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XU PING

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PHD THESIS

ANTI-INFLAMMATORY EFFECT AND MECHANISM OF CHLOROGENIC ACID EXTRACT FROM *TARAXACUM OFFICINALE* ON LTA-INDUCED MASTITIS IN DAIRY COWS

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The dissertation contains the results of own research. The use of ideas, results and texts of other authors have references to the relevant source XU PING **Ping Xu**

Scientific supervisor (consultant): **Hanna Fotina**, Doctor of Veterinary Sciences, Professor

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ANNOTATION

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Based on scientific demonstration and experimental research, this thesis is devoted to studying Anti-inflammatory effect and mechanism of Chlorogenic acid extract from *Taraxacum officinale* on LTA-induced mastitis in dairy cows.

Mastitis is one of the costliest diseases affecting dairy cattle. Direct economic costs associated with mastitis include reduced milk yield and quality, increased veterinary costs, discarded milk (during the course of treatment) and somatic cell count (SCC) penalties.

When analyzing literary sources, we established that mastitis still remains a pressing problem for many countries, including China and Ukraine.

And extracts from plant have obvious therapeutic effects and play an inportent role in the treatment of mastitis in dairy cows. They can not only avoid the production of drug-resistant bacteria caused by the application of antibiotics, but also avoid problems such as drug residues.

Therefore, the application of plant extracts has always been high hopes in the prevention and treatment of dairy cow mastitis. Therefore, 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.

It has long been known that mastitis resistance or susceptibility is a complex trait that is comprehensively affected by the environmental, physiological and genetic factors.

Analyzing plans for carrying out treatment and preventive measures in dairy at present, antibiotics are mainly used for the treatment of cow mastitis.

However, the abuse of antibiotics has lead to increased resistance to pathogenic bacteria and serious drug residues.

Chlorogenic acid (3-O-caffeoylquinic acid) is the main active ingredient of an extract from Dandelion (*Taraxacum officinale*), which has played an important pharmacological role in the study of many inflammatory diseases.

However, the molecular mechanism underlying the formation of resistance is poorly understood.

This research takes chlorogenic acid as the research object, which is a natural ingredient in the Chinese herbal medicine *Taraxacum officinale*. Firstly, the optimal extraction conditions of chlorogenic acid in dandelion were determined by orthogonal experiment. A high performance liquid chromatography (HPLC) method for the detection of chlorogenic acid in dandelion was established. Secondly, detection of the antibacterial mechanism of phenolic acid extract (include chlorogenic acid) against *Staphylococcus aureus*. Lastly, the mammary epithelial cells were isolated and cultured from milk, and obtained pure primary cells; BMEC inflammatory model was established by Lipoteichoic acid (LTA); CCK-8, ELISA, qRT-PCR and WB were used to analyze the protective and anti-inflammatory effect of chlorogenic acid on LTA-induced inflammatory lesions. In order to provide a reference for the Chinese herbs treatment of dairy cow mastitis, it provides scientific basis for the research and development of Chinese herbs and related basic research.

In the study of detection and extraction process of chlorogenic acid extract from *Taraxacum officinale* was conducted. 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 ^{0}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 HPLC 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. After the action of dandelion phenolic extract, the growth of *S. aureus* was obviously inhibited entering into the decay phase early. Furthermore, after the action of dandelion, the extracellular AKP (alkaline phosphatase) contents of *S. aureus,* the electrical conductivity and the extracellular protein contents were all increased. The phenolic extract also affects the normal reproduction of *S. aureus*.

When analysis the content of phenolic extract from *T. Officinale,* the dandelion was extracted by ultrasonic assisted extraction method with 70% methanol-water (v/v) as a solvent. Subsequent identification and quantification of phenol in extract was carried out using High Performance Liquid Chromatography (HPLC). The results showed that chlorogenic acid (1.34 mg/g) was present in higher concentration, followed by luteolin (1.08 mg/g), ferulic acid (0.22 mg/g), caffeic acid (0.21 mg/g), and rutin (0.19 mg/g) in the dandelion phenolic extract.

When studying the establishment of bovine mammary epithelial cells (BMEC) from the milk of Ukraine Holstein dairy cows in order to develop a general in vitro model. The whole growth process is different when culturing epithelial cells from tissue. Single adherent cells appear on the culture dish after culturing for 3-4 days. Bovine mammary epithelial cells formed islands when cultured at low density after 5-7 days.

Primary epithelial cells were elongated after culturing for 8-10 days. Mammary epithelial cells developed into different shapes, including oval, typical cobblestone, and irregular polygon. Most of the isolated cells that extended from the milk had an irregular polygon shape. The cells obtained after freezing and thawing maintained normal morphology and growth characteristics. In the selection of frozen storage medium, the frozen solution containing 90% FBS and 10% DMSO was more

conducive to the survival of cryopreserved BMECs than DMEM/F12: FBS: DMSO= 5:4:1. For subsequent research, FBS containing 10% DMSO was used for preservation of frozen cells.

The characteristics of BMECs were examined using real time cell assay (RTCA), immunocytochemistry (ICC), reverse transcription- polymerase chain reaction (RT-PCR), and Western blot (WB). RTCA provides a remarkable method for real time monitoring of cell viability. The result showed that the best seeding density for the proliferation of BMECs was 1×10^4 cells. The BMECs 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. RT-PCR was used to determine the mRNA expression and WB was used to determine the milk proteins expression in the BMEC. And the results confirmed the ability of the isolated cells to synthesize milk proteins. It is very important that MECs express milk proteins and can mimic the *in vivo* system.

CCK-8 assay carried out to examine the viability of cells. The viability of BMECs infected with LTA were lower than that of the normal BMECs. RTAC was used to detect the effect of different concentrations of LTA on the proliferation of BMECs, the results were shown that with the increase of LTA concentration, the proliferation activity of BMEC cells was inhibited. According to the change of cell index value and different proliferation curves, the dynamic detection after LTA treatment of cells was found. The effects of LTA at different concentrations (0, 10, 20, 40, and 80 μg / mL) on BMEC were observed at different time points (12, 24 and 48 h); It was found that with the increase of LTA dose, the inhibition rate of cell vitality increased gradually, indicating that LTA of certain concentration would cause the damage of BMEC. According to 24 h of LTA action cells of different concentrations, the inhibition rate of concentration of 20 μg / mL and 40 μg / mL was significantly increased. The results of ELISA and qRT-PCR showed that the treatment of BMEC with LTA at the concentration of 20 μg / mL for 24 h obviously induced TNF-α and IL-6 protein and gene expression levels.

In the last, the anti-inflammatory effect and mechanism of chlorogenic acid extract were detected in LTA-stimulated bovine mammary epithelial cells. The effect of chlorogenic acid extract on the viability of bovine mammary epithelial cell was evaluated using the CCK-8 and RTCA assay. The effects of chlorogenic acid extract on NF-κB activation was detected by Western blotting.

The effects of chlorogenic acid extract on LTA-induced inflammatory cytokines expression were detected by ELISA and qRT-PCR. The results showed that the cell viability was not affected by chlorogenic acid extract at the dose of 12.5, 25, 50, 100, 200, and 400 μg/mL. The IC_{50} of the CCK-8 and RTCA analysis methods were 326.8 and 320.4 ug/mL.

Meanwhile, chlorogenic acid extract inhibited LTA-induced TNF-α, IL-1β, and IL-6 expression. Western blot analysis showed that chlorogenic acid extract suppressed LTA-induced NF-κB activation in a dose-dependent manner.

This paper investigates the anti-inflammatory mechanism of chlorogenic acid extract from *Taraxacum officinale* in inflammatory signaling pathways. It provides a basis for reference to the study of drug prevention and treatment of mastitis in dairy cows.

We 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 (HIST).

We can recommend that at farm level, dandelion extract may use as a new type of feed additive in production. And new therapies with CGA as a drug should be developed in future.

Key words: chlorogenic acid, Bovine mammary epithelial cells, Lipoteichoic acid, cytotoxicity, mastitis of dairy cows, herbal preparations, antimicrobial agent, cow, milk, somatic cells, inflammation of the breast, mastitis pathogens, somatic cells, milk productivity, microflora, milk quality, lactation, bacterial contamination, milk fat, PCR, enzyme immunoassay, antibody.

АНОТАЦІЯ

Сюй Пінь «Протизапальний ефект і механізм дії екстракту хлорогенової кислоти з *Taraxacum officinale* на LTA-індукований мастит у дійних корів». – Кваліфікаційна наукова праця на правах рукопису. Дисертація на здобуття наукового ступеня доктора філософії за спеціальністю 211 «Ветеринарна медицина» − Сумський національний аграрний університет, м. Суми.

На основі наукових, експериментальних досліджень дисертація присвячена вивченню протизапального ефекту та механізму дії екстракту хлорогенової кислоти з *Taraxacum officinale* на LTA - індукований мастит у молочних корів.

Мастит – одне з найдорожчих захворювань молочної худоби. Прямі економічні витрати, пов'язані з маститом, включають зниження надоїв і якості молока, збільшення ветеринарних витрат, вибраковане молоко (під час лікування) та штрафи за підвищену кількість соматичних клітин (SCC).

Аналізуючи літературні джерела, ми встановили, що мастит досі залишається актуальною проблемою для багатьох країн, у тому числі Китаю та України.

А екстракти з рослини мають очевидну терапевтичну дію і відіграють важливу роль у лікуванні маститу у дійних корів. Вони можуть не тільки уникнути утворення стійких до ліків бактерій, спричинених застосуванням антибіотиків, але й уникнути таких проблем, як залишки ветеринарних препаратів в продуктах тваринництва.

Тому на застосування рослинних екстрактів завжди покладалися великі надії в профілактиці та лікуванні маститу дійних корів.

Саме тому сучасні дослідження потребують розуміння антибактеріального механізмуі дії рослинних екстрактів проти патогенних бактерій, таких як *Staphylococcus aureus і Escherichia coli*, і виявлення захисного механізму рослинних екстрактів проти маститу у молочних корів.

Давно відомо, що резистентність або сприйнятливість до маститу є комплексною ознакою, на яку впливає сукупність екологічних, фізіологічних та генетичних факторів.

Аналізуючи плани проведення лікувально-профілактичних заходів на молочних фермах України та Китаю, ми встановили, що на даний час для лікування маститу корів в господарствах переважно використовують антибіотики.

Однак зловживання антибіотиками призвело до підвищення резистентності патогенних бактерій і накопиченню залишків ветеринарних препаратів в молочних продуктах та м'ясі.

Хлорогенова кислота (3-О-кофеоілхінова кислота) є основним діючим інгредієнтом екстракту кульбаби (*Taraxacum officinale*), яка відіграває важливу фармакологічну роль у вивченні багатьох запальних захворювань.

Однак молекулярний механізм, що лежить в основі формування резистентності, недостатньо вивчений.

В дисертаційній роботі в якості об’єкта дослідження береться саме хлорогенова кислота, яка є природним інгредієнтом китайського фітопрепарату *Taraxacum officinale*.

По - перше, проведеними експериментами, було визначено оптимальні умови екстракції хлорогенової кислоти в кульбабі.

В наслідок наших досліджень було створено метод високоефективної рідинної хроматографії (HPLC) для виявлення хлорогенової кислоти в кульбабі.

По - друге, було виявлено антибактеріальний ефект екстракту фенолової кислоти (включаючи хлорогенову кислоту) проти *Staphylococcus aureus*. Нарешті, були виділені та культивовані з молока епітеліальні клітини молочної залози і отримані чисті первинні клітини. Запальну модель епітеліальних клітин молочної залози великої рогатої худоби (BMEC) створювали за допомогою ліпотейхоєвої кислоти (LTA); CCK-8, ELISA, qRT-PCR та WB використовувалися для аналізу захисної та протизапальної дії хлорогенової кислоти на запальні ураження, спричинені LTA. Для того, щоб надати посилання на лікування китайськими травами маститу молочних корів, наша робота

забезпечує наукову основу для досліджень і розробок препаратів на основі китайських трав і відповідних фундаментальних досліджень.

Було проведено дослідження процесу виявлення та екстракції екстракту хлорогенової кислоти з Taraxacum officinale. Технологія вилучення хлорогенової кислоти була досліджена з багатьох факторів та отримані найкращі параметри технології, а саме: температура ультразвуку становила 80 ^{0}C, співвідношення твердої речовини до рідини становило 1: 30, оптимальна концентрація розчинника 50%, а час ультразвуку був 40 хв, при таких параметрах швидкість вилучення може досягати 1,921%.

Також використовували метод високоефективної рідинної хроматографії (HPLC) для виявлення хлорогенової кислоти з *Taraxacum officinale*: 5% метанол, умови елюції 0 ~ 5 хв, 5–15% умови метанолу, умови 5 ~ 15 хв, 15–5% умови елюції метанолу, 15 ~ 20 хв, умови елюції 5 % метанолу 20 ~ 25 хв, а буферна сіль була 1‰ водним розчином фосфорної кислоти, а довжина хвилі становила 350 нм. Цей метод виявився стабільним і надійним.

У процесі антибактеріального експерименту було встановлено, що фенольний екстракт *T. Officinale* має пригнічувальну дію на *S. aureus*, а механізм дії полягає в порушенні цілісності його клітинних стінок і клітинних мембран. Мінімальна інгібуюча концентрація (МІК) фенольного екстракту кульбаби проти *S. aureus* становила 12,5 мг/мл. Аналіз антибактеріальної кінетичної кривої показав, що інгібуючий ефект фенольного екстракту кульбаби на *S. aureus* був переважно у фазі експоненціального росту. Після дії фенольного екстракту кульбаби ріст *S. aureus* був явно пригнічений, вступаючи у фазу розпаду рано. Крім того, після дії кульбаби, позаклітинний вміст (лужної фосфатази) АКР *S. aureus*, електропровідність і вміст позаклітинного білка були збільшені. Фенольний екстракт також впливає на нормальне розмноження *S. aureus*.

При аналізі вмісту фенольного екстракту з *T. Officinale* кульбаба була екстрагована ультразвуковим методом екстракції з використанням 70% метанол-вода (об./об.) як розчинник. Подальшу ідентифікацію та кількісне визначення фенолу в екстракті проводили за допомогою високоефективної

рідинної хроматографії (HPLC). Результати показали, що хлорогенова кислота (1,34 мг/г) була присутня у вищій концентрації, потім лютеолін (1,08 мг/г), ферулова кислота (0,22 мг/г), кавова кислота (0,21 мг/г) і рутин (0,19). мг/г) у фенольному екстракті кульбаби.

Було проведено вивчення процесу утворення епітеліальних клітин молочної залози великої рогатої худоби (BMEC) з молока молочних корів голштинської породи України з метою розробки загальної моделі *in vitro*. Весь процес росту відрізняється при культивуванні епітеліальних клітин із тканини. Після культивування протягом 3-4 днів на чашці з культурою з'являються поодинокі приклеєні клітини. Епітеліальні клітини молочної залози великої рогатої худоби утворювали острівці при культивуванні при низькій щільності через 5-7 днів. Первинні епітеліальні клітини подовжувалися після культивування протягом 8-10 днів. Епітеліальні клітини молочної залози набули різних форм, включаючи овальну, типову бруківку та неправильний багатокутник. Більшість ізольованих клітин, які відбирали з молока, мали форму неправильного багатокутника. Клітини, отримані після заморожування та відтавання, зберігали нормальну морфологію та характеристики росту. При виборі замороженого середовища для зберігання використовували розчин, що містить 90% FBS (ембріональна сироватка великої рогатої худоби) і 10% DMSO (диметилсульфоксид (ДМСО) , був більш сприятливим для виживання кріоконсервованих епітеліальних клітин молочної залози великої рогатої худоби (BMEC), ніж DMEM/F12: FBS: DMSO= 5:4:1. Для подальших досліджень для збереження заморожених клітин використовували FBS, що містить 10% DMSO.

