland induced by LPS, suppressed the activity of myeloperoxidase, reduced the levels of TNFα and IL-1β in mice with mastitis. It was shown that baicalin, by inhibiting the nuclear factor-kB (NF-kB) and phosphorylation of p38, reduces the expression of cytokines such as TNF-α, IL-β, and IL-6 in the mammary gland with mastitis (Guo et al., 2013). Further studies revealed that baicalin inhibits apoptosis induced by *Staphylococcus aureus* in the mouse mammary glands through the reduction of TLR2 expression and p53 phosphorylation, and the regulation of apoptosis-related factors like BCL-2, BAX, and CASP-3 (Guo et al., 2014). Other studies demonstrated that baicalin binds to lysozyme and increases its antibacterial activity on *S. aureus* in mammary glands in mice (Gao et al., 2017). Inhibitory effect of baicalin on *E. coli in vitro* and the effects of baicalin treatment on antimicrobial resistance of isolates from mastitis milk were also shown (Zhao et al., 2018). Moreover, homologous flavonoid – quercetin showed a significant impact on somatic cell count (SCC) after intramammary administration (Burmańczuk et al., 2018).

The literature describes the separation and detection methods for baicalin, and their application mainly in rat plasma or plant products (Wang et al., 2009; Lu et al., 2014; Ji et al., 2017). A chromatographic method for the quantitative analysis of baicalin in human plasma was also reported (Wang et al., 2009; Ji et al., 2017). However, no analytical method has been described for the analysis of flavonoid in milk. In the presented study, the presence of baicalin in milk was determined by the sensi-

tive ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) method, developed in this study.

Based on previous studies it appears that baicalin may have activity against the causative agents of mastitis. Therefore, the aim of this study was the analysis of the possible impact of baicalin on somatic cell count (SCC) after multiple intramammary (IMM) treatments of dairy cows with mastitis.

Material and methods

Dosage and drug preparation

The solution of baicalin (Sigma-Aldrich; Poland) in phosphate-buffered saline (PBS) (Biomed-Lublin, Poland) was prepared by sonication (Sonic-2 ultrasound bath, POLSONIC Pałczyński, Poland) and shaking for 30 min at 37°C. Final doses were infused using a solution of 30 mg of baicalin in 5 mL of PBS (Biomed-Lublin, Poland). All doses in the study were given immediately after morning milking at 9:00 am. Because immunomodulatory response after IMM administration is difficult to limit to the effect located only in one quarter, baicalin was administered to all quarters in all animals. Thirty mg/quarter/day dose was proposed (120 mg/udder/day). Treatment was continued between the first and eighth day dose (T1–T8). After baseline monitoring (B1–B3) SCC was analyzed within seven days after the last dose (T8) in the recovery period (R1–R8).

Animals

Animals were cared for according to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals, based on SCC and clinical investigation. The control group was excluded by "the 3Rs" principle and the Ethics Committee recommendation, to avoid unnecessary use of experimental animals. Instead, control group baseline effect analysis was taken as a benchmark. The permission for the study was issued by the bioethical commission in Lublin (decision number 33/2017).

Twelve dairy cows with clinical mastitis of one quarter were selected for the pilot study. SCC>1,500,000 cells/mL in one of 4 quarters was taken as an inclusion criterion. Only cows with only one inflamed quarter were included in the study. The study was conducted on Polish Holstein-Friesian Black and White cows with daily milk yield 33–34 L, weighing ≈650 kg each, cows were between fourth to sixth lactations. The animals were fed with farming feed concentrates and fodder (oats, barley) grain alternated with raw corn, pasture grazing grass silage, green forage, straw, and meadow hay with water access *ad libitum*. The analyses were carried out in one selected farm (Agromarina Sp. z o.o., Poland). Before the drug administration and milk delivery, each udder was disinfected. Milk samples for analyses (0.5 L) were collected once per day from the inflamed quarter of each cow, immediately before daily morning milking. Milk samples (19 per each cow) were collected at baseline (B1–B3), treatment period (T1–T8), and within the recovery period (R1–R8).

