

## **RESPONSE**

official opponent for the dissertation work of Ping Xu "Anti-inflammatory effect and mechanism of Chlorogenic acid extract from *Taraxacum officinale* on LTA-induced mastitis in dairy cows", submitted for obtaining the scientific degree of Doctor of Philosophy in the field of knowledge 21 - "Veterinary Medicine" in the specialty 211 - "Veterinary Medicine"

### **Relevance of the topic of the work**

Mastitis remains a major problem in dairy farming. Mastitis brings great losses not only in connection with the decline of milk productivity, but also due to premature culling of cows, morbidity and death of newborn calves.

Antibacterial drugs, such as mastisan, demast, mastiet forte, masticid etc., are widely used in the treatment of mastitis. Despite the fact that the treatment can be quite effective - in the future, antibiotic residues are found in milk, which makes it unsuitable for human consumption. The remaining amount of antibiotics is excreted with milk for 3-5 days or more after the last administration of the drug. After the treatment of mastitis with antibiotics, the productivity of the animal decreases, and the causative agents of mastitis acquire antibiotic resistance. Improper prescription of antibiotics, long courses can lead to undesirable consequences. Some drugs for the treatment of mastitis, based on a number of antibiotics, have low therapeutic efficiency, as a result of which the administration period is extended, the disease transitions into a subacute and chronic course, against the background of which atrophy of the udder quarters and culling of dairy cows occur.

In recent years, the problem of antibiotic resistance, as well as the resistance of pathogens to other drugs (disinfectants, antivirals), has globally faced the world's leading countries, and they have begun to develop containment strategies. The use of phytopreparations has a significant advantage over other antimastitis agents.

Therefore, the relevance of Xu Ping's dissertation on the study of the anti-inflammatory effect and the mechanism of action of the extract of chlorogenic acid from dandelion on the inflammatory model of epithelial cells of the mammary gland of cattle is beyond doubt.

### **Connection of work with scientific programs, plans, topics**

The dissertation work is carried out in accordance with the programs of research work of Sumy National Agrarian University: "System of monitoring methods of control and veterinary and sanitary measures, regarding the quality and safety of livestock products in diseases of infectious etiology" (state registration

No. 0114U005551, 2014-2019); "Forecasting the risks of cross-border introduction and spread of particularly dangerous animal diseases and the development of scientifically based disinfection systems based on innovative import-substitutable highly effective means" (state registration No. 0115U001342, 2018-2023).

The work was carried out for the period from 2018 to 2022 at the departments of veterinary examination, microbiology, zoohygiene and safety and quality of animals products, the department of epizootology and parasitology and the department of virology, pathanatomy and bird diseases of Sumy National Agrarian University. In addition, veterinary studies were conducted at Henan, China.

### **The degree of validity of scientific statements**

The aim of this study is to explore the anti-inflammatory effect of Chlorogenic acid on LTA-induced Bovine Mammary Epithelial Cells (BMEC) and its mechanism, especially on the pathway. To provide theoretical guidance and help for the clinical application of Chlorogenic acid in veterinary medicine and the development of dairy cow mastitis prevention and treatment drugs.

For the purpose were assigned the following 5 tasks: extraction and detection of chlorogenic acid in *Taraxacum officinale*; Antibiofilm activity against *Staphylococcus aureus* and content analysis of *Taraxacum officinale* phenolic extract; in vitro culture and evaluation of bovine mammary epithelial cells from Ukraine dairy cows; establishment of inflammatory model of bovine mammary epithelial cells induced by lipoteichoic acid; effect of chlorogenic acid on inflammatory response induced by lipoteichoic acid in bovine mammary epithelial cells.

Ping Xu applied various and modern research methods: clinical (history taking, clinical examination), microbiological (microscopic, biological), bacteriological (the antibacterial efficiency of the drug), pharmacological, toxicological (degree of toxicity and harmlessness of the drug), immunological (drugs cause changes in gene and protein levels of inflammatory factors) and statistical (processing of research results).

The dissertation student conducted research in compliance with biological safety norms and principles of bioethics.

The methods used in the work correspond to the tasks and provide an opportunity to conduct modern research at a high scientific level.

Conclusions, proposals for production are fully substantiated and correspond to the obtained results of own research of the dissertation work.

Scientific novelty of the obtained results. For the first time, chlorogenic acid in dandelion (*Taraxacum officinale*) was conducted extraction and detection. A high performance liquid chromatography method was established for simultaneous

detection of five phenolic acids in dandelion, and the method was efficient and stable. Antibiofilm activity against *Staphylococcus aureus* of *Taraxacum officinale* phenolic extract was determined. In vitro culture and evaluation of bovine mammary epithelial cells from Ukraine dairy cows was conducted. Inflammatory model of bovine mammary epithelial cells induced by lipoteichoic acid was established. At last, the effect of chlorogenic acid extract on inflammatory response induced by lipoteichoic acid in bovine mammary epithelial cells was studied.