Характеристики BMEC досліджували за допомогою клітинного аналізу в реальному часі (RTCA), імуноцитохімії (ICC), полімеразної ланцюгової реакції зворотної транскрипції (RT-PCR) і Вестерн-блот (WB). RTCA забезпечує надійний метод моніторингу життєздатності клітин у реальному часі. Результат показав, що найкраща щільність посіву для проліферації BMEC була 1 × 104 клітин. Культура BMEC повільно росла протягом перших 3 днів, і клітини увійшли в стабільну фазу з найкращою щільністю посіву. Клітини продемонстрували сильне позитивне фарбування на цитокератин 18, який є

специфічним для епітеліальних клітин, що вказує на те, що культивовані клітини володіють властивостями епітеліальних клітин.

RT-PCR використовували для визначення експресії мРНК, а WB використовували для визначення експресії молочних білків у BMEC. Результати підтвердили здатність ізольованих клітин синтезувати білки молока. Дуже важливо, щоб епітеліальні клітини молочної залози (MEC) експресували білки молока і могли імітувати систему *in vivo*.

Був проведений аналіз CCK-8, з метою перевірки життєздатності клітин. Життєздатність BMEC, інфікованих LTA, була нижчою, ніж у звичайних BMEC.

RTAC використовувався для виявлення впливу різних концентрацій LTA на проліферацію BMEC, результати показали, що зі збільшенням концентрації LTA активність проліферації клітин BMEC пригнічувалася. Відповідно до зміни значення клітинного індексу та різних кривих проліферації виявлено динамічну наявність після обробки клітин LTA. Вплив LTA в різних концентраціях (0, 10, 20, 40 і 80 мкг / мл) на BMEC спостерігався в різні моменти часу (12, 24 і 48 годин). Було виявлено, що зі збільшенням дози LTA швидкість інгібування життєдіяльності клітин поступово зростала, що вказує на те, що LTA певної концентрації спричиняє пошкодження BMEC. Згідно з 24-годинною дією клітин LTA різних концентрацій швидкість інгібування концентрації 20 мкг/мл та 40 мкг/мл була значно збільшена. Результати ELISA та qRT-PCR показали, що обробка BMEC LTA у концентрації 20 мкг/мл протягом 24 год, очевидно, індукувала TNF-α та IL-6 білки та рівні експресії генів.

Матеріали дисертаційної роботи використовуються при вивченні курсів «Ветеринарна мікробіологія», «Внутрішні хвороби тварин» та «Ветеринарно-санітарна експертиза» для магістрів факультету ветеринарної медицини Сумського НАУ, а також для курсів «Внутрішні хвороби тварин» та «Китайська традиційна ветеринарія» для магістрів Хенанського інституту науки і технологій (HIST).

Екстракт кульбаби, як новий вид кормової добавки, рекомендовано використовувати у агропромислових господарства при виробництві молока.

Перспктивним є розробка нових засобів лікування з використанням CGA.

Ключові слова: хлорогенова кислота, епітеліальні клітини молочної залози, ліпотейхоєва кислота, цитотоксичність, мастит молочних корів, рослинні препарати, протимікробний засіб, корова, молоко, соматичні клітини, запалення молочної залози, збудники маститу, соматичні клітини, молочна продуктивність, мікрофлора, якість молока, лактація, бактеріальна контамінація, молочний жир, ПЛР, імуноферментний аналіз, антитіла.

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CONTENT

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LIST OF CONDITIONAL ABBREVIATIONS

- AP - Ammonium persulfate
- BCA - Bicinchoninic acid
- BMEC - Bovine mammary epithelial cells
- BSA - Bovine Serum Albumin
- cDNA - Complementary DNA
- CGA - Chlorogenic acid
- DAPI - 4,6-diamino-2-phenyl indole
- DEPC - Diethy pyrocarbonate
- DMEM/F 12 - Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
- DMSO - Dimethyl sulfoxide
- EDTA - Ethylene Diamine Tetraacetic Acid
- EGF - Epithelial growth factor
- ELISA - Enzyme-linked immunosorbent assay
- FBS - Fetal bovine serum
- FITC - Fluoresceine Isothiocyanate
- HRP - Horseradish Peroxidase
- ICC - Immunocytochemistry
- IGF - Insulin like growth factor
- IL-1 β - Interleukin-1 β
- IL-6 - Interleukin-6
- IκBα - Inhibitor of NF-κB
- LTA - Lipoteichoic acid
- MAC-T - Mammary alveolar cell-T
- MEC - Mammary epithelial cells
- NC - Nitrocellulose
- NF-κB - Nuclear factor κB
- NO - Niitric oxide
- OD - Optical density

- PBS - Phosphate Buffered Saline
- PBST - Phosphate Buffered Saline-Tween20
- PI - 3,8-Diamino-5-[3-(diethylmethylammonio) propyl]-6-phenylphenanthridinium diiodide
- PVDF - Polyvinylidene fluoride
- qRT-PCR - Quantitative realtime PCR
- RIPA - Radio Immunoprecipitation Assay
- RTCA - Real-time cell assay
- RT-PCR - Reverse Transcription-Polymerase Chain Reaction
- SDS- PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- TBS - Tris-HCl Buffered Saline
- TBST- Tris-HCl Buffered Saline-Tween 20
- TEMED - N,N,N',N'-Tetramethylenediamine
- TLR2 - Toll-like Receptor 2
- TNF α - Tumor Necrosis Factor α
- WB - Western blot

INTRODUCTION

Actuality of theme. Mastitis is one of the costliest diseases affecting dairy cattle. Direct economic costs associated with mastitis include reduced milk yield and quality, increased veterinary costs, discarded milk (during the course of treatment) and somatic cell count (SCC) penalties. The main indirect costs, which are usually underestimated by farmers, are the increased risk of culling and reduced fertility. On average, each case of clinical mastitis causes an estimated loss of around 200 Euros. The cost of subclinical mastitis depends on the number of cows with an increased SCC and is mainly attributed to losses in milk production.

Mastitis has a wide range of incidence, occurring in dairy farms in Ukraine, China, and even all over the world. Antibiotics play an important role in the prevention and treatment of mastitis. However, antibiotics are often accompanied by drug residue problems and are harmful to human health. Therefore, people are forced to find safer drugs to prevent and treat mastitis in dairy cows. Traditional Chinese medicine has a history of thousand years of clinical application and has been used for the prevention and treatment of breast diseases. Among them, the Chinese herbal medicine *Taraxacum officinale* is often used as an important ingredient in certain rented houses and participates in the treatment of mastitis. As one of the important effective ingredients in dandelion, dandelion chlorogenic acid has been proven to have anti-inflammatory, antibacterial and anti-tumor effects, and has attracted increasing attention from scientific researchers.

Chlorogenic acid, a phenolic acid isolated from *Taraxacum officinale*, has been used in this study. Firstly, we determined the optimal extraction conditions of chlorogenic acid in dandelion by orthogonal experiment. And we established the high performance liquid chromatography (HPLC) method for the detection of chlorogenic acid in dandelion. Secondly, we investigated the anti-bacterial effects of phenolic acid extract (include chlorogenic acid) against *Staphylococcus aureus.* Lastly, the mammary epithelial cells were isolated and cultured from milk, and obtained pure primary cells; BMEC inflammatory model was established by Lipoteichoic acid (LTA); CCK-8, ELISA, qRT-PCR and WB were used to analyze the protective and

anti-inflammatory effect of chlorogenic acid on LTA-induced inflammatory lesions. In order to provide a reference for the Chinese herbs treatment of dairy cow mastitis, it provides scientific basis for the research and development of Chinese herbs and related basic research.

Connection of work with scientific programs, plans, topics. The materials of the dissertation work are part of comprehensive scientific research of the Department of Veterinary Expertise, Microbiology, Zoohygiene and Safety and Quality of Livestock Products of the Sumy National Agrarian University according to the following thematic plans of research works: "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 aim and objectives of the study. 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 tasks:

1. Extraction and detection of chlorogenic acid（CGA） in *Taraxacum officinale*.

2. Antibiofilm activity against *Staphylococcus aureus* and content analysis of *Taraxacum Officinale* phenolic extract.

3. In vitro culture and evaluation of bovine mammary epithelial cells from Ukraine dairy cows.

4. Establishment of inflammatory model of bovine mammary epithelial cells（BMEC) induced by LTA.

5. Effect of chlorogenic acid (CGA) on inflammatory response induced by

LTA in BMEC.

Object of study antibacterial mechanism of chlorogenic acid in *Taraxacum officinale,* anti-inflammatory effect and mechanism of chlorogenic acid extract from *Taraxacum officinale* in the LTA-induced bovine mammary epithelial cell in vitro inflammation model, Mammary epithelial cells of dairy cow mastitis caused by Staphylococcus aureus LTA stimulation, Holstein breed.

Subject of study Antibiofilm activity against *Staphylococcus aureus,* content analysis of *Taraxacum Officinale* Phenolic extracts, in vitro culture and evaluation of bovine mammary epithelial cells from dairy cows, inflammatory model of bovine mammary epithelial cells（BMEC) induced by LTA, Effect of Chlorogenic acid (CGA) on inflammatory response induced by LTA in BMEC.

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).

Scientific novelty of the obtained results. For the first time, chlorogenic acid (CGA) 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 (BMEC) induced by LTA was established. At last, the effect of chlorogenic acid extract on inflammatory response induced by LTA in BMEC was studied.

The practical significance of the obtained results. We 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 (HIST).

We can recommend that at farm level, dandelion extract may use as a new type of feed additive in production. And new therapies with CGA as a drug should be developed in future.

The main provisions of the dissertation were included in the Methodological Recommendations of Modern methods of cow mastitis diagnostic and prevention, for laboratory, practical classes and independent work for master's students of veterinary department from disciplines "Veterinary Microbiology"and "Veterinary Zoohygiene", specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise". (approved by the Scientific Council of SNAU, protocol No. 5, dated December 29, 2021).

Research results are introduced to the "Veterinary internal medicine" and Veterinary sanitary examination" sections when creating educational and methodological complexes and are used in distance learning of students based on the "Moodle" platform.

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 author expresses his gratitude to the heads and employees of laboratories, institutes of dairy farms, for their help during the dissertation work.

Approbation of dissertation results. 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 7th International Symposium on Dairy Cow Nutrition and Milk Quality, Beijing (2021), Food Quality and Safety, Health and Nutrition Congress, Ohrid, Macedonia (2019 and 2021).

Publications. 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 Chines journal – 3, in conference materials– 9, and 1 methodical recommendation.

The structure and scope of the dissertation. The dissertation is presented on 146 pages of computer text, illustrated with 15 tables and 38 figures and consists of annotation, introduction, review of literature, materials and methods, results of own research, generalization, analysis and discussion of research results, conclusions, proposals, list used sources, applications. The list of used sources of literature includes 182 names.

CHAPTER 1

LITERATURE REVIEW ON THE TOPIC AND CHOICE OF RESEARCH DIRECTIONS

1.1 Research progress of dairy cow mastitis

Mastitis is an inflammatory disease of the mammary gland, which has a significant economic impact and is an animal welfare concern. Mammary gland development and regression was directly related with cow lactation. Many different microbial and environmental factors can induce mastitis. *Escherichia coli* and *Staphylococcus aureus* were the mian cause of mastitis. The treatment of clinical and subclinical mastitis mainly focuses on the use of antibiotics. At present, although some progress has been made in the clinical diagnosis, antibiotic treatment, and pathogenesis control of dairy cow mastitis, the molecular mechanism of the pathogenicity of dairy cow mastitis was still not very clear. So, it is important to understand the mechanisms controlling the immune response at the molecular level. A deeper understanding of the molecular mechanisms of mastitis in dairy cows will help to discover new ways to reduce the harm that mastitis poses to dairy farming.

Etiology and treatment of the dairy cow mastitis. Dairy cow mastitis is an inflammatory reaction caused by a variety of pathogens. It is also one of the most serious diseases in dairy farming. The occurrence of cow mastitis not only reduces the yield and quality of milk, but also lead to prolonged estrus time and pregnancy time after delivery of the dairy cows. Disorders can cause cows to be eliminated or even die in severe cases, causing huge economic losses to the dairy farming industry [123]. In the past few decades, the economic losses caused by dairy cow mastitis to the world dairy industry have remained basically unchanged [47]. A deeper understanding of the molecular mechanisms of mastitis in dairy cows will help to discover new ways to reduce the harm that mastitis poses to dairy farming.

Pathogenic microorganisms causing mastitis in dairy cows have been found to have at least 150 species, among which *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Mycoplasma* [8]. Among them, *Staphylococcus aureus* and *Escherichia coli* are the most common pathogen causing mastitis in dairy cows and has been widely used to inducing pathogens in subclinical and clinical mastitis models [13]. *S. aureus* intramammary infection (IMI) often causes slight clinical symptoms; in contrast, *E. coli* IMI often leads to severe clinical mastitis, especially during calving, which seriously affects the health of the cow [31]. In the past few decades, people have been able to stably and effectively reveal specific pathogen-induced effects pathological mechanism of mastitis in dairy cows through in vivo infection experiments [150]. It has been shown that *E. coli* and *S. aureus* are effective substitutes for inducing clinical and subclinical mastitis, respectively. In addition, *S. aureus* secretes a variety of virulence factors during the process of infection to cause local suppurative inflammation, damage of skin and soft tissues.

In the past, antibiotic therapy was used in the treatment of mastitis in cows. However, due to the increasing resistance of pathogenic microorganisms, the effectiveness of antibiotic therapy is getting worse and worse, and antibiotic residues in dairy products are also affecting human health. Therefore, the prevention and control of the mastitis of dairy cows has always been the focus and hotspot of research.

With the development of technology, modern molecular analysis methods have provided new methods for detecting transcriptional regulation of immune-related factors in experiments over the past decade. This will help to dissect the relevant molecular mechanisms in the broader field of dairy mastitis and provide a basis for developing more effective ways to prevent and treat mastitis in dairy cows.

Immune mechanism of the dairy cow mastitis. When bacteria invade the mammary gland through the nipple sphincter and local catheter or through the epidermal wound, bacterial PAMPs such as LPS further activate the immune defense of the mammary gland, and neutrophils, macrophages and lymphocytes become chemotaxis into the inflammatory area to perform the immune system function [164]. In addition to exerting a barrier function, mammary epithelia l cells are also stimulated by antigen to produce an inflammatory response [43]. Interstitial fibroblasts are also

involved in the process of inflammation [169]. When the mammary gland encounters bacterial invasion, macrophages in the breast tissue and milk recognize the invading pathogens and initiate an inflammatory response, while secreting various pro-inflammatory factors to recruit neutrophils to the mammary gland inflammation center area to resist the bacteria. The health mammary gland cells distributed in the breast tissue and the milk are mainly breast epithelial cells and macrophages, while the diseased tissues and the milk they secrete are mainly changed macrophages into neutrophils.

First, macrophages by releasing neutrophils and other pro-inflammatory factors to kill bacteria, such as TNF-α, IL-1β. while enhancing the release of prostaglandins and leukotrienes to exacerbate the inflammatory reaction. These could quickly and powerfully against the pathogen. When the pathogenic bacteria adhered the mammary epithelial cells, the mammary epithelial cells were continuously induced by toxicological components of pathogenic bacteria to produce TNF-α, IL-6 and IL-8. Vascular endothelial cells, under the action of pro-inflammatory factors and chemokines induced by macrophages and mammary epithelial cells, secrete cell-binding molecules to attract neutrophils in the blood to the inside of the vascular endothelium. Neutrophils migrate through the gap between the epithelial cells and the subcutaneous matrix through deformation and eventually reach the infected area. Eventually most of the mastitis-causing bacteria will be recruited to the reactive oxygen species, low molecular weight antimicrobial peptides and defensins produced by neutrophils in the center of the infected area, and then the bacterial fragments will be endocytosed by phagocytes and further digested and degraded by intracellular lysosomes. When the inflammation is overreacted or out of control [168], normal cells are damaged, and the breast tissue is not restored in time.