Adverse drug reactions monitoring

Signs of intolerance or adverse drug reactions related to the IMM administered baicalin were monitored during the study. No other medicines were given to the animals during the study. The animal's health and udder conditions were examined daily by a veterinarian.

Chemicals and reagents

Analytical grade reagents and HPLC grade solvents were used in the study. Methanol, acetonitrile, and formic acid were obtained from J.T. Baker (Deventer, The Netherlands). Water was deionised (>18 MΩ/cm) in-house by Millipore system (Millipore, France). Syringe filters, 0.22 µm hydrophilic polyvinylidene fluoride, were from Restek (USA). Analytical reference standards of baicalin were obtained from Interforum Pharma (China) and sulfaphenazole used as an internal standard (IS), was purchased from Sigma Aldrich (USA).

Analytical method

The analysis of baicalin in milk was conducted by ultra-high performance liquid chromatography with mass spectrometry (UHPLC-MS/MS) Shimadzu Nexera X2 (Shimadzu, Japan) system connected to the QTRAP® 4500 triple quadrupole mass spectrometer (AB Sciex Framingham, MA, USA) controlled by Analyst 1.6.3 software. The mass spectrometer operated in the positive ion electrospray ionization mode and MS data acquisition was performed in the multiple reaction monitoring mode. The precursor → product ion pairs were 447→271 (ion 1), 447→123 (ion 2) for baicalin, and 315→158 for IS. The following values for MS/MS system were set: declustering potential (DP) – 40 V, collision energy (CE) ion 1, 30 V and ion 2, 70 V, cell exit potential (CXP), 13 V for baicalin and DP 100 V, CE 15 V, CXP 15 V for IS. The separation was performed on an analytical column ZORBAX SB-C18, 50 mm × 2.1 mm × 1.8 µm (Agilent, USA) with an octadecyl guard column, 2 × 4 mm (Phenomenex, USA). The column temperature was set at 35°C. The chromatographic analyses were carried out using a mobile phase consisting of solvent A: methanol and solvent B: 0.5% formic acid in the gradient elution mode. The mobile phase starting conditions were 15% of solvent A (0–2.0 min), increased to 80% of solvent A (2.01–3.30 min), and then decreased to 15% of solvent A (3.31–5.0 min) within 5 min. The flow rate of the mobile phase was set as 0.5 mL/min. with the injection volume of 20 µL.

Sample preparation

A weight of 500 µg of milk was directly transferred into the 1.5 mL microcentrifuge tube and milk was fortified with 15 µL internal standard fortification solution (2 µg/mL). Then, 1 mL of 0.5% formic acid in acetonitrile was added, vortex-mixed, and centrifuged at 9447 × g for 5 min at 4°C. After centrifugation, the supernatant was filtered through a 0.22 µm syringe filter into the chromatographic vials.

Analytical method validation

The method used in the study was validated and the following parameters such as linearity, precision (repeatability and within-laboratory reproducibility), accuracy,

and the lower limit of quantification (LLOQ) were established. The linearity was checked by preparing three matrix-matched calibration curves at the concentrations range of 0.1–500 µg/kg, 500–5000 µg/kg, and 5000–60,000 µg/kg. Precision (repeatability and within-laboratory reproducibility) was determined by the repeated analysis (n=6) of milk samples spiked with baicalin at three concentrations corresponding to 10, 100, and 1000 µg/kg, from run to run during 1 day and 3 days, respectively. The recoveries were evaluated in the same experiment as precision by comparing the mean measured concentration with the concentrations of the analyte in the fortified samples in relation to the matrix-matched calibration curve. The specificity of the method was evaluated by the repeated analysis of 10 blank milk samples originating from different sources. The LLOQ was determined as the lowest level of the matrix-matched calibration curve. The method was validated according to current guidelines (FDA, 2018; EMA, 2011).