The high level of scientific results and conclusions presented in the dissertation is confirmed by the objectivity and totality of the material, experimental studies using bacteriological, pharmacological, toxicological, biochemical, immunological and statistical methods.

#### **Importance for science and practice of the results obtained by the author of the dissertation and ways of their use**

According to the results of the experiments, Ping Xu determined the optimal conditions for the extraction of chlorogenic acid in dandelion. The extraction technology of chlorogenic acid was researched from multiple factors, the best parameters of the technology were obtained that the ultrasonic temperature was 80 °C, the solid-liquid ratio was 1 : 30, the solvent concentration was 50%, and the ultrasonic time was 40 min, then the extraction rate can reach 1.921%.

The high performance liquid chromatography detection method of chlorogenic acid from *Taraxacum officinale* was 5% methanol elution condition 0 ~ 5 min, 5 - 15% methanol elution condition 5 ~ 15 min, 15 - 5% methanol elution condition 15 ~ 20 min, 5 % methanol elution condition 20 ~ 25 min, and the buffer salt was 1‰ phosphoric acid aqueous solution, and the wavelength was 350 nm. The method was stable and reliable.

In the process of the antibacterial experiment, it was found that phenolic extract from *T. officinale* has an inhibitory effect on *S. aureus*, and the mechanism of action was to destroy the integrity of its cell walls and cell membranes. The minimum inhibitory concentration (MIC) of dandelion phenolic extract exhibited against *S. aureus* was 12.5 mg/mL. The antibacterial kinetic curve analysis showed that the inhibitory effect of dandelion phenolic extract on *S. aureus* was mainly in the exponential growth phase.

When studying the establishment of bovine mammary epithelial cells from the milk of Ukraine Holstein dairy cows in order to develop a general in vitro model. Epithelial cells of the mammary gland were isolated and cultured from milk by student and pure primary cells were obtained. Such cell culture can be widely

used by many scientists when studying the mechanism of action of various means for milking, for the prevention and treatment of mastitis in cows.

The characteristics of bovine mammary epithelial cells were examined using real time cell assay, immunocytochemistry, reverse transcription- polymerase chain reaction, and Western blot. Real time cell assay provides a remarkable method for real time monitoring of cell viability. The result showed that the best seeding density for the proliferation of bovine mammary epithelial cells was  $1 \times 10^4$  cells. The bovine mammary epithelial cells culture slowly grew within the first 3 days and cells entered the stable phase in the best seeding density. Cells exhibited strong positive staining for cytokeratin 18, which is specific for epithelial cells, indicating that the cultured cells possessed the properties of epithelial cells. And the results confirmed the ability of the isolated cells to synthesize milk proteins.

The viability of bovine mammary epithelial cells infected with lipoteichoic acid LTA were lower than that of the normal bovine mammary epithelial cells.

It was found that with the increase of lipoteichoic acid dose, the inhibition rate of cell vitality increased gradually, indicating that lipoteichoic acid of certain concentration would cause the damage of bovine mammary epithelial cells.

The author recommended extract of *Taraxacum officinale*, as a new type of feed additive to be used for dairy cows.

The results of the conducted research are a prerequisite for the development of new means of treatment using chlorogenic acid.

The main provisions of the dissertation were included in the Methodical guidelines "Modern methods of cow mastitis diagnostic and prevention", approved by the Scientific Council of SNAU, protocol № 5, dated December 29, 2021).

Ping Xu recommend using the materials of the dissertation work when studying the courses " Veterinary microbiology", "Veterinary internal medicine" and "Veterinary sanitary examination" for masters of the Faculty of Veterinary Medicine of Sumy NAU. And for the courses "Veterinary internal medicine" and "Chinese traditional veterinary science" for masters of the Henan Institute of Science and Technology.

Xu Ping's dissertation is a completed research paper. The results obtained by her using effective modern research methods are highly informative and reliable.

### **The completeness of teaching the results of the dissertation in published works, their quantity and quality**

For the materials of the dissertation, 20 scientific works were published, including: in scientific and professional publications of Ukraine - 4, Scopus

publication 3, publications in Chinese journal – 3, in conference materials– 9, and 1 methodical recommendation.

The main provisions and results of the research were reported and received general scientific approval at the annual scientific reports and conferences of faculty and graduate students of Sumy National Agrarian University, Faculty of Veterinary Medicine (2018-2021); Fifth Annual Regional Scientific Symposium One Health Concept, Kyiv (2019); Twentieth Chinese National Conference on Animal Genetics & Breeding, Guangzhou (2019); The 2021 Academic Forum of the Veterinary Microbiology Committee of the Chinese Society of Microbiology, Zhengzhou (2021); The 7<sup>th</sup> International Symposium on Dairy Cow Nutrition and Milk Quality, Beijing (2021), Food Quality and Safety, Health and Nutrition Congress, Ohrid, Macedonia (2019 and 2021).