Research status of the dairy cow mastitis. In dairy farming, antibiotics are the first choice in clinical practice for the treatment of dairy cow mastitis. In particular, antibiotics can have a very obvious therapeutic effect in the treatment of acute clinical mastitis, and it is necessary for the conversion of acute clinical mastitis to chronic or even sepsis. Can play an effective therapeutic role.

At present, antibiotics used in the treatment of dairy cow mastitis in clinical practice mainly include penicillin, streptomycin, sulfa drugs and kanamycin and other antibiotics. Although antibiotics can effectively treat mastitis in dairy cows, they often cause some side effects on the animal body, such as the decline of the body's immune function, the loss of animal appetite, and the imbalance of the flora in the animal body. Moreover, long-term use of antibiotics will also cause bacteria to develop drug resistance, which will weaken the therapeutic effect of antibiotics.

The long-term use of a large number of antibiotics will cause drug residues in the animal's body, causing a large amount of antibiotics in the milk of dairy cows, which not only greatly reduces the quality of milk and dairy products, but also seriously endangers human health. In veterinary clinical practice, antibiotics are still mainly used to treat dairy cow mastitis, but they can no longer be used as an effective strategy to treat dairy cow mastitis. Therefore, safer and more effective methods for treating mastitis have become the main research hotspot of researchers.

In our country, the use of Chinese herbal medicine to treat dairy cow mastitis has a long history. Medical studies have confirmed that a variety of traditional Chinese herbal medicines and their active ingredients have good effects on antibacterial, anti-inflammatory, and improving immunity. In the process of clinical application, it has the advantages of low toxicity, less residue, and resistance to bacteria. Therefore, in the treatment of mastitis in dairy cows, traditional Chinese herbal medicine and its effective ingredients have very broad application prospects and great research value. At present, researchers have developed a variety of Chinese herbal medicine compound preparations and Chinese herbal medicine extracts for the prevention and treatment of mastitis in dairy cows. It has been reported that Chinese herbal medicines such as aloe and houttuynia have good effects on the treatment of mastitis in dairy cows. Zhang et al. [173] confirmed that the use of Chinese medicine with honeysuckle and dandelion as the main components can have a good therapeutic effect on diseased dairy cows, and its effect is significantly better than that of cefazolin sodium. Geng et al. [35] showed that the extract of Chinese herbal medicine Sanguis sylvestris is significantly better than cephalexin in the treatment of mastitis in dairy cows. Therefore, our country's traditional Chinese herbal medicine and its effective

ingredients have great advantages and potentials in the treatment of dairy cow mastitis in clinical practice.

At present, such as antimicrobial peptides, lysozyme, cytokines and transgenic technology, etc., have become the research hotspots of domestic and foreign researchers in the treatment of dairy cow mastitis. In the production of dairy products, it will bring serious economic losses. Therefore, it is necessary to study effective, safe, and alternative methods.

1.2 Research progress of LTA-induced inflammation

Staphylococcus aureus mastitis remains a major pathology of dairy ruminants. It poses unsolved problems owing to the long persistence of infections and the poor success rate of treatments with antibiotics [6]. A number of studies have resorted to intramammary infections with *S. aureus* to explore the host/pathogen interactions in the mammary gland, and a great deal of information has accumulated on the immune and inflflammatory responses of ruminants and on the virulence factors of *S. aureus* [10, 12, 134]. In spite of their usefulness, experimental infections have two major drawbacks: they are costly because they last long and may result in the culling of the experimental animals, and the complexity of the interactions between host defences and the array of the bacterial virulence factors and modulins defifies the analysis [142]. Also, the infection foci usually involve small and scattered portions of the mammary tissue. This complicates investigations based on the analysis of tissue samples (PCR and immunohistochemistry). A more controlled bacterial stimulus than live bacteria would be of help. In the case of *Escherichia coli* mastitis, the lipopolysaccharide (LPS) is used as a surro gate for infection. This Gram-negative bacteria outer-membrane component has been extensively used to mimic mastitis due to *E. coli*, and it has been shown that the responses it provokes are in many respects representative of those induced by the infection [13]. This makes LPS a relevant substitute for E. coli mastitis. A surrogate has yet to be defifined for *S. aureus* mastitis. Progress in the knowledge and the availability of pathogen-associated molecular patterns (PAMP) for Gram positive bacteria, and in particular *S. aureus* [29], makes it feasible to research the role

of defifined molecules in the early inflflammatory response induced in the mammary gland through interaction with the innate immune system. One of these molecules is lipoteichoic acid (LTA) of *S. aureus*, which is anchored to the cytoplasmic membrane and is exposed at the surface of the bacteria: LTA has been shown to be an important pattern for immune recognition of *S. aureus* [142]. Until recently, the effects of LTA on immune and inflflammatory cells have been controversial, because most preparations of LTA were contaminated with endotoxin and other immunostimulatory components [29,95]. Moreover, the isolation procedure yielded partially degraded LTA with much reduced immunostimulatory properties [94]. A new procedure was developed to obtain pure and active LTA, which proved to be a potent stimulus for cytokine release and neutrophil recruitment [94,96,143]. The availability of a commercial preparation based on this improved isolation procedure makes it possible to investigate the effects of *S. aureus* LTA in the mammary gland. One of the advantages of LTA as a tool to model inflflammation is that it is a defifined bacterial PAMP which targets identifified pattern recognition receptors (PRR) and increasingly defifined accessory molecules for recognition and for the signaling cascade. The recognition complex includes TLR2, TLR6 and CD14 [46,120]. Other receptors such as the platelet activating factor (PAF) receptor or the scavenger receptor CD36 are also involved, maybe in a tissue-specifific way, as well as the adaptor molecule LPS binding protein (LBP).

The response of the bovine mammary gland to lipoteichoic acid (LTA), which is a major pathogen-associated molecular pattern of Gram-positive bacteria. Purified LTA looks promising as a convenient tool to investigate the inflammatory and immune responses of the mammary gland to *S. aureus*.

1.3 Research progress on the prevention and treatment of dairy cow mastitis with plant-derived drugs

As a natural substance, plant-derived drugs contain a variety of effective biologically active ingredients. It has the characteristics of antibacterial, anti-inflammatory, anti-drug resistance, low toxicity and low residue [136], and has the

dual functions of medicine and nutrition. In recent years, experts at home and abroad have conducted a lot of research on the use of plant-derived drugs to treat dairy cow mastitis, playing an irreplaceable role in preventing and treating dairy cow mastitis, achieving green farming, and producing animal products and related products without drug residues [63]. It can be seen from the examination and approval of preparations for cow mastitis in China that plant-derived pharmaceutical products have a place [62].

The author summarizes the action mechanism of the plant-derived plants, main active ingredients, and plant extracts for preventing and treating cow mastitis in recent years, and prospects for the future development of traditional Chinese medicine treatment of cow mastitis.

Types of plant-derived medicines and their chemical components. Published literature shows that more than 30 kinds of plant extracts have an inhibitory effect on dairy cow mastitis in vivo or in vitro, such as, musk, stevia, scutellaria, rhubarb, astragalus, dandelion, honeysuckle, forsythia, licorice and so on. Many active ingredients in plants have strong bactericidal effects. For example, phenolic acids, alkaloids, flavonoids, terpenes, volatile oils and other drugs. They can directly or indirectly inhibit pathogenic bacteria killing and exert anti-inflammatory effects. Finding new anti-pathogenic bacteria ingredients from plant extracts is great significance to scientific research.

Phenolic acids. The phenolic acid extracts of various plants have protective effects on different types of dairy cow mastitis, which mainly include chlorogenic acid, caffeic acid, tea polyphenol compounds and so on. Gao et al. [34] have reported that anti-inflammatory effects of chlorogenic acid against LPS-induced mastitis may be due to its ability to inhibit TLR4-mediated NF-kB signaling pathway. CGA significantly reduced TNF- α, IL-1β and IL-6 production compared with LPS group. Liu et al. [80] showed that the protective effect of caffeic acid on LPS-induced inflammation injury in bMEC was at least partly achieved by the decreased by the effect of reducing the kB inhibitor α degradation and p65 phosphorylation in the NF-kB pathway. The use of caffeic acid would be beneficial in dairy cows during *Escherichia coli* mastitis as a safe and natural anti-inflammatory drug. Total phenol extract from *Clerodendranthus spicatus* could effectively scavenge DPPH free

radicals, reduced the production of NO and TNF-α in RAW264.7 cells induced by LPS, down-regulated the expression of IL-1β and IL-2, up-regulated the expression of IL-10, and increased cell viability of the breast epithelium under oxidative stress [146].

Alkaloids. Alkaloids plays an important role in the treatment of many chronic diseases and exhibits strong anti-bacterial and anti-inflammatory activity. A study reported that, chelerythrine isolated from root of *Toddalia asiatica* (Linn) Lam possesses antibacterial activities through destruction of bacterial cell wall and cell membrance and inhibition of protein biosynthesis. Chelerythrine showed strong antibacterial activities against Gram-positive bacteria, Staphylococcus aureus (SA), Methicillin-resistant S. aureus (MRSA), and extended spectrum β-lactamase *S. aureus* (ESBLs-SA) [45]. Lai et al. [62] Found that indirubin can inhibit the expression of TLR4 in a dose-dependent manner, and play a therapeutic role in LPS-induced MMECs inflammation and mouse mastitis. *Staphylococcus epidermidis* (*S. epidermidis*) is an opportunistic pathogen with low pathogenicity and a cause of the repeated outbreak of bovine mastitis in veterinary clinical settings. Li et al. [72] suggested that total alkaloids of *Sophora alopecuroides* has an inhibitory effect on biofilm formation of clinic *S. epidermidis*, which may be a potential agent warranted for further study on the treatment prevention of infection related to *S. epidermidis* in bovine mastitis.

Flavonoids. It has been reported that flavonoids possess a number of biological properties, such as anti-inflammatory, anti-virus, anti-bacteria, anti-tumor, and immunosuppressive properties. Astragalin, a main flavonoid component isolated from Chinese herbs, which has several medical functions including treating allergy, antiatopic dermatitis, and anti-inflammatory effects. Li et al. [68] showed that astragalin suppressed the expression of TNF- α, IL-6 and NO in a does-dependent manner in mouse mammary epithelial cells (mMECs), the expression of inducible nitric oxide synthase and cyclooxygenase-2 was also inhibited. Besides, astragalin efficiently decreased LPS-induced TLR4 expression, NF-κB activation, IκBα degradation, and the phosphorylation of p38, extracellular signal-regulated kinase in BMECs. It may be a potential therapeutic agent for bovine mastitis. Baicalin, one of

the major flavonoids in *Scutellaria baicalensis*, has natural antioxidant and anti-inflammatory properties in various cell types. Baicalin exerts protective antioxidant effects on bovine mammary cells, which suggests that it could be used to prevent oxidative metabolic disorders in dairy cows [106]. Emodin is an anthraquinone derivative from the Chinese herb *Radix et Rhizoma Rhei*. Emodin has protective effect against lipopolysaccharide (LPS)-induced mastitis in a mouse model by reduced MPO, IL-6, IL-1 β and TNF- α. It acts on mastitis through the NF-KB pathway like other flavonoids [67].

Terpenoids. Geniposide is a medicine isolated from *Gardenia jasminoides* Ellis. Song et al. [129] use a lipopolysaccharide (LPS)-induced mouse mastitis model and LPS-stimulated primary mouse mammary epithelial cells (mMECs) to explore the anti-inflammatory effect and the mechanism of action of geniposide. The results showed that geniposide significantly reduced the infiltration of inflammatory cells and downregulated the production of TNF-α, IL-1β, and IL-6. Then, geniposide exerted its anti-inflammatory effect by regulating TLR4 expression, which affected the downstream NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways. Stevioside is isolated from Stevia rebaudiana, which reduced the expression of TNF-α, IL-1β, IL-6 and TLR2 by inhibiting the phosphorylation of proteins in the NF-κB and MAPK signaling pathways dose-dependently in the *S. aureus*-infected mouse mammary gland and mouse mammary epithelial cells (MMECs), as well as caspase-3 and Bax [147,148].

1.4 The mechanism of plant-derived medicines on dairy cow mastitis

Preventive and therapeutic effects. Dairy cow mastitis has a huge impact on the dairy industry. The key to its treatment is prevention. Its preventive measures mainly include scientific feeding management, excellent hygienic conditions, scientific milking methods, nipple medicine baths, and vaccine prevention. The studies found that mammary gland epithelial cells will repair themselves by fine-tuning death caused by pathogenic bacteria and other factors. According to further research, cell apoptosis

is the main method in the early onset of mastitis. When mastitis is aggravated, cell necrosis is the main method. Therefore, in order to avoid the occurrence and development of inflammation, the body increases the apoptosis of epithelial cells in the inflammatory reaction of breast, which is a self-protection mechanism to protect the integrity of the breast to the maximum extent. Chen et al. [16] found that astragalus polysaccharide (APS) has the effect of inducing apoptosis of tumor cells, which can have reduced the number of cells in S phase and increased the number of cells in G0-G1 and G2-M phases. The increased of polysaccharide dose stayed in the G2-M phase, indicating that inducing apoptosis of tumor cells is an anti-tumor way of astragalus polysaccharides. Zhong et al. [177] studied the effect of astragalus polysaccharides on *E. coli* endotoxin (LPS) -induced experimental mastitis in goats, cows and rats. The results showed that local infusion of APS in the breast or feeding animals can alleviate the effect of LPS on animal breast tissue. Briefly, the destruction has a certain protective effect on animal breast tissue. The traditional Chinese medicine and prescriptions for the treatment of mastitis were: synthetic Houttuynia cordata, propolis mixture, Xianfang Huoming Yin, Gongying Shanjia Tang, Erhua Zaozi Yin, Ruyan San, etc [145]. Studies by Zhang et al. [172] have confirmed that the use of plant-derived drugs with honeysuckle and dandelion as the main components to treat diseased cows can play a good therapeutic effect, and its effect is significantly better than cefazolin sodium. Geng [36] have shown that the extract of the plant-derived medicine Ulmus pumila is better than cefalexin in the treatment of dairy cow mastitis. Zhang et al. [167] selected Chinese herbal medicines such as angelica, chuanxiong, astragalus, dandelion, salvia miltiorrhiza, motherwort to feed dairy cows with latent mastitis, and detected the lymphocyte stimulation index (SI) and neutrophil phagocytosis of dairy cows. The results showed that the additive directly strengthens the phagocytic power of neutrophils and stimulates the proliferation of lymphocytes. Through the action of antibodies and complements, the phagocytic power of neutrophils is further strengthened. It had obvious therapeutic effect. Therefore, Chinese traditional plant-derived drugs and their active ingredients have great advantages and potential in clinical treatment of cow mastitis.

Inhibition of pathogenic bacteria. According to the reports, there are more than 130 pathogenic microorganisms that can cause mastitis in dairy cows, and even more than 20 kinds are common. The pathogens with the highest detection rate are *Staphylococcus aureus*, *Streptococcus* and *Escherichia coli*. Mastitis caused by a variety of pathogens can account for 90%. Therefore, the antibacterial activity of plant-derived drugs is the important indicator of their effectiveness [113]. Luan et al. [86] used eight Chinese herbal medicines, including Daqingye and Coptis, to detected their resistance to β-lactamase-producing *E. coli*. Inhibition screening study found that Scutellaria baicalensis had the most obvious inhibitory effect, followed by Coptis and Daqingye. Liu et al. [81] used five Chinese herbal medicines to test the drug resistance inhibition screening of drug resistant strains producing extended-spectrum β-lactamase and sustained high yield AmpC enzyme, such as Forsythia suspensa, Senecio, Scutellaria baicalensis, etc. As a result, it was found that 5 kinds of traditional Chinese medicines inhibited the production of extended-spectrum β-lactamase and AmpC enzyme strains to varying degrees. Among them, the effect of Scutellaria baicalensis was more obvious, followed by Coptis chinensis and Senecio. Honeysuckle is known as "Chinese medicine penicillin" and has inhibitory effects on a variety of bacteria, including *S. aureus*, *E. coli*, *Vibrio cholerae*, and *hemolytic streptococcus*, etc [128].