Data analysis and statistical methods

The baseline value of SCC was determined every 24 h for all cows three days before the first dose (B1–B3). SCC analysis was performed using the Bentley BactoCount IBCm analyser (Bentley Instruments Inc.). Analyses were performed immediately after milk sampling. Half-life effect – time to reach a 50% reduction of SCC ($t_{0.5,SCC}$) based on the slope of the SCC median was calculated based on the first-order process equation $t_{0.5,SCC}$ = Ln(2)/slope. The slope value based on a linear fit was calculated by ThothPro 4.1 software (ThothPro, LLC).

The pharmacokinetic analyses were performed based on raw data using Phoenix® WinNonlin® 8.0 software (Certara L.P., US) and ThothPro v 4.1 (ThothPro LLC). The calculations were based on the slope, height, area, and moment analysis after IMM administration. Key pharmacokinetic parameters were included in the comparative analysis, i.e. AUC_{0-t} – area under the curve calculated between zero and the last sampling point, $AUMC_{0-t}$ – area under the first moment curve calculated between zero and the last sampling point, MRT_{0-t} – mean residence time calculated for the last sampling point, $t_{0.5kel}$ – elimination half-life, C_{max} – maximal concentration, k_{el} – elimination rate constant, CL clearance and V_{ss} – the volume of distribution at steady state.

The analysis of raw data was conducted with the use of GraphPad Prism® v. 6.01 (GraphPad Software Inc.). Unpaired two-tailed T-test was used for data comparison in subsequent groups and chi^2 test was used for median SCC trend analysis. The first group was the SCC arithmetic mean representing baseline (B1–B3), the second one was the SCC value on a particular day of treatment or recovery phase. The differences with $P<0.05$ were considered as statistically significant.

Results

SCC levels in milk samples

The SCC changes in milk in baseline and during baicalin administration after IMM treatment at a single dose of 120 mg/udder/day are shown in Figure 1. SCC

arithmetic mean in baseline was 2,701,257±657,174 cells/mL. Starting from T1 and continuing to T8, a decrease of SCC in relation to baseline was characterized by a declining trend (P<0.0001). After 3 days of baicalin administration in a 120 mg/udder/day (T1–T3) dose, SCC decreased by 31.99% with reference to the arithmetic mean of baseline (P>0.05). The slope of the median for SCC in the T1–T8 phase pointed out that $t_{0.5,SCC}$≈131.41 h. Significant changes in SCC values were observed only at the recovery phase (R1–R8) (Figure 1). After 8 days of baicalin administration in a 120 mg/udder dose, SCC decreased by 42.55–53.56% (R1–R3, P<0.001), 39.13–51.28 (R4–R5, P<0.05) and 56.90–100.34% (R6–R8, P<0.001), with reference to the arithmetic mean of baseline.

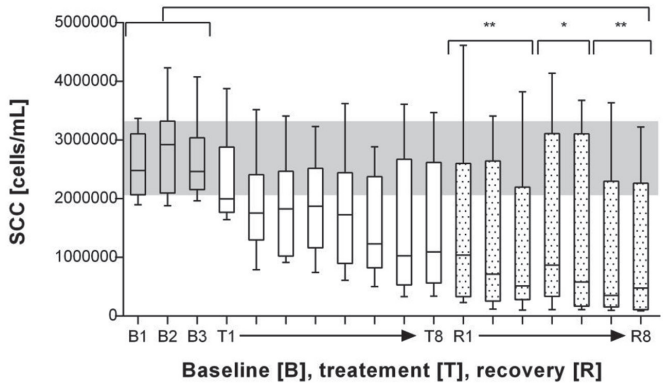

Figure 1. Daily changes of somatic cell count (SCC) in the milk of cows after multiple intramammary dosing of quercetin every 24 h, within 8 days. B1–B3 – baseline analyzed within 3 subsequent days before the first dose; T1–T8 – baicalin treatment (120 mg/udder/day); R1–R8 – recovery period. * – P<0.05 and ** – P<0.001, arithmetic mean of baseline vs. subsequent day.
Shadow area – range of SCC variability at baseline; line in shadow and white boxes – median value; boxes borders – 50% percentiles; whiskers – range from 10 to 90% percentiles