#### **Personal contribution of the applicant**

The author took part in the implementation of scientific programs, which are the basis of the dissertation; developed schemes and methods of conducting experiments in laboratory and production conditions; performed experimental and analytical studies; conducted the analysis and generalization of the obtained results; conclusions and practical recommendations are substantiated. Personally or in co-authorship, with the consent of the co-authors, scientific works have been prepared for publication, in which the main material of the dissertation is presented.

#### **The recipient's knowledge of the results of scientific research by other scientists on the chosen topic of the dissertation and the comparability of these results with the results of the recipient's scientific positions**

The author of the dissertation is sufficiently familiar with the results of scientific research by other scientists on the topic of the dissertation. When writing her dissertation, Xu Ping processed 182 sources of scientific literature. The dissertation student successfully compared the obtained data with the results of other researchers, which are given in the 4th chapter of the dissertation "Summary and analyses of results", which occupies 9 pages of computer text.

#### **General assessment of the essence of the dissertation, its value and shortcomings in terms of content and design**

The dissertation work of Ping Xu "Anti-inflammatory effect and mechanism of Chlorogenic acid extract from *Taraxacum officinale* on LTA-induced mastitis in dairy cows" meets the requirements for dissertation works in Ukraine: a relevant topic of research is chosen, the conducted scientific research corresponds to the

most modern level, valuable results are obtained scientific results that have theoretical and practical significance for science and veterinary medicine specialists. At the same time, Xu Ping's dissertation has minor shortcomings, some remarks and wishes:

1. "Fat" is declared as one of the key words, but this indicator is not found even once in the chapter "results of own research".
2. Some grammatical and typographical errors "Перспективним" (p. 12) "literature reveur" (p. 125), unfortunate expressions "In order to provide a reference to the Chinese herbal treatment of mastitis in dairy cows, our work provides a scientific basis for research and development of drugs based on Chinese herbs and relevant fundamental research" (p. 8).
3. There are some typographical errors in the bibliography. For example, DIO is written instead of DOI. In literature sources № 71, 72, 89, 113, 117, 157, the pages are not indicated.

***In the process of reviewing the dissertation, some questions arose:***

1. One of the tasks in the work: creation of cell culture in vitro and assessment of mammary epithelial cells of Ukrainian dairy cows. Please tell us more about the isolation of epithelial cells from milk.
2. In the conclusion from literature review, you indicate that "... research needs to understand the antibacterial mechanism of plant extracts against pathogenic bacteria such as *Staphylococcus aureus* and *Escherichia coli*, and reveal the protective mechanism of plant extracts against mastitis in dairy cows." But the research is devoted only to *S. aureus*. Has there been any research done on *E. coli*?
3. You used ultrasonic extraction and high performance liquid chromatography to extract chlorogenic acid from *Taraxacum officinale*. Please compare these two methods, indicate their advantages and disadvantages.
4. Based on which results did you conclude that best seeding density for the proliferation of bovine mammary epithelial cells was  $1 \times 10^4$  cells?
5. Why did you choose lipoteichoic acid to induce a model of inflammation in bovine mammary epithelial cells?
6. What compounds are inflammatory cytokines and what is their meaning?
7. You propose to use dandelion extract as a new type of feed additive in production. In your opinion, the protozoa found in ruminants' stomachs will not use chlorogenic acid for their own needs? Are you sure that this substance will reach the epithelial cells of the mammary gland?

However, despite some minor shortcomings, the dissertation made a very positive impression. The identified shortcomings do not reduce the scientific value and practical importance of the dissertation work.

## Conclusion

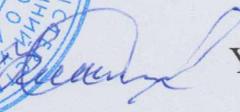
Ping Xu's dissertation on the topic: "Anti-inflammatory effect and mechanism of Chlorogenic acid extract from *Taraxacum officinale* on LTA-induced mastitis in dairy cows" issued in accordance with the order of the Ministry of Education and Science of Ukraine dated January 12, 2017 No. 40 "On approval of the Requirements for the preparation of a dissertation" and the Ministry of Education and Science of Ukraine dated May 31, 2019 No. 759 with changes and additions, is a completed scientific research work, which according to the relevance of the chosen topic, scientific novelty, theoretical and practical significance of the obtained results, the level and scope of the performed research, fully meets the requirements stipulated by the Procedure for awarding the degree of Doctor of Philosophy approved by the Resolution of the cabinet of Ministers of Ukraine dated 12.01.2022 No. 44, and its author deserves the award of the educational and scientific degree of Doctor of Philosophy in the field of knowledge 21 "Veterinary medicine" in specialty 211 "Veterinary medicine".

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