In vitro antibacterial experiments of 10 Chinese herbal medicines showed that, the antibacterial effects of Chinese herbal medicines on *E. coli* were: Myrobalan, Viola and Houttuynia cordata, which were moderately sensitive; Prunella vulgaris, Scutellaria, Senecio, Astragalus, Gorgon, Teasel were followed by low sensitivity. The antibacterial effect on *S. aureus* were: Myrobalan, Scutellaria, Pomegranate Peel and Rhubarb had the best effects, which were the highly sensitive; Forsythia, Chuanxiong and Shegan were second, and were the moderately sensitive; Prunella vulgaris, Xanthium grass, Fried gardenia, and Rhubarb were low sensitivity. The antibacterial effect of *Streptococcus agalactiae* were: Astragalus and pomegranate peel were the highly sensitive; Ligusticum chuanxiong, Shegan, Phellodendron amurense and Houttuynia cordata were moderately sensitive; Forsythia, Myrobalan, Radix Scutellariae, Viola Ding, Rhubarb and Wangbu Staying were low sensitivity [88]. In

summary, Chinese herbal medicine has a good inhibitory effect on *E. coli*, *S. aureus* and *Streptococcus agalactiae*.

Mechanism of action on inflammation. In recent years, the role of non-professional immune cells such as dairy cow mammary epithelial cells in resisting pathogens from invading the cow's mammary gland has received attention. When pathogenic bacteria invaded the mammary gland of dairy cows, epithelial cells would first synthesize and secrete a variety of immunologically active substances to resisted the infection of pathogenic bacteria and reduced or even relieved the inflammatory response. After pathogenic microorganisms were infected, its lipoteichoic acid, peptidoglycan and lipopolysaccharide could trigger the natural immune system of the mammary gland, activated intracellular signal transduction pathways such as NF-kB, MAPKs and JAK/STAT, and finally led to chemokines and inflammation factor release. Most scholars use pathogenic microorganisms or their products to stimulate breast tissue or breast epithelial cells, established in vivo and in vitro models of mastitis, and use Chinese herbal medicine or its main active ingredients to detect inflammatory factors such as IL-1β, IL-6 and TNF-α, TLRs, NF-kB, MAPKs and JAK/STAT signal pathway changes. Studies have shown that chlorogenic acid [34] and caffeic acid [80] in honeysuckle and dandelion, emodin in rhubarb [67], thymol in musk [149], indirubin in Indigo Naturalis [62], and berberine hydrochloride in Coptis [163], Astragalus glycosides in Astragalus vulgaris [68], Geniposide in Gardenia [129], flavonoids baicalin [106], Kidney tea total phenols [146], Dandelion sterols [117], Astragalus polysaccharides [106], all of them could inhibited NF-kB, MAPKs and JAK/STAT pathways, reduced the expression level of inflammatory factors and played a protective effect on breast cells or animals.

Improve immune function. The mammary glands of dairy cows contained the necessary components for immune response to invading pathogenic microorganisms. When the low content of immunoglobulin and complement in breast secretions and the existence of certain inhibitory factors, the immune function of the breast was suppressed [49]. According to the reports, a traditional Chinese medicine consisting of astragalus, angelica, salvia, dandelion, etc. can increased animal antibody production and promoted lymphocyte transformation [89]. Shang et al [125] added Chinese herbal

medicine additived to the diets of normal dairy cows at the early stage of lactation, and the results showed that the addition of Chinese herbal medicines in the early stage of lactation can significantly increased the milk production of dairy cows by 7.4 ($P < 0.01$), it also improved the milk composition, and increased the milk fat rate by 11.7 ($P < 0.05$). That means the Chinese herbal medicine can reduce the incidence of non-clinical mastitis, and enhanced the immunity of dairy cattle. Another study has found that Astragalus polysaccharides can enhanced the ability of phagocytes, activated macrophages, promoted cell differentiation and the secretion of IL-2, thereby enhancing the ability of macrophages to kill bacteria and disease, and enhance the immune system of dairy cows. It could stimulate the release of cytokines, affect the neuroendocrine-immune system [82]. The use of Chinese herbal medicine to prevent and treat dairy cow mastitis has the advantages of no drug residues and high economic benefits, and has broad application prospects in the dairy industry. Although the prevention and treatment of mastitis by traditional Chinese medicine was indeed effective, the current research and development efforts were not strong. It was manifested that there were few varieties of traditional Chinese medicine preparations for the prevention and treatment of mastitis. Most of them were powders and decoctions. The effective ingredients, content and structure have not been researched clearly, which restricts its wide application in clinic and application effect. Further research is needed on the method of separating and extracting the active ingredients of traditional Chinese medicine, the efficacy and mechanism of traditional Chinese medicine. On this basis, we will develop efficient, safe and stable Chinese medicine preparations so that Chinese medicine can play a greater role in the prevention and treatment of cow mastitis.

1.5 The chemical constituents and pharmacological effects of *Taraxacum officinale*

Taraxacum officinale, commonly called dandelion is herbaceous perennial plant which belongs to the family of Asteraceae (Compositae). It grows in the intemperate regions of the world and is found mostly in lawns, roadsides, disturbed banks, shores

of water ways and other areas with moist soils. Because dandelion has a strong ability to grow and reproduce, its raw materials are abundant and widely distributed. It grows widely in Europe and North America, and is almost all over China except for the Southeastern and Southern Provinces. Although, dandelion is considered a weedy species, but it has been used in many herbal medical systems. In recent years, a large number of scholars have studied the biologically active components of dandelion and found that dandelion contains a variety of active ingredients. Yang [162] and others researchers have showed that triterpenes, polysaccharides, carotenes, sterols, fatty acids, choline, vitamins, flavonoids, sugars, minerals, proteins and other ingredients were rich in the herbs of the same genus as *T. officinale*. Choi et al. [20] were successfully extracted triterpenes, flavonoids, carotene, phenolic acids, taraxsterol and a variety of fatty acids. Scholars successfully obtained a new pigment - KinobeonA, from the *T. officinale* plant through tissue culture [122]. Tumbarski [140] detected 20 kinds of flavonoids in *T. officinale* flowers, and identified 10 of them by the chromatographic analysis method. *T. officinale* mainly contains many medicinal ingredients, such as chlorogenic acid, caffeic acid, total flavonoids, alkaloids, and polysaccharides [77]. Chlorogenic acid is a kind of depsip acid, a phenolic compound, which can scavenge free radicals [152].

Antibacterial, anti-inflammatory and antioxidant effects. Chlorogenic acid is a secondary metabolite that has inhibitory activity on pathogenic bacteria and shows different inhibitory effects on a variety of pathogenic bacteria. Chlorogenic acid extracted from sirloin leaves has certain inhibitory effects on *Shigella dysenteriae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, B*acillus subtilis*, and *Escherichia coli*, and it has certain inhibitory effects on *Shigella dysenteriae* and *Streptococcus pneumoniae* [85]. Terry [139] showed that as the concentration of chlorogenic acid increases, strawberries are less susceptible to infection by *Botrytis cinerea*. Liu [80] has proved that dandelion has antibacterial effect on *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtili*s, *Aspergillus niger*, *Paecilomyces variabilis*, and the antibacterial effect is stronger than fungi. Zhang [167] used reflux extraction method to prepare dandelion extract, and determined its antibacterial power, thermal stability and minimum inhibitory concentration. The research results showed that the

dandelion extract have a strong inhibitory effect on *Staphylococcus aureus* and *Proteus*. Inflammation is a defensive response of humans or animals to pathogenic factors that invade the body. Inflammation is divided into infectious inflammation and non-infectious inflammation according to whether it is infected or not. Although the general chemical drugs have good anti-inflammatory effects, they still have certain drug residues and have relatively large adverse reactions. However, Chinese herbal medicine has the advantages of unique naturalness, small adverse reactions, long-lasting efficacy, and two-way regulation [36, 54, 101, 127]. *T. officinale* is rich in flavonoids, such as luteolin, quercetin, coumarin and scopolamine, which have antioxidant and anti-inflammatory activities. Qiao [112] used mice as the research object to study the effects of dandelion extract on inflammation, early risk changes of atherosclerosis, oxidative stress, and recorded the data and concluded that dandelion extract has an effect on atherosclerosis. It has a good inhibitory effect, and has a good anti-oxidation and anti-inflammatory effect. Chlorogenic acid has a strong free radical scavenging ability and can effectively scavenger DPPH (diphenylpicrylhydrazine) free radicals. Therefore, it can be used as an antioxidant and is widely used in the fields of daily chemical and biological pharmacy. He [44] found that 40.09 μg/mL apple-derived chlorogenic acid can scavenge 50% of DPPH free radicals, and significantly improve the oxidative stability of soybean oil, and its antioxidant effect is equivalent to the synthetic antioxidant TBHQ (tert-butyl hydroquinone) ，and higher than that of Vc. Zhou [180] found that chlorogenic acid helps to improve the antioxidant capacity of the intestinal tract in rats; DPPH free radical scavenging method and FRAP (iron ion antioxidant capacity) method were used to characterize the antioxidant activity of honeysuckle extracts. The results show that chlorogenic acid plays a major role in the antioxidant activity. It can be considered that the higher the content of chlorogenic acid, the stronger the antioxidant activity [154].

Antiviral, and anti-tumor effects. Chlorogenic acid is an important component with antiviral activity in traditional Chinese herbal medicine. The quality control standard of honeysuckle, which is a heat-clearing and detoxifying medicinal material, is generally based on the content of chlorogenic acid. Xing [157] showed that

the natural compound chlorogenic acid can resist pig reproduction and respiration in vitro. PRRSV has a strong effect, which may be achieved by directly inactivating and inhibiting cell apoptosis caused by PRRSV. The effect of chlorogenic acid in inhibiting enterovirus 71 (EV71) in vitro is also very significant, mainly by interfering with the expression of 2AmRNA and protein translation in the early stage of EV71 replication to achieve antiviral effects [71]. In addition, chlorogenic acid can inhibit HBV-DNA replication to fight hepatitis B disease (HBV) [144]. Chlorogenic acid is an important secondary metabolite in plants, which can produce anti-tumor effects through a variety of ways, such as regulating cell cycle, inducing cell apoptosis, inhibiting cell growth, etc. It is considered to be an effective anti-tumor natural extract. Studies have shown that chlorogenic acid can regulate the apoptosis of non-small cell lung cancer by reducing the expression of VEGF (vascular endothelial growth factor), down-regulating the level of Delta, and blocking Notch1 signaling pathway [70]. Zhou [179] found that chlorogenic acid can induce glioma cells to up-regulate the expression of p53 and Bax, down-regulate the expression of Livin and Bcl-2, and finally activate the activity of Caspase-3 protein, thereby promoting the apoptosis of glioma cells. In addition, in view of its good liver protection effect, the combined application of chlorogenic acid and anti-tumor chemotherapy drugs can not only reduce the liver toxicity of chemotherapy drugs, but also exert a synergistic anti-tumor effect. As early as 1993, Japanese scholars pointed out that chlorogenic acid, which is naturally present in plants, can inhibit 4-nitroquinoline-1-oxide (4-NQO)-induced tongue cancer, and can be used to prevent tongue cancer chemotherapy [137]. Zhang [166] used interleukin-13 to induce the M2 polarization model of macrophages in vitro, and detected the expression of M2 marker CD206 (specific marker), and found that compared with lapatinib alone, it inhibited the combined use of chlorogenic acid. CD206 expression is better ($P<0.05$), which proves that the combination of lapatinib and chlorogenic acid can inhibit the M2 polarization of macrophages and inhibit the metastasis of breast cancer.

Other effects. *T. officinale* not only has the above pharmacological effects, but also has the functions of reducing endotoxin, promoting insulin secretion, treating edema, resisting fertility and reproduction, and promoting milk. Hussain [52] studied

the effect of dandelion extract on the release of INS-1 cells on insulin secretion by alcohol extraction of dandelion in vitro, and found that dandelion extract affects insulin secretion, and different concentrations have different effects. When the concentration of dandelion extract is 40g/mL, insulin secretion can be enhanced by using dandelion extract. Zhu [182] has proved that dandelion leaves have a dredging effect on blocked mammary ducts and can improve milk secretion.

1.6 Extraction of chlorogenic acid

Chlorogenic acid is a kind of phenylpropanoids formed by plants through the shikimic acid pathway during aerobic respiration [32]. It has a variety of plant sources, mainly in the genus *Lonicera* and *Artemisia*. The content is higher especially in eucommia, honeysuckle, sunflower meal, and coffee. The leaves of *Eucommia ulraoides*, *Arctium lappa* and *Nicotiana tobacco* are rich in chlorogenic acid, and the content of chlorogenic acid in Eucommia ulraoides leaves can reach 2%~5% [18, 42, 51, 111]. Chlorogenic acid can be extracted from the roots, stems, leaves and flowers of *Lonicera japonica*, and the content in flower buds is the highest [55]. At present, chlorogenic acid is mainly extracted from Eucommia, honeysuckle, tobacco and other plants in China, while coffee is used more as a raw material for extracting chlorogenic acid in foreign countries [41, 65, 147]. Comprehensive extraction method of chlorogenic acid can be roughly divided into water extraction method, organic solvent extraction method, microwave-assisted extraction method, and ultrasonic-assisted extraction method, etc (Table 1.1).

Water extraction method. Chlorogenic acid is a highly polar phenolic acid. Based on the principle of similar compatibility, water can be used as a solvent to extract chlorogenic acid. Stela [56] used subcritical water (referring to the water heated to above the boiling point and below the critical point, and controlling the system pressure to keep the water liquid) to extract chlorogenic acid from tobacco. They found that too high temperature and too long extraction time would lead to Decomposition of chlorogenic acid. Consistent with this result, Jeszka-Skowron [90] study showed that extraction conditions such as temperature, time, and

material-to-liquid ratio have significant effects on the extraction of chlorogenic acid from plants. Response surface methodology (RSM) was used to optimize the water content of chlorogenic acid in tea. After extraction, the content of chlorogenic acid can be as high as 6.66 ± 0.58 μg/mL. The optimal solution for extracting chlorogenic acid from tobacco leaves with distilled water as solvent is the extraction temperature of 60 ℃, the ratio of material to liquid 1:16 g/mL, and the extraction time of 60 min. The extraction rate under this condition can reach 2.33% [161].

Table 1.1

Extraction methods and extraction rate of plant chlorogenic acid

Extraction method	**Raw material**	**The best extraction method**	**Withdrawal rate**
Water extraction [144]	Tobacco leaves	T 60 ℃, t 60 min, m/L 1:16 (w/v)	2.33%
Organic solvent extraction [130]	Sweet potato leaves	pH 5.0, t 90 min, m/L 1:2 (w/v), Ec 30%	3.517%
Microwave-assisted extraction [171]	Lonicera japonica flower buds	T 60 ℃, t 20 min, m/L 16:1 (w/v), Ec 50%	6.14%
Ultrasonic-assisted extraction [55]	Eucommia ulmoides leaves	pH 5.0, t 90 min, m/L 1:2 (w/v), Ec 30%	6.338%
Cellulase extraction [41]	Corn silk	1.4% cellulase, t 1.25 h, Ec 70%	8.94 mg/g
Extra-high pressure extraction [141]	Honeysuckle flowers	Pressure 325 MPa, holding time 10 min, t 2 h, m/L 1:10 (w/v), Ec 60%	4.872%
Low eutectic solvent extraction [90]	Honeysuckle	T 70 ℃, t 60 min, m/L 1:25 (w/v)	--
Super critical CO2 fluid extraction [14]	Oiled-type sunflower seeds	Extraction pressure 63.94 MPa, T 72 ℃, t 3 h, Ec 15%	44.14%

Note：T: Extraction temperature, t: extraction time, m / L: material / liquid ratio, Ec: ethanol concentration, w: Ultrasonic power.