Pharmacokinetics

As shown in Figure 2 general profile of baicalin disposition after single IMM administration is typical for one compartment model. The observed plateau between 20 and 80 h after last dose (T8) represents concentrations close to 100 ng/mL. As shown, C_{max} analysis within the T1–T8 period (Figure 3) cumulation was not confirmed. Everyday C_{max} concentrations were 12.57±6.55 µg/mL of milk (Figure 3). C_{max} after the last dose was 25,166.67±10,210.72 ng/mL immediately after drug administration. The k_{el} calculated was 0.1150±0.0326 h^{-1} and $t_{0.5kel}$ 6.67±2.37 h, AUC_{0-t} value was 117,967.12±52,567.34 µg×h/L, $AUMC_{0-t}$ value was 1,318,441.58±700,241.79 µg×h^2/L, MRT_{0-t} 11.10±3.74 h, CL 0.31±0.12 L/h and V_{ss} 3.33±1.70 L.

Adverse reactions monitoring

Administration of 120 mg baicalin per udder within 8 days was well tolerated by the animals within the treatment and recovery period (T1–R8). No swelling, redness, or any other local or general adverse drug reaction occurred after baicalin administration in the proposed regimen.

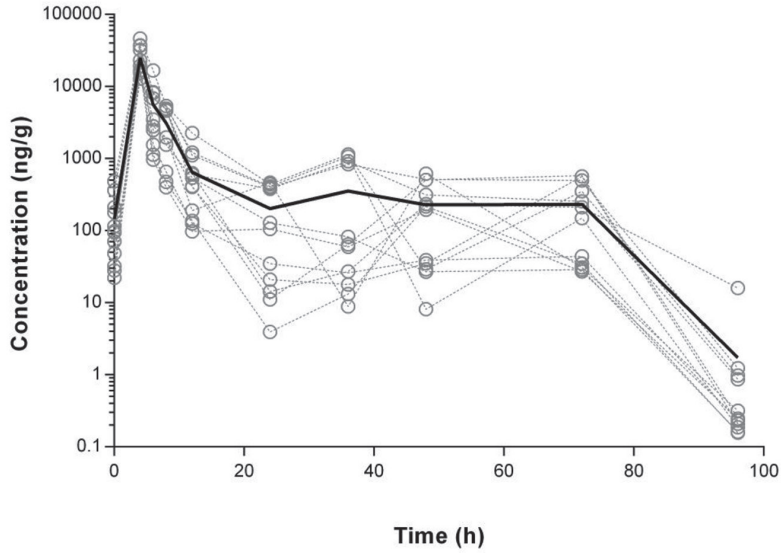

Figure 2. Pharmacokinetics of baicalin after the last dose in day 8 (T8) after the last intramammary administration of the drug at a dose of 120 mg/udder/day.
Black line – arithmetic mean; dotted lines – observed values from subsequent animals

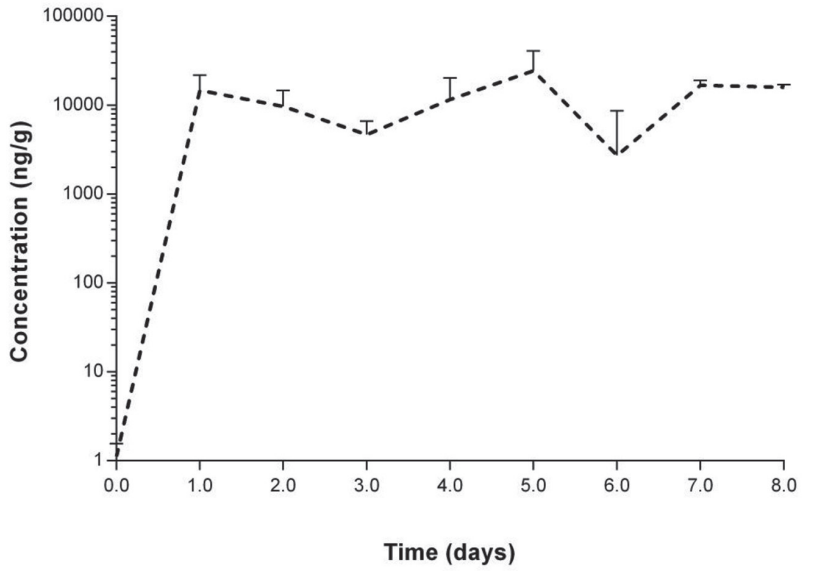

Figure 3. Variability of baicalin concentrations was measured immediately after each dose between first-day dose (T1) and day 7 (T8).
Dotted line – arithmetic mean; vertical bars – 95% confidence interval