Considering economic benefits and environmental safety, water is the most ideal solvent. Leker [65] also believe that the method of using subcritical water to extract chlorogenic acid from green coffee beans is more economical and safer than organic solvents. But it’s worth noting that the high boiling point of water will cause too much impurities in the extract. It is difficult to separate chlorogenic acid.

Organic solvent extraction method. The organic solvent extraction method utilizes the chlorogenic acid's ability to dissolve in organic solvents, such as methanol and ethanol for extraction. Studies have shown that methanol as a solvent can extract antioxidant phenolic compounds chlorogenic acid from waste coffee grounds [41]. After extracting ginger leaves four times with 30% ethanol, the yield of chlorogenic acid is 234 ± 25 mg/100 g [14]. Chlorogenic acid can also be obtained by extracting fermented plant extracts (FPE) with ethyl acetate [98]. Compared with the water decocting method, the ethanol reflux method can significantly increase the extraction rate of chlorogenic acid in honeysuckle. The extraction rate is as high as 7.2%, while the water decocting method is only 2.6% [73]. Li [69] optimized the extraction method of chlorogenic acid in sweet potato leaves through orthogonal experiments.

When the extraction process is 30% alcohol concentration, material-to-liquid ratio 1:2 (w/v), pH 5.0, and extraction four times, after 90 minutes of distillation, the extraction rate can reach 3.517%. Moreover, the extract obtained by this extraction process is similar to the natural state, which is conducive to further purification.

Ultrasonic assisted extraction method. The principle of ultrasonic-assisted extraction is to use ultrasonic waves to break the cell wall, so that the intracellular components leak into the solution, so as to achieve the purpose of separation faster. Pandey [102] used RSM to optimize the ultrasonic-assisted extraction of phenolic compounds from rhubarb rhizomes, the extraction concentration of chlorogenic acid reached 26.68 mg/g after only ultrasonic extraction for 30 min. Chen [17] also used RSM to optimize the ultrasonic-assisted extraction method of phenolic compounds in black wolfberry.

Under the optimal conditions, the concentration of chlorogenic acid was 6.48 ± 0.16 mg/g. These research results show that the ultrasonic assisted extraction method

not only shortens the extraction time, but also increases the extraction efficiency. The optimization of the ultrasonic-assisted water extraction process solves most of the conventional technical shortcomings mentioned in the literature, including the degradation of chlorogenic acid caused by excessive extraction time in the extraction process, the waste of energy caused by excessively high extraction temperature, and the purity of chlorogenic acid. Therefore, the ultrasonic-assisted water extraction process can be used as an environmentally friendly, safe and economical alternative to the traditional extraction process.

Other methods. In addition to the above extraction methods, the extraction of chlorogenic acid also involves Soxhlet extraction, ultra-high pressure extraction, hot reflux extraction, enzyme extraction, supercritical CO_2 extraction and other methods. Liu [83] explored an enzyme-assisted extraction method of chlorogenic acid based on ionic liquids. This method mainly uses cellulase to decompose the cell wall to reduce the mass transfer barrier and obtain a high extraction rate. Compared with other conventional extraction techniques, this novel extraction method has more advantages in terms of yield and efficiency. The honeysuckle is extracted with ethanol after ultra-high pressure treatment. The extraction rate of chlorogenic acid under the optimal extraction conditions can reach 4.872% ± 0.049%, which is better than ultrasonic extraction and heat reflux extraction, and has low energy consumption and short time consumption [109].

Molecular imprinting technology is a technology that can create a specific cavity complementary to the template molecule in shape, size and chemical function to achieve adsorption. Some scholars have developed a new type of hydrophilic magnetic molecularly imprinted nanoparticles based on this technology for selective separation and determination of water-soluble chlorogenic acid. This method is more effective in separating coexisting compounds with similar functional groups (caffeic acid, gallic acid, etc.).

In addition, eutectic solvent extraction, hollow fiber solid phase microextraction and supercritical CO_2 fluid extraction methods can also be used for the extraction of plant chlorogenic acid [40, 53, 87, 178].

At present, eutectic solvent extraction has not been widely used, and hollow fiber solid phase microextraction is still in its infancy. Although the extraction rate of supercritical CO_2 fluid is high, the technology industrialization research is relatively weak and the equipment is expensive, and the development is relatively slow [155].

1.7 Conclusions from literature review

A review of the literature shows that mastitis remains a pressing problem for many countries, including China and Ukraine. Plant extracts have obvious therapeutic effects in the treatment of mastitis in dairy cows. They can not only avoid the production of drug-resistant bacteria caused by the application of antibiotics, but also avoid problems such as drug residues. Therefore, the application of plant extracts has always been high hopes in the prevention and treatment of dairy cow mastitis. Therefore, 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.

CHAPTER 2

2.1 Research materials

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, zooghygiene and safety and quality of animals products, the department of epizootology and parazitology and the department of virology, pathanatomy and bird diseases of Sumy National Agrarian University. In addition, veterinary studies were conducted at Henan, China.

Object of study antibacterial mechanism of chlorogenic acid in *Taraxacum officinale,* anti-inflammatory effect and mechanism of chlorogenic acid extract from *Taraxacum officinale* in the LTA-induced bovine mammary epithelial cell in vitro inflammation model, Mammary epithelial cells of dairy cow mastitis caused by Staphylococcus aureus LTA stimulation, Holstein breed.

Subject of study Antibiofilm activity against *Staphylococcus aureus,* content analysis of *Taraxacum Officinale* Phenolic extracts, In vitro culture and evaluation of bovine mammary epithelial cells from dairy cows, inflammatory model of bovine mammary epithelial cells（BMEC) induced by LTA, Effect of Chlorogenic acid (CGA) on inflammatory response induced by LTA in BMEC.

2.2. Research methods

Research on the topic of the dissertation was carried out according to the scheme presented in fig. 2.1.

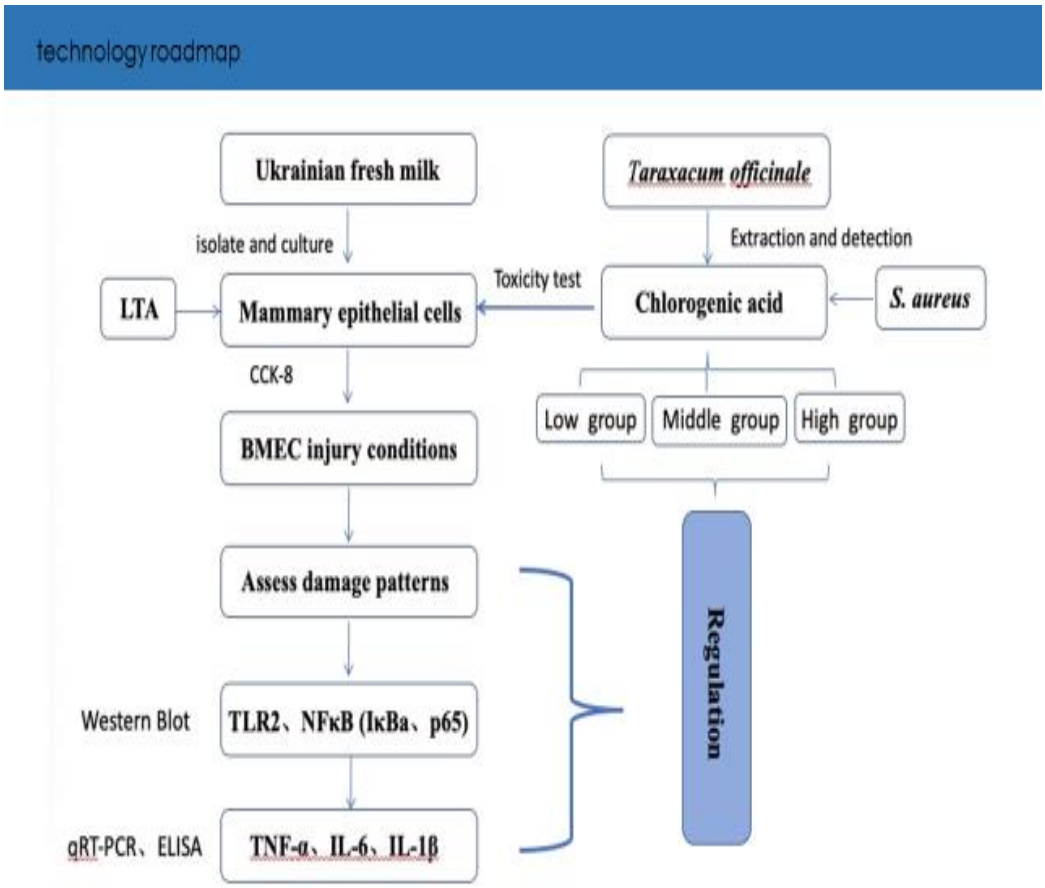


Fig. 2.1. General scheme of conducting research

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) research methods were used in the work.

Consumables – Gloves, masks, shoe covers. Alkaline Phosphatase Assay Kit (Colorimetric, Abcam Cambridge, UK, ab83369). DNA extraction kit (TIANGEN

BIOTECH Co., Ltd, Beijing, P. R. China, cat. KG203). Cytokeratin 18 (Abcam, UK, Cambridge, cat.10830-1-AP).

Fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-mouse IgG (Sigma, USA, Louis, MO, cat. F4143). PureLinkTM RNA Mini kit (ThermoFisher, USA, Waltham MA, cat.12183018A). Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12, Gibco, USA, New York, cat.12400-024). Fetal bovine serum (FBS, Biological Industries, Israel, Kibbutz Beit-Haemek, cat.04-011-1A/B) Epidermal growth factor (EGF, Sigma, USA, Louis, MO, cat. E4127). Lipoteichoic acid (LTA, Inviogen, Carlsbad, CA, USA, cat. tlrl-slta). RNAiso Plus (TaKaRa, Dalian, P. R. China, cat. 9109). PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, P. R. China, cat. RR047A). Cell counting KIT-8 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, P. R. China, cat.CK04). TB Green Premix Ex TaqTM II (TaKaRa, Dalian, P. R. China, cat#RR820B). Griess reagent Nitric Oxide Assay Kit (Beyotime Biotechnology, Shanghai, P. R. China, cat. S0023). BCA Protein Assay Kit (Solarbio Science & Technology Co., Ltd., Beijing, P. R. China, cat. PC0020). 0.45 μm membrane (Millipore, Carrigtwohill, Co. Cork, Ireland). Cell culture dish, Cell culture 6-well plate, Cell culture 12-well plate, centrifuge tube (Corning, USA, New York, cat.430639).

Equipment. 1. Micropipette (Eppendorf Germany); 2. Autoclave (Hirayama Corporation); 3. Electric heating constant temperature drying oven (Shanghai sunshine co., ltd.); 4. Inverted microscope (Zeiss, LSM7800, Germany, Oberkochen); 5. Confocal Laser Scanning Microscope (Zeiss Germany); 6. Constant temperature magnetic stirrer (Heilongjiang Dongming Medical Instrument Factory); 7. SDS-PAGE vertical electrophoresis instrument (Becton, Dickinson and Company, USA); 8. Film transfer instrument (Becton, Dickinson and Company, USA); 9. Horizontal electrophoresis system (Becton, Dickinson and Company, USA); 10. Microplate reader (Bio-Rad, Hercules, CA); 11. Flow cytometer (Becton, Dickinson and Company, USA); 12. Electronic balance (Sartorius, Germany); 13. Gel imaging system (Shanghai Tianneng Biological Company); 14. 7500 Real-Time PCR system (Applied Biosystems Inc., Foster City, CA); 15. PCR instrument (TaKaRa, Dalian, P. R. China); 16. Ultraviolet spectrophotometer (Beijing Junyi-Dongfang

Electrophoresis Equipment Co., Ltd China, Beijing, JY04S-3E); 17. Ultra Clean Desk (Wujiang Purification Equipment General Factory); 18. CO_2 cell incubator (Thermo Fisher Germany); 19. Centrifuge (Thermo Fisher Germany); 20. High-speed refrigerated centrifuge (Thermo Fisher Germany); 21. Water bath (Shanghai Yiheng Instrument Co., Ltd.); 22. Ice maker (Shanghai Bilang Instrument Co., Ltd.); 23. Shaker (North Tongzheng Technology Co., Ltd.); 24.pH meter (Shanghai INESA Scientific Instrument Co., Ltd. Beijing, P. R. China); 25. RTAC DP Analyzer (San Diego, CA 92121, USA); 26. High performance liquid chromatography (Shimadzu，Japan); 27. Ultrasonic cleaner (Liuyi Equipment Co., Ltd China, Beijing); 28. Lieci conductivity meter (Shanghai INESA Scientific Instrument Co., Ltd. Beijing, P. R. China, ca. DDSJ-308F); 29. NanoDrop 1000 (Thermo Scientific, Co., Ltd., P. R. China); 30. Freeze dryer (Labconco, FreeZone 4.5 L, USA) .

Chemicals and solvents. 1. Ethanol (Tianjin Deen Chemical Reagent Co., Ltd.); 2. Methanol (Tianjin Deen Chemical Reagent Co., Ltd.); 3. Phosphoric acid (Tianjin Deen Chemical Reagent Co., Ltd.); 4. Chloroform (Tianjin Deen Chemical Reagent Co., Ltd.); 5. Ammonium acetate (Tianjin Deen Chemical Reagent Co., Ltd.); 6. hydrochloric acid (Tianjin Deen Chemical Reagent Co., Ltd.); 7. Methanol (99%, PA, Fisher Scientific, Lisbon, Portugal); 8. Acetonitrile (99.9%, HPLC grade, Fisher Scientific, Lisbon, Portugal); 9. Triton X-100 (Solarbio Science & Technology Co., Ltd., Beijing, P. R. China); 10. Chlorogenic acid (98%, Sigma Aldrich St. Louis, MO, USA); 11. Caffeic acid (98%, Sigma Aldrich St. Louis, MO, USA); 12. Rutin (98%, Sigma Aldrich St. Louis, MO, USA); 13. Ferulic acid (98%, Sigma Aldrich St. Louis, MO, USA); 14. Luteolin (98%, Sigma Aldrich St. Louis, MO, USA); 15. Bovine serum albumin (Solarbio Science & Technology Co., Ltd., Beijing, P. R. China); 16. Coomassie Brilliant Blue G250 (Solarbio Science & Technology Co., Ltd., Beijing, P. R. China); 17. Dextran gel (Solarbio Science & Technology Co., Ltd., Beijing, P. R. China); 18. Phosphate buffered saline (Solarbio Science & Technology Co., Ltd., Beijing, P. R. China); 19. Antibiotic-antimycotic (Gibco, USA, New York, cat.15240-062); 20. DAPI (4,6-diamino-2-phenyl indole, Solarbio Science & Technology Co., Ltd., Beijing, P. R. China); 21. Biowest Agarose (Gene company LTD, Hong Kong).

2.2.1 Collection of plant material

The whole plant of *Taraxacum Officinale* were collected from the campus of Sumy National Agrarian University, Sumy, Ukraine, in May 2018 and 2019 and identified by Professor Li MENG, Henan Institute of Science and Technology, Xinxiang, China.

The voucher specimen was stored at the Institute of Chinese Materia Medica, Henan University (Kaifeng, Henan, China).

The whole plants were cleaned, dried in shade for several days and pulverized in a laboratory crusher.

2.2.2 Determination method of chlorogenic acid content in *T. officinale* Chromatographic Conditions and Instrumentation

Analysis was performed on a Shimadzu Acquity HPLC system (LC-20A, Shimadzu, Japan). An Inertsil ODS-3 C18 column (5 μm, 250*4.6 mm) was applied for all analyses. The mobile phase was composed of A (MeOH) and B (1‰ Phosphoric acid solution, adjusted to pH 8.0 with ammonia-water) with a gradient elution: 0-5 min, 5% A; 5-15 min, 5%-15% A; 15-20 min, 15%-5% A; 20-25 min 5% A. The flow rate of the mobile phase was 1.0 mL/min. And the column temperature was maintained at 30 ℃. Detection wavelength was set at 350 nm. Target peak was identified by comparing their retention time of the respective standard. A standard graph of chlorogenic acid was prepared by plotting concentration versus peak area.

Standard Preparation 10 mg chlorogenic acid standard was weighed, and then was dissolved in a 10 mL one-mark volumetric flask with 10% methanol to form the 1 mg/mL stock solution. Draw 5 mL of 1 mg/mL chlorogenic acid solution, add 10% methanol to a constant volume to 10 mL, and then sequentially dilute to obtain 6 concentration levels of the reference mixture 1, 0.5, 0.25, 0.125, 0.0625, 0.0313 mg/mL chlorogenic acid standard solution. The standard solutions were filtered through a 0.45μm membrane prior to injection. All solutions were stored in a refrigerator at 4 ℃ before analysis.

Extraction Preparation of Sample of *T. officinale* Accurately weigh 1.0 g of sample powder and add 50 mL of extraction solvent 60% ethanol. Ultrasonic (power 250 W, frequency 35 KHz) extraction for 40 min, suction filtration, repeat the extraction operation 3 times, the extracts 3 times were combined, concentrated under reduced pressure at 50 ℃ by rotary evaporator (N-1300D, EYELA, Japan), made up to 25 mL with 60% ethanol, and stored at 4 ℃ in the refrigerator. Filter with 0.45 μm filter before injection.

Method Validation Through determining the linearity, LOD, LOQ, precision, repeatability, stability, and accuracy additive recovery rate, the established HPLC method was validated.

Calculation of chlorogenic acid extraction rate

$$\text{Extraction rat}\ (\%) = \frac{c \times v \times a}{M}$$

"v" is the volume of the extract; "c" is the concentration of chlorogenic acid in the extract; "a" is the dilution factor; "M" is the weight of the sample.

2.2.3 Single factor experiment for chlorogenic acid content in *T. officinale*

The effect of different temperature (40 ℃, 50 ℃, 60 ℃, 70 ℃ and 80 ℃) on extraction rate were tested, while the ethanol volume fraction for extraction was 70%, the material-liquid ratio was 1:20 (g/mL), ultrasonic time was 40 min. After confirming that 70°C of ultrasonic temperature of was better, the effect of the material-liquid ratios (1:15, 1:20, 1:25, 1:30, and 1:35) on extraction was compared under the fllowing conditions: the ethanol volume fraction was 70%, and the ultrasound time was 40 min. After the material-liquid ratio of 1:30 was found better, the effect of ethanol volume fractions (20%, 40%, 60%, 80% and 100%) on extraction rate was tested. While ultrasonic time was 40 min, the ultrasonic temperature was 70 °C and the material-liquid ratio was 1:30. When the better volume fraction of ethanol was determined, the effects of extraction of ultrasonic time (10 min, 20 min, 30 min, 40 min and 50 min) was tested under the conditions as followings: the ultrasonic

temperature was 70℃ and the material-liquid ratio was 1:30. Finally the optimal level of each factor were determined. The dandelion sample was 1.0 g for each test.

2.2.4 Orthogonal experiment design

According to the single-factor test results, ultrasonic temperature (A), material-liquid ratio (B), ethanol volume fraction (C) and ultrasonic time (D) were selected as the investigation factors with the extraction rate of dandelion chlorogenic acid as the evaluation index. Orthogonal experiment design L9(34) was applied for obtaining the best extraction process.

Statistical Analyses All statistical analyses, including the design of orthogonal test and independent-sample t test, were carried out with EXCEL 2010 and PASW (IBM SPSS Statistics) statistical software (version 19.0).

2.2.5 Quantification of five phenolic compounds in extract by High Performance Liquid Chromatography (HPLC)

HPLC conditions The analysis was performed on Shimadzu LC-20A with Inertsil ODS-3 C18 (5 μm, 250 mm×4.6 mm). Acetonitrile and 2% acetic acid aqueous solutions were used as mobile phase with the ratio of 80/20 flow rate of 1.0 mL/min. Detection wavelength was 320 nm and the column oven temperature was 35 ℃。

Standard curve Precisely weighed the 5 standard products (10 mg/each), put them into a 10 mL volumetric flask, dissolved in 70% methanol water ultrasonically, and diluted to the mark.

Drawn 1 mL of the 5 kinds of mother liquors from the 10 mL volumetric flasks, and diluted to the mark to make 5 kinds of mixed standard solutions with a concentration of 0.1 mg/mL. Inject 2, 4, 6, 8, and 10 μL, respectively and analyzed them according to the above-mentioned high performance liquid chromatography conditions with 3 repetitions. Used the formula to calculate and drawn a standard curve related to the standard injection volume and the peak area.

Preparation of phenolic extract Phenolic extract was obtained from plant sample (1 g) with 30 mL of methanol: water (70:30, v/v) mixture at 30 ℃ for 30 min. Three times collected extract were combined and concentrated under reduced pressure to a constant volume of 10 mL. Sample was passed through 0.45 μm membrane before sample injection.

2.2.6 Bacterial strains and growth condition

The pathogenic bacterial strain *S. aureus* (ATCC 25923) was obtained from the American Type Culture Collection (ATCC), which was grown on Nutrient Broth (NB) and incubated overnight at 37 ℃ under aerobic condition.

2.2.7 Inhibition assay–Minimum Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) against *S. aureus* was determined by microbroth dilution technique with some modifications. Dandelion phenolic extract was diluted in sterile water to the concentration of 500 mg/mL, further dilutions were made up to the concentration of 25, 20, 15, 10, 5 mg/mL.

Tested pathogenic microorganisms were cultured in MHB at 37 ℃ for 24 h. Initially, the cultures were diluted to match the turbidity of 0.5 McFarland standard; thereafter, further dilutions with sterile MHB made it possible to obtain a suspension of about 5×10^5 CFU/mL. Aliquot of bacterial suspensions (50 μL) were added to a sterile 96-wells plate containing 100 μL of MHB and 100 μL of dandelion phenolic extract dilutions.

A positive control (without dandelion phenolic extract) was included on each microplate. The plates were incubated in aerobic conditions at 37 ℃ for 24 h. A microplate reader (Bio-Rad, Hercules, CA) was used to record the optical density (OD) at 600 nm. The MIC was defined as the lowest concentration of dandelion phenolic extract able to inhibit the microorganism's growth.

2.2.8 Microbial growth

S. aureus was cultured to the logarithmic growth phase and 2% (V/V) inoculum was added to the medium. The dandelion phenolic extract was added with the final concentration of 1/2MIC and 1MIC inhibitory concentration and a blank was set as control group. Under aerobic conditions at 37 °C in a shaking incubator at 120 rpm/min. Samples were taken at different times to determine the absorbance value, and to draw the growth curve of *S. aureus*.

2.2.9 Antimicrobial activity

S. aureus was cultured to the logarithmic growth phase and 2% (V/V) inoculum was added to the medium. The dandelion phenolic extract was added with the final concentration of 1/2MIC and 1MIC inhibitory concentration, with a blank used as control group. Incubated in aerobic conditions and 37 °C in a shaking incubator at 120 rpm/min.

Effect of plant extract on the *S. aureus* cell wall permeability Samples collected at different time intervals were centrifuged at 4500 rpm/min for 10 minutes, and the supernatant was taken to test the alkaline phosphatase activity in accordance with the operating steps of alkaline phosphatase kit. All assays were performed in triplicate and three independent experiments were performed.

Effect of plant extracts on the integrity of *S. aureus* biofilms Samples collected at different time intervals were centrifuged at 4500 rpm/min for 10 minutes. The supernatant was taken to test the solution which was diluted to measure the conductivity with a Lieci conductivity meter (Shanghai INESA Scientific Instrument Co., Ltd. Beijing, P. R. China, ca. DDSJ-308F). All assays were performed in triplicate and three independent experiments were performed.

Effect of plant extract on extracellular protein content The protein standard curve was drawn with bovine serum albumin as a standard solution. The samples were added to Coomassie Brilliant Blue G250 which were remained in standing position for 10 min. The absorbance was measured at a wavelength of 595 nm.

Interaction between bacterial DNA and plant extract *S. aureus* was cultured to the logarithmic growth phase and 2% (V/V) inoculum was added to the medium. Incubated in aerobic conditions at 37 °C in a shaking incubator at 120 rpm/min for overnight. DNA extraction kit (TIANGEN BIOTECH Co., Ltd, Beijing, P. R. China, cat. KG203) was used to extract the DNA of *S. aureus*. The same amount of DNA extracted and different concentrations of dandelion extract were mixed. The mixture was reacted for 20 minutes, and then 1% dextran gel electrophoresis was performed.

Statistical analysis The results are expressed as means ± SD. Statistical differences were analyzed using a t-test for independent groups. The ANOVA was performed using GraphPad Prism version 6.01 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was declared as *P < 0.05, **P < 0.01, and ***P < 0.001. Each experiment was repeated at least 3 times.

2.2.10 Cell preparation, culture, and frozen

Milk was obtained from several Ukrainian dairy cows in middle lactation (150-250 days after parturition). The nipples were scrubbed with sterile water, and the milk was squeezed by hand. The first 50 mL of milk was discarded, then approximately 150 mL was collected in a sterile centrifuge tube. The tube was sealed over a fire, brought back to the laboratory, stored at 37 °C, and treated within 2 h. The milk was centrifuged at 1000 g for 20 min and the upper layer of milk was removed as much as possible. The bottom turbid liquid was washed several times with phosphate buffered saline (PBS) (1:1) solution containing antibiotic-antimycotic (Gibco, USA, New York, cat.15240-062) until the solution was pellucid and did not contain milk. The bottom pellet was mixed with fresh medium and transferred to an empty plastic cell culture dish (Corning, USA, New York, cat.430639). The culture dishes were incubated at 37 °C under 5% CO2. The basal medium was replaced with a fresh medium every 48 h until the cells were distributed across the bottom of the dish. The cells were subcultured and frozen when they were distributed across 80% of the bottom.

2.2.11 Growth characteristics

The real-time cell assay (RTCA) was used to monitor the growth of the third-generation mammary gland epithelial cells. Totals of 4×10^4, 2×10^4, 1×10^4, 5000, 2500, 1250, and 625 cells per well were seeded on E-Plate, 16 plates, 150 μL medium per well.

Real time detection of cells was performed to plot cell growth curves while optimizing optimal plate density.

2.2.12 Immunocytochemistry

The expression of cytokeratin 18 (Abcam, UK, Cambridge, cat.10830-1-AP) was examined by seeding 1×10^4 cells/well in 12-well flat-bottom culture plates. Cytostructural protein expression was examined on day 3 after seeding cells that were cultured in induction media. Before staining, the cells were washed with PBS solution and fixed with ice-cold methanol.

Cells were incubated in PBS containing 0.2% Triton X-100 at 37 °C for 5 min, then rinsed with PBS. Nonspecific reactivity was blocked with 5% bovine serum albumin (BSA) for 30 min at room temperature. First, anti-cytokeratin 18, was diluted 1/100 in PBS, added to the cells, and incubated for 1 h at room temperature.

The cells were then washed three times for 5 min each time with Phosphate Buffered Saline-Tween20 (PBST). Secondary antibody, fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-mouse IgG (Sigma, USA, Louis, MO, cat. F4143), was diluted 1/50 in PBS, added to the cells, and incubated in the dark for 0.5 h. Cells were then washed three times for 5 min each time with PBS.

DAPI (4,6-diamino-2-phenyl indole) was used as a nuclear counterstain. Finally, the slides were washed three times and visualized with a microscopy characterization facility (Zeiss, LSM7800, Germany, Oberkochen).

2.2.13 RT-PCR

Total RNA from mammary tissue, BMECs cultured with the induction media, and BMECs, and mammary alveolar cell-T (MAC-T) cells cultured with basal media were isolated using a PureLinkTM RNA Mini kit (ThermoFisher, USA, Waltham MA, cat.12183018A). The expressions of CSN2, BTN1A1, and GAPDH were determined by RT-PCR. The integrity and concentration of the RNA was verified via analysis of 5 µL of each sample on a 1% agarose gel using an ultraviolet spectrophotometer (Beijing Junyi-Dongfang Electrophoresis Equipment Co., Ltd China, Beijing, JY04S-3E). The RT system (SuperScript@) was purchased from Invitrogen. CSN2, BTN1A1, and GAPDH primers were designed with Primer 5.0 (Table 2.1) and synthesized by Shanghai Sangon Biological Engineering Technology and Services Co. Ltd., China.

Table 2.1

Primer sequences for RT-PCR on identification of BMECs

Gene	Primers forward/reverse	Product length (bp)	Melting temperature (°C)
CSN2	F:5'AGGAACAGCAGCAAACAG3' R:5'TTTCCAGTCGCAGTCAAT3'	579	55
BTN1A1	F:5'TGTGTTGCTGCTGATAGAGT3' R:5' CCTCCAAGTTCCTTTATGGGAT3'	305	53
GADPH	F:5'GGCAAGTTCAACGGCACA3' R:5'ACCACATACTCAGCACCAGCA3'	128	56

Table 2.2

The reaction system of PCR (Total 20 µL)

Reagent	Usage amount
10 x buffer	2.5 µL
Forward primer (10 µM)	1.0 µL
Reverse primer (10 µM)	1.0 µL
dNTP	2.0 µL
Taq	1.0 µL
DNA	1.0 µL
RNase Free H_2O	Up to 25 µL

2.2.14 Western-blot (WB)

Total protein was isolated from BMECs, MAC-T, and mammary tissues using Radio Immunoprecipitation Assay (RIPA) (Servicebio, China, Wuhan, cat. G2002). The proteins were run through a 12% polyacrylamide gel under reducing conditions, and transferred onto polyvinylidene fluoride (PVDF) (0.45 μm, GE Healthcare Life Sciences, Pittsburgh, PA, USA). After blocking in Tris-HCl Buffered Saline (TBS) (20 mM Tris and 137 mM NaCl) containing 5% BSA, the membrane was incubated overnight at 4 °C with rabbit anti-bovine casein antibody (Jingmei, Jiangsu, China, cat. F030106). The membrane was washed three times for 5 min each with Tris-HCl Buffered Saline-Tween20 (TBST), and incubated with Horseradish Peroxidase (HRP) conjugated mouse anti-rabbit IgG (Beyotime, Shanghai, China, cat.TR-1003) for 1 h at 37 °C.

2.2.15 Cultivation and treatment of BMECs for the inflammatory model

The BMECs harvested from mid-lactation dairy cow milk were isolated by our laboratory. Briefly, the base medium for this cell is DMEM/F-12 (Gibco, USA, cat.12400-024). The complete growth medium included 10% fetal bovine serum (Biological Industries, Israel, cat.04-011-1A/B), DMEM/F-12, and 10 ng/mL epidermal growth factor (Sigma, USA, cat. E4127). Cells were maintained at 37℃in an incubator containing 5% CO2. When cells grew to 80% confluency, the cells were rinsed twice with PBS, and then the primary mammary epithelial cells were trypsinized with 0.25% trypsin plus 0.02% EDTA and passaged.