HPLC method optimization and validation

Various solvents like acetonitrile, acetonitrile with methanol (50:50, v/v), 0.5% formic acid in acetonitrile, and 0.5% formic acid in methanol were tested to achieve the most effective extraction procedure. Finally, the use of 0.5% formic acid in acetonitrile for the extraction of baicalin from milk samples was found to be the most efficient. For the elimination of matrix impurities, 0.22 µm syringe filters were used before the injection of the samples on the UHPLC-MS/MS system. The successful validation shows high accuracy and precision of the described method, with a good matrix-matched calibration curve linearity (r^2>0.99). No interfering peaks from endogenous compounds in the retention time of the target analyte in milk samples were observed. The recoveries were in the range of 92.6–103%, depending on the fortification level with relative standard deviation in the range of 5.5–8.9% for repeatability and 9.9–13.1% for within-laboratory reproducibility at all fortification levels, indicating good precision of the method. The satisfactory sensitivity with LLOQ=0.1 µg/kg was obtained.

Discussion

In the current study, the potential effect after baicalin administration at the level of SCC in the milk of cows with mastitis was verified. The only report concerning intramammary administration of other polyphenols concerns quercetin (Burmańczuk et al., 2018). Current research on baicalin is consistent with the previously reported effects of quercetin after intramammary administration. Moreover the performed analyses confirmed the results of many previous studies on the mechanism of baicalin action. These studies indicated that baicalin possesses anti-inflammatory, antiviral, antioxidative, anticancer, and antithrombotic properties (Ma et al., 2005; Li-Weber, 2009; Li et al., 2011; Guo et al., 2013, 2014; He et al., 2015). It was also found that baicalin has antioxidant effects on bovine mammary cells (Perruchot et al., 2019). The multidirectional influence of baicalin on cytokines, interleukins, and other factors, which take a part in communication between immunocompetent cells is compatible with the results of the presented research. So far, the effect of baicalin on SCC levels after IMM administration in cows has not been studied. The starting point for the proposed dose were previous studies with intramammary quercetin administration to dairy cows. The result of the presented work is both the determination of the two dose levels and its interval at which a therapeutic effect can be expected. A similar effect was observed in the case of the IMM administration of quercetin in dairy cows (Burmańczuk et al., 2018). However, it should be noted that quercetin was less tolerated, therefore a correction of the dose from 30 mg/quarter/day to 10 mg/quarter/day was required. In the case of baicalin, the 30 mg/quarter day dose for 7 days was well tolerated by the animals and did not cause any swelling or redness or other local reactions within the mammary gland in general. Although the downward trend for SCC was present after the first baicalin administrations (as in the case of quercetin), a significant decrease in SCC was noted only in R1–R8 ($P<0.05$). The cause of the delayed effect, which was observed

after the end of baicalin administration may be due to the specific effect of baicalin on the immune system. Baicalin is an immunotropic substance (Schapoval et al., 1998; Shen et al., 2003; Guo et al., 2013). The characteristic of the dynamics of the observed pharmacodynamic effect seems to confirm the significant effect of this component on the level of SCC in cow's milk.

To determine the potential accumulation of baicalin in milk after multiple administration, a novel UHPLC-MS/MS method for the quantitative analysis of tested flavonoid was developed. For the effective isolation of baicalin, a liquid/liquid extraction (LLE) was applied. Various solvents like acetonitrile, acetonitrile with methanol (50:50, v/v), 0.5% formic acid in acetonitrile, and 0.5% formic acid in methanol were tested. Finally, the use of 0.5% formic acid in acetonitrile for the extraction of baicalin from milk samples was determined to be the most efficient. Instead of commonly reported solid-phase extraction (SPE) for clean-up procedure in quantitative methods, the purification of extracts with filters only was carried out in a developed method (de Rijke et al., 2006). For the elimination of matrix impurities, 0.22 μm syringe filters were used before injection on the UHPLC-MS/MS system. The less complex sample preparation step and UHPLC technique used in the presented method, allow for fast and labor-effective analysis of many samples, which is particularly important in a pharmacokinetic study.