The BMECs were seeded into 6-well plated overnight at 37℃. Then, the cells were treated with different concentration (0, 10, 20, 40, 80 ng/μL) lipoteichoic acid (LTA) (Inviogen, Carlsbad, CA, USA, cat. tlrl-slta). After 12 h, 24 h, and 48 h of stimulation, the BMECs were harvested for subsequent analyses.

Extraction and purification of total RNA Total RNA was extracted from adherent BMECs using RNAiso Plus (TaKaRa, Dalian, P. R. China, cat. 9109) in

accordance with the manufacturer's instructions. The assessment of the quantity and quality of RNA was verified using a NanoDrop 1000 (Thermo Scientific, Co., Ltd., P. R. China). The 260:280 nm optical density value was between 1.8 and 2.0. Then, the first-strand cDNA was synthesized using PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, P. R. China, cat. RR047A).

Table 2.3

Reaction system for removing genomic DNA

Reagent	Usage amount
5 x gDNA Eraser buffer	2.0 μL
gDNA Eraser	1.0 μL
Total RNA	1.0 μL
RNase Free H_2O	Up to 10 μL

Table 2.4

Reaction components of reverse transcription

Reagent	Usage amount
5 x RT buffer	4.0 μL
RT Enzyme Mix	1.0 μL
RT primer	1.0 μL
The product of Table 2-3	10 μL
RNase Free H_2O	4.0 μL

Cell Counting Kit-8 Assay

BMECs were seeded at a concentration of 1x104 cell per well in 96-well plates with eight replicates per condition. At the indicated timepoint, Cell counting KIT-8 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, P. R. China, cat.CK04) solution at a medium dilution of 1:10 diluted was added to each well and the plate was incubated at 37℃ for 3 h.

The absorbance was measured at a wavelength of 450 nm by a micoplate reader (Bio-Rad, Hercules, CA), and the proliferation of each groups was calcuted using the equation:

[(AS - Ab)/ (AC - Ab)]x100%.

AS: The absorbance value of the wells with cells, LTA, CCK-8;

AC: The absorbance value of the wells with cells, CCK-8;

Ab: The absorbance value of the wells without cells.

Real-Time Cell Assay (RTCA)

The Real-Time Cell Assay (RTCA) was used to detect the effect of different concentrations (0, 10, 20, 40, 80 ng/μL) of LTA on BMEC proliferation. The CI value is directly proportional to the number of cells. RTCA was determine the CI value by measuring the impedance record.

Enzyme-linked immunosorbent assay (ELISA)

BMECs were cultured for 12h, 24 h, and 48h in fresh serum-free medium after treatment with LTA. The medium was collected and centrifuged at 12,000 rpm for 5 min to remove celldebris.

The levels of tumor necrosis factor α (TNF-α), and Interleukin-6 (IL-6) in the supernatants of BMECs were detected according to the ELISA kit instructions (Jiangsu Mei Biao Biological Technology Co., Ltd., Jiangsu, P. R. China, cat. MB-4838A\MB-4837A).

RT-qPCR analysis

Real-time PCR primers for amplification of mRNA were designed using Primer Premier 5.0 and synthesized by Sangon Biotech (Shanghai, P. R. China, Co., Ltd.). The primers used are in Table 2.2.

Real-time quantitative PCR was performed using TB Green Premix Ex TaqTM II (TaKaRa, Dalian, P. R. China, cat#RR820B) on a 7500 Real-Time PCR system (Applied Biosystems Inc., Foster City, CA). GAPDH was used as a reference gene. The relative gene expression was calculated using the $2^{-\triangle\triangle Ct}$ method.

Table 2.5

Real-time quantitative PCR Primer Information

Gene	Accession	Sequence	Product size (bp)
TNF- α	NM_173966.3	F:5'GGTGGTGGGACTCGTATGCCAATGC3' R:5'GTGAGGAACAAGGGGGTGG3'	151
IL-6	NM_173923.2	F:5'ACAGCTATGAACTCCCGCTT3' R:5' TCTCACATATCTCCTTTCTCATTGC3'	226
GADPH	NM_001034034.2	F:5'AGATGGTGAAGGTCGGAGTG3' R:5'CGTTCTCTGCCTTGACTGTG3'	189

Table 2.6

The reaction system of qRT-PCR

Reagent	Usage amount
5 x RT buffer	7.5 μL
Forward primer (10 μM)	0.5 μL
Reverse primer (10 μM)	0.5 μL
cDNA	2.0 μL
RNase Free H_2O	Up to 15 μL

Statistical analysis

The results are expressed as means ± SD. Statistical differences were analyzed using a t-test for independent groups. The ANOVA was performed using GraphPad Prism version 6.01 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was declared as *P< 0.05, **P< 0.01, and ***P< 0.001. Each experiment was repeated at least 3 times.

2.2.16 Effect of CGA on LTA-Stimulated BMECs

Cells Culture and Treatment Bovine mammary epithelial cells were isolated by our laboratory from mid-lactation dairy cow milk. Briefly, the BMECs were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) (Gibco,

USA, New York, cat.12400-024) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel, Kibbutz Beit-Haemek, cat.04-011-1A/B) and 10 ng/mL epidermal growth factor (EGF) (Sigma, USA, Louis, MO, cat. E4127). Cells were maintained at 37 ℃ in an incubator containing 5% CO2. Cells were routinely passaged at a rate of 70-80% for all the experiments. Cells were pretreated with different concentrations of CGA (25, 50, 100 ng/μL) for 5 h followed by incubation with 20 ng/μL lipoteichoic acid (LTA) (Inviogen, Carlsbad, CA, USA, cat. tlrl-slta) for 24 h. Phosphate Buffer Saline (PBS) was used as a positive control. Total prteins, supernatants and mRNA were extracted from cells at specified time intervals.

CCK-8 Assay of Cell Viability The toxic effects of CGA on BMECs were determined using the Cell counting Kit-8 (Solarbio Science & Technology Co., Ltd., Beijing, P. R. China, cat. CK04). The cells were seeded at a concentration of $1x10^4$ cell per well in 96-well plates with eight replicates per condition, and stimulated with CGA (12.5, 25, 50, 100, 200, and 400 ng/μL) for 24 h. Another group stimulated with CGA (25, 50, 100 ng/μL), then treated with LTA (20 ng/μL) for 24 h. At the indicated timepoint, CCK-8 solution at a medium dilution of 1:10 diluted was added to each well and the plate was incubated at 37℃ for 3 h. Finally, the absorbance was measured at a wavelength of 450 nm by a microplate reader.

Real-Time Cell Assay (RTCA) of Cell Viability

The Real-Time Cell Assay (RTCA) was used to detect the effect of different concentrations (0, 12.5, 25, 50, 100 μg/mL) of CGA extract on BMEC proliferation. The CI value is directly proportional to the number of cells. RTCA was determine the CI value by measuring the impedance record.

Quantitative Real-Time PCR Analysis

Total RNA was extracted from the cells using RNAiso Plus (TaKaRa, Dalian, P. R. China, cat. 9109) in accordance with the manufacturer's instructions. The assessment of the quantity and quality of RNA was verified using a NanoDrop 1000 (Thermo Scientific, Co., Ltd., P. R. China). The 260 : 280 nm optical density value was between 1.8 and 2.0. Then, the first-strand cDNA was synthesized using PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, P. R. China, cat. RR047A). Quantitativ real-time PCR was performed using TB Green Premix Ex

TaqTM II (TaKaRa, Dalian, P. R. China, cat. RR820B) on a 7500 Real-Time PCR system (Applied Biosystems Inc., Foster City, CA). GAPDH was used as a reference gene.

The relative gene expression was calculated using the $2^{-\triangle\triangle Ct}$ method. The primers were designed using Primer Premier 5.0 and synthesized by Sangon Biotech (Shanghai, P. R. China, Co., Ltd.). The primers used are listed in Table 2.7.

Table 2.7

Quantitative real-time PCR Primer Information

Gene	Accession	Sequence	Product size (bp)
TNF- α	NM_173966.3	F:5'GGTGGTGGGACTCGTATGCCAATGC3' R:5'GTGAGGAACAAGGGGGTGG3'	151
IL-6	NM_173923.2	F:5'ACAGCTATGAACTCCCGCTT3' R:5'TCTCACATATCTCCTTTCTCATTGC3'	226
IL-1β	XM_005889988.2	F:5'TCGAAACGTCCTCCGACGAG3' R:5'TGAGAGGAGGTGGAGAGCCT3'	131
GADPH	NM_001034034.2	F:5'AGATGGTGAAGGTCGGAGTG3' R:5'CGTTCTCTGCCTTGACTGTG3'	189

Enzyme-linked Immunosorbent Assay (ELISA)

The BMEC cells were pre-treated with CGA 5 h before LTA stimulation, and 24 h later, the supernatants were collected. The concentrations of TNF-α, IL-6, and IL-1β in the supernatants were measured by ELISA kits (Jiangsu Mei Biao Biological Technology Co., Ltd., Jiangsu, P. R. China, cat. MB-4838A\MB-4905A\MB-4837A).

NO Assay

The cells were pre-treated with CGA 5h before CGA stimulation for 24 h. Then, the supernatants were collected. The concentration of NO in the supernatants were measured using Griess reagent Nitric Oxide Assay Kit (Beyotime Biotechnology, Shanghai, P. R. China, cat. S0023) through detecting nitrite level. Nitrite level represents the NO production.

Western Blot Analysis

The whole cell proteins were isolated using RIPA buffer containing a protease inhibitor mixture.

The concentration of protein in the extract from BMEC cells was determined using a BCA Protein Assay Kit(Solarbio Science & Technology Co., Ltd., Beijing, P. R. China, cat. PC0020).

Then, 40 μg proteins were separated on 12% SDS-PAGE and transferred onto PVDF membranes and blocked with 5% non-fat dry milk in TBST for 2 h. The membranes were then incubated with primary antibody (Abcam, Cambridge, UK, ca. ab213676\ab140751\ab32518) overnight at 4 ℃.

The membranes were incubated with primary antibodies. Then, the membranes were incubated with HRP-conjugated secondary antibodies. β-Actin was used as the control.

The protein levels of specific target genes expressed by electrophoresis were detected by a 3,3'-diaminobenzidine (DAB) substrate chromogenic assay (n=3).

Table 2.8

Dilution method of BCA standard samples

NO.	Diluent volume (μL)	2 mL/mL BSA Standard (μL)	BSA final concentration (μg/ mL)
A	0	120	2000
B	30	90	1500
C	60	60	1000
D	75	45	750
E	90	30	500
F	105	15	250
G	150	10	125
H	120	0	0 (Blank)

Table 2.9

Preparation system of 12% separation adhesive

Reagent	Volume
H_2O	3.3 mL
30% Acrylamide (29:1)	4 mL
1.5 M TRIS-Hcl (Ph=8.8)	2.5 mL
10% SDS	0.1 mL
10% Ammonium persulfate	0.1 mL
TEMED	0.004 mL

Table 2.10

Preparation system of 5% concentrated adhesive

Reagent	Volume
H_2O	3.42 mL
30% Acrylamide (29:1)	0.83 mL
1.5 M TRIS-Hcl (Ph=8.8)	0.625 mL
10% SDS	50 µL
10% Ammonium persulfate	75 µL
TEMED	75 µL

Statistical Analysis

Analysis was performed using GraphPad Prism 7.02 Software (GraphPad Software, Inc., USA). Differences between mean values of normally distributed data were analyzed using one-way analysis of variance (ANOVA) multiple comparisons. $p<0.05$ or $p<0.01$ was considered statistically signficant. All data were obtained from three independent experiment.

CHAPTER 3
RESULTS OF OWN RESEARCH

3.1 Determination and extraction process of chlorogenic acid in *T. officinale*

Chlorogenic acid is one of the important medicinal ingredients of *Taraxacum officinale*. It is a phenolic compound with antibacterial, antioxidant and antiviral effects. At present, the research on extraction of chlorogenic acid mainly focuses on natural products such as Eucommia ulmoides and honeysuckle.

The *Taraxacum officinale* is more widely distributed and produces. However, there were few studies on extraction technology of chlorogenic acid from *Taraxacum officinale*. At present, the research of chlorogenic acid in dandelion is mostly focused on the research of active function, and there are few research methods using its detection and extraction process.

Due to the special biological effect of ultrasound, choosing appropriate ultrasound parameters can form more pores between the cell walls of the plant, thereby enhancing the permeability and selectivity of the cell membrane, and is now widely used to extract biologically active ingredients in plants. Ultrasonic extraction method has been used in the extraction of active ingredients of Eucommia ulmoides, *Lonicera japonica Thunb*. and other plants.

Therefore, in this study resource-rich *Taraxacum officinale* was used as materials for extracting chlorogenic acid, a high-performance liquid chromatography method was used to establish the determination method of chlorogenic acid in *T. officinale*. On the basis of single factor experiment, orthogonal experiment design was used to investigate the effect of the ethanol volume fraction, material-liquid ratio, extraction time, and extraction temperature on extraction rate of chlorogenic acid in *T. officinale*.

3.1.1 High performance liquid chromatography for determination of chlorogenic acid

In the experiment of detecting chlorogenic acid content by high performance liquid chromatography, all the analysis was performed on a Shimadzu Acquity HPLC system (LC-20A, Shimadzu, Japan). And an Inertsil ODS-3 C18 column（5 μm, 250*4.6 mm）was applied for all analyses. After many repeated tests, the mobile phase was final determinationed as compose of A (MeOH) and B (1‰ Phosphoric acid solution, adjusted to pH 8.0 with ammonia-water) with a gradient elution: 0-5 min, 5% A; 5-15 min, 5-15% A; 15-20 min, 15%-5% A; 20-5 min 5% A. The flow rate of the mobile phase was 1.0 mL/min.and the column temperature was maintained at 30℃. Detection wavelength was set at 350 nm. Under this method, the peak shape was good. The detection object and other components were well separated. The high performance liquid chromatogram of chlorogenic acid standard was shown in Figure 3.1.1. The standard curve for chlorogenic acid was y = 1.4624 x - 2.5424, and the linear range was 0.00692~0.44 μg/mL. The linear relationship was good (R2=0.999). The high performance liquid chromatogram of chlorogenic acid sample was shown in Figure 3.1.2.

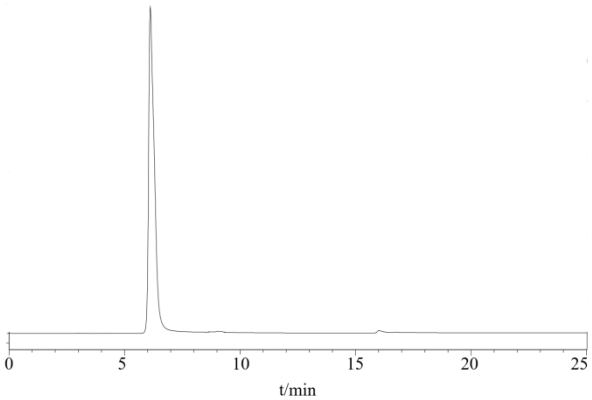


Fig. 3.1.1 High performance liquid chromatogram of chlorogenic acid standard

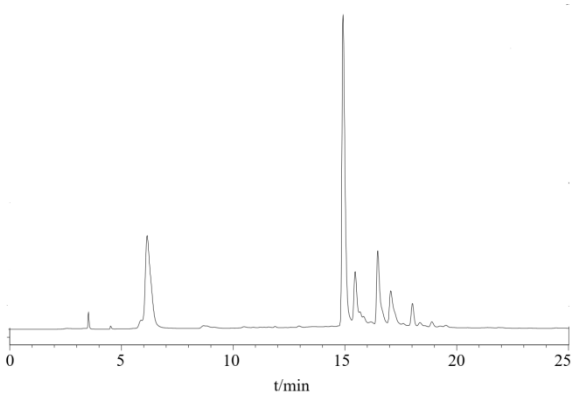


Fig. 3.1.2 High-performance liquid chromatogram of dandelion sample

The method was examined from the precision test, stability test, repeatability test, and recovery rate. Take the reference solution and inject 6 times continuously with a volume of 10 μL. The RSD of the chlorogenic acid peak area was 1.32%, which indicated that the instrument precision was good.