In the present study, baicalin was repeatedly administered without obtaining steady-state in a single dose. During 8 days of once a day baicalin dosing of 30 mg/quarter/day referring to the C_{max} on individual days, no accumulation of baicalin was found in the tested milk. After 8 days of baicalin administration, its concentration was tested for 96 h after the last administration. After 24–72 h of the last administration, a plateau was found, which may indicate some saturation of the elimination process. The presented results allowed for the confirmation of the significant influence of baicalin on the reduction of SCC in mastitis in dairy cows after 8 days of therapy ($P<0.05$). Therefore, based on previous studies it appears that baicalin may have an action against mastitis and is potentially useful for the treatment of mastitis. After 8 days of baicalin administration in a 120 mg/udder/day dose, significant changes in SCC values were observed at the recovery phase (R1–R8). The presented results allowed for the confirmation of the significant influence of baicalin on the reduction of SCC in mastitis in dairy cows after 8 days of therapy ($P<0.05$). Therefore based on previous studies, it appears that baicalin may have an action against mastitis and is potentially useful for the treatment of mastitis. After 8 days of baicalin administration in a 120 mg/udder/day dose, significant changes in SCC values were observed at the recovery phase (R1–R8).

The conducted research allows us to hope for the use of polyphenols in the treatment of mastitis because some of them, such as quercetin, are consumed in very high amounts in plant-based products. The use of flavones and other polyphenols in the treatment of mastitis could therefore mean very short withdrawal periods and very low risk for consumers. This marks a new direction for intramammary drugs. The presented studies have some weaknesses. They result, for example, from the structure typical for pilot studies. The current research must certainly be expanded both

in terms of the dose-effect, stage of disease development and the sample size. But the authors believe that the current results are an excellent basis for future research.

List of abbreviations used in the manuscript:

IMM, intramammary
SCC, somatic cell count
B1–B3, baseline
T1–T8, treatment period
R1–R8, within recovery period
PGE2, prostaglandin E2
LTB4/LTC4, leukotriene B4 C4
COX, cyclooxygenase
Th1, t helper lymphocytes
IL-1, interleukin 1
IL-4, interleukin 4
IL-6, interleukin 6
IL-10, interleukin 10
IL-12, interleukin 12
IL-25, interleukin 25
IL-33, interleukin 33
MCP-1, monocyte chemoattractant protein-1
NF-kB, nuclear factor-kB
VEGF-A, vascular endothelial growth factor A
5-LOX, 5-lipoxygenase
iNOS, inducible nitric oxide synthase
NO, nitrogen oxide
CRP, C-reactive protein
TNF, tumor necrosis factor
IgE, immunoglobulin E
VCAM-1, vascular cell adhesion molecule 1
ICAM-1, intercellular adhesion molecule 1
MIP-2, macrophage inflammatory protein 2
LPS, lipopolysaccharide
TLR2, toll-like receptor 2
p53, tumor suppressor protein
BCL-2, B-cell lymphoma 2
BAX, bcl-2-like protein 4
CASP-3, caspase 3
UHPLC-MS/MS, liquid chromatography with tandem mass spectrometry
PBS, phosphate buffered saline
LLOQ, the lower limit of quantification
$t_{0.5,SCC}$, half-life effect – time to reach 50% reduction of SCC
AUC_{0-t}, area under the curve calculated between zero and the last sampling point
$AUMC_{0-t}$, area under the first moment curve calculated between zero and the last sampling point

MRT_{0-t}, mean residence time calculated for the last sampling point
$t_{0.5kel}$, elimination half-life
C_{max}, maximal concentration
k_{el}, elimination rate constant
CL, clearance
V_{ss}, volume of distribution at steady state

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