Take the dandelion sample for the test sample solution and inject them at 0, 2, 4, 8, 12, and 24 h, respectively, and measured the peak area according to the method of "2.2.2". The RSD of the chlorogenic acid peak area was 1.02%, indicating that the test sample solution is stable within 24 h.

Take 6 dandelion sample solutions to determine the content of chlorogenic acid in the sample. The RSDs of the measured contents were 1.14%, indicating good repeatability. Through the high-medium-low-concentration chlorogenic acid reference substance addition recovery test, the recovery rate obtained was between 93.8-97.6%.

3.1.2 Single factor analysis

The single factor experiment was used to find the effects of solvent concentration, microwave-assisted extraction (MAE) temperature and time, and liquid/solid (ratio) on extraction of chlorogenic acid.

3.1.2.1 Effect of extraction temperature on extraction rate of chlorogenic acid

Through 40, 50, 60, 70, 80 degrees Celsius, five different temperatures were used to explore the effect of extraction temperature on the extraction rate of chlorogenic acid. As shown in Figure 3.1.3, the extraction rate of chlorogenic acid increased first and then decreases slightly with ultrasonic temperature increasing. When the extraction temperature was 70°C, the extraction rate of chlorogenic acid was the highest with the rate of 1.41%. When the temperature exceeded 70°C, the extraction rate decreased. High temperature is conducive to the dissolution of compound besides the chlorogenic acid, and the temperature is too high, the active ingredients are easily damaged. At the same time solvent lose easily [15].

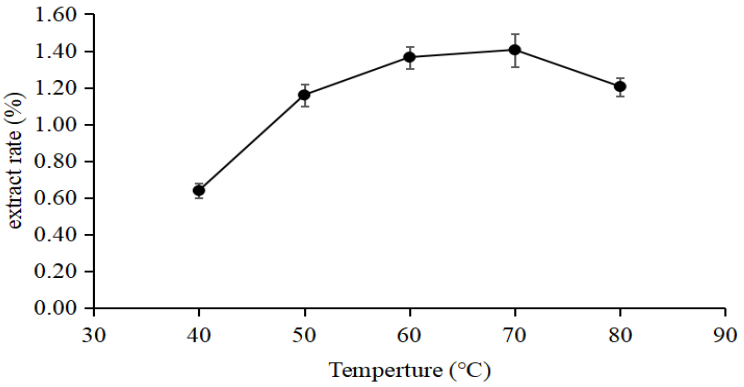


Fig. 3.1.3 The effect of ultrasonic temperature on the extraction rate

3.1.2.2 Effect of solid-liquid ratio (right) on the extraction rate of chlorogenic acid

Solid-liquid ratio of 1:10, 1:20, 1:30, 1:40, and 1:50, were used to explore the effect of solid-liquid ratio (right) on the extraction rate of chlorogenic acid. It can be seen from Figure 3.1.4 that the chlorogenic acid extraction rate of dandelion rises when the material-liquid ratio was increased at the beginning. The chlorogenic acid of dandelion had basically dissolved out when the solid-liquid ratio reached at 1:30 g/mL, and then tends to be stable. For cost considerations, the solid-liquid ratio was

1:30 g/mL. When the solid-liquid ratio was 1:50 g/mL, the extraction rate decreased slightly. As the amount of solvent increasing, the dissolved amount of alcohol-soluble and fat-soluble components in dandelion increased, which may effect the dissolution of organic acid components. However, thereby reducing the extraction rate of chlorogenic acid in dandelion. Finally, the extraction rate of chlorogenic acid in dandelion reduced.

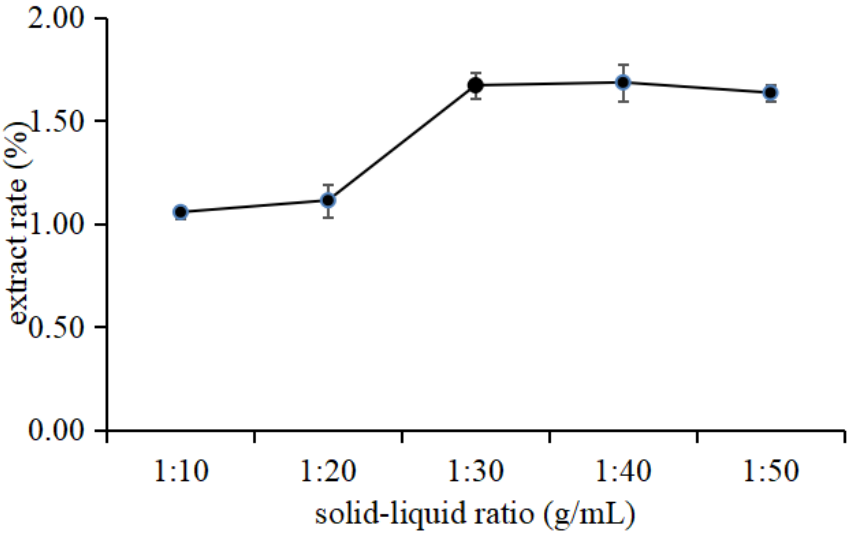


Fig. 3.1.4 Effect of solid-liquid ratio (right) on the extraction rate

3.1.3 Effect of ethanol content on the extraction rate of chlorogenic acid

Here, ethanol content of 20%, 40%, 60%, 80%, and 100%, were used to explore the effect of ethanol content on the extraction rate of chlorogenic acid. The result was shown in Figure 3.1.5, at the beginning, the extraction rate of dandelion chlorogenic acid increased significantly with the increasing of ethanol content in the extraction solvent.

When the ethanol content was 60 % (volume: volume), the extraction rate of dandelion chlorogenic acid reached the highest with the value of 1.51%. However, as the ethanol content continued to increase, the extraction rate of dandelion chlorogenic acid showed a downward trend. When the ethanol content was 80%, the extraction rate decreases slightly. It was speculated that with the ethanol content

increasing, the dissolution amount of some alcohol-soluble and fat-soluble in dandelion increased. In addition, the viscosity of the solution increases, the diffusion resistance of chlorogenic acid in the dandelion to the solvent increases, the diffusion slowed down, and the extraction rate decreased [126].

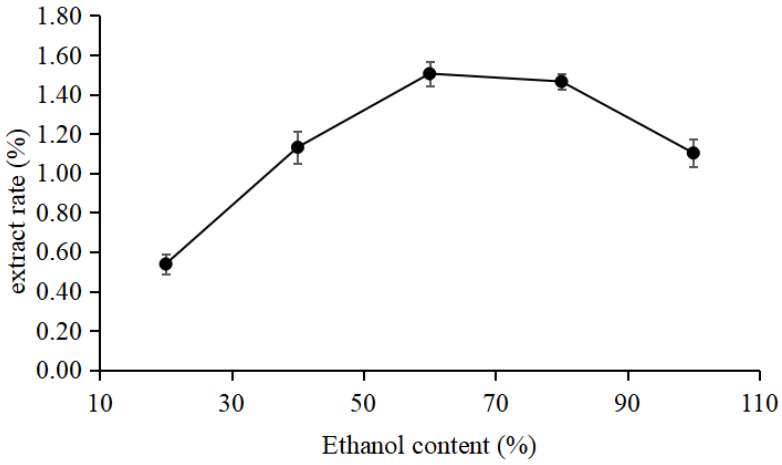


Fig. 3.1.5 The effect of ethanol content on the extraction rate

3.1.4 Effect of ultrasonic time on the extraction rate of chlorogenic acid

The effect of ultrasonic time on the extraction rate of chlorogenic acid was tested for 25, 35, 40, 45, and 50 minutes of ultrasonic treatment, respectively. As shown in Figure 3.1.6, at the beginning, the extraction rate gradually increased as the extraction time increased. When the extraction time was 45 min, the highest extraction rate was 1.67%. However, with the extraction time continual increasing the extraction rate began to decline. It indicated that when the extraction time is sufficient, chlorogenic acid in dandelion had basically dissolved out. If the ultrasonic time was too long, a large amount of non-organic acids will be dissolved out, resulting in a reduction in the extraction rate of organic acids and increased energy consumption.

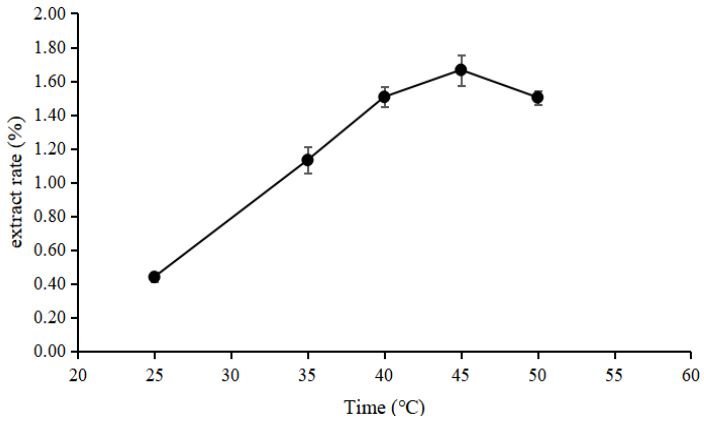


Fig. 3.1.6 The effect of ultrasonic time on the extraction rate

3.1.5 Orthogonal experiment result

Based on the single-factor experimental results, L9 (34) orthogonal test was performed on the ultrasonic time, ultrasonic temperature, material-liquid ratio and ethanol volume fraction. The orthogonal test design was shown in Table 3.1.1, and the results were shown in Table 3.1.2.

Table 3.1.1

Factors and levels

Level	Factor			
	A Temperture (℃)	B S/L ratio	C Ethanol content (%)	D Time (min)
1	50	1:25	50	35
2	60	1:30	60	40
3	70	1:35	70	45

The range (R) of the orthogonal test was B>C>A=D, which indicated that the order of the influence of various factors on the extraction rate of dandelion chlorogenic acid was from large to small in order: material-liquid ratio > ethanol content > ultrasonic temperature = ultrasound time. The optimal process conditions

for dandelion chlorogenic acid extraction were A2B2C1D2 (solid-liquid ratio was 1:30, the ethanol content was 50%, the ultrasonic temperature was 60°C, and the ultrasonic time was 40 min). Since this optimal combination did not appear in the orthogonal test, a verification test should be performed on the obtained optimal combination. The dandelion chlorogenic acid extraction rate test was repeated 5 times. The dandelion chlorogenic acid extraction rate was 1.92%, which was higher than the highest chlorogenic acid extraction rate (1.89%) in Table 3.1.2. The relative standard deviation was 0.83%, which proved that the process conditions were stable, and suitable for the extraction of dandelion chlorogenic acid.

Table 3.1.2

L9 (34) arrangement and results of orthogonal test

NO	A	B	C	D	Extraction rate (%)
1	1	1	1	1	1.41
2	1	2	2	2	1.67
3	1	3	3	3	1.58
4	2	1	2	3	1.39
5	2	2	3	1	1.89
6	2	3	1	2	1.81
7	3	1	3	2	1.53
8	3	2	1	3	1.85
9	3	3	2	1	1.12
K1	4.66	4.33	5.07	4.42	
K2	5.09	5.41	4.19	4.81	
K3	4.50	4.51	5.00	4.82	
k1	1.55	1.44	1.69	1.47	
k2	1.70	1.80	1.40	1.67	
k3	1.50	1.50	1.66	1.61	
R	0.20	0.36	0.29	0.20	

Note: “K” represents the sum of chlorogenic acid extraction rate at the same level of each factor; “k” represents the average value of chlorogenic acid extraction rate at the same level of each factor; “R” determines the magnitude of the influence of factors on the test results.

In this part experiment, the results showed that the HPLC 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. 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 ^{0}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%.

This study obtained the detection method and optimized the extraction process of chlorogenic acid in *Taraxacum officinale*, and it provided a research basis for the development and utilization of *Taraxacum officinale* medicinal value.

3.2 Antibiofilm activity against *Staphylococcus aureus* and content analysis of *Taraxacum Officinale* Phenolic extracts

Staphylococcus aureus is a common zoonotic pathogen that exists widely in nature. Among the main pathogenic bacteria causing diseases, *E. coli and S. aureus* are ranked second. *S. aureus* can cause a wide range of infections and diseases including purulent, sepsis, endocarditis, meningitis and others. It is also one of the main pathogenic bacteria in dairy cow mastitis, which is quite common problem for animal health and dairy industry. At the same time, it can cause food poisoning of humans and animals and thus endanger the health of animals and humans. With the use of western medicine, bacterial resistance has developed which has increased the

toxic effects on animals as well as made it more difficult to choose anti-*Staphylococcus aureus* drugs in clinical practice.

Several studies demonstrated the antimicrobial activity of dietary polyphenols, and their activity on bacterial growth is mainly related to the strain, polyphenol structure and dosage assayed.

Plant extracts are rich in polyphenols which are reported to inhibit the biofilm formation by *S. aureus*, including methicillin-resistant *Staphylococcus aureus* MRSA, *Escherichia coli* and *Pseudomonas aeruginosa*.

However, five types of microbial strains (*Streptococcus mutans*, *Streptococcus pyogenes*, *Streptococcus pneumonia*, *Streptococcus aureus*, and *Pseudomonas aeruginosa*) have been used for the estimation of antimicrobial effect of *T. officinale*. Among all the plant extracts, the methanol extract was found to have the antimicrobial potential against most bacterial strains. But the antibacterial mechanism of *T. officinale* extract was still unclear.

Considering the common and serious pathogenic characteristics of *S. aureus* in the areas of food industry and safety, we selected *T. officinale* phenolic extract as a potential active compound, the effect of phenolic contents as well as the antibiofilm activity against *Staphylococcus* aureus of phenolic extract from *T. Officinale* were evaluated in vitro.

With 70% methanol-water (v/v) as a solvent, the dandelion was extracted by ultrasonic assisted extraction method. Subsequent identification and quantification of phenol in extract was carried out using High Performance Liquid Chromatography (HPLC). The minimum inhibitory concentration and antibacterial kinetic curve of dandelion phenolic extract were analyzed by spectrophotometry.

Changes in extracellular alkaline phosphatase (AKP) contents, electrical conductivity, intracellular protein contents, and DNA of *S.* aureus after the action of dandelion phenolic extract were determined to study its effect on the permeability of *S. aureus* cell wall and cell membrane. The results were shown as following:

3.2.1 Five phenolic acid contents of *T. Officinale* phenolic extract (TPE)

Phenolic acid has antibacterial and anti -inflammatory biological activity, which is related to the content of phenolic acid. Generally, not only the total phenolic acid content but also its composition of has the impact on its bioactivities. In this study, the phenolic acid composition from *T. Officinale* was analyzed by HPLC. All the analysis was performed on a Shimadzu Acquity HPLC system (LC-20A, Shimadzu, Japan). And an Inertsil ODS-3 C18 column（5 μm, 250*4.6 mm）was applied for all analyses. After many repeated tests, the mobile phase was final determinationed as compose of acetonitrile and 2% acetic acid aqueous solutions with the ratio of 80/20(v/v), and with a gradient elution: 0-5 min, 5% A; 5-15 min, 5-15% A; 15-20 min, 15%-5% A; 20-5 min 5% A.

The flow rate of the mobile phase was 1.0 mL/min. And the column temperature was maintained at 35℃. Detection wavelength was set at 320 nm. Each of the five standard compounds (10.0 mg) was dissolved in methanol-water (70:30, v/v) in a 10 mL volumetric flask to make the individual standard stock solutions. The standard curve for each compound is shown in Figuer 3.2.1. The standard curve for chlorogenic acid was y = 423,717 x + 1,972 (Figure 3.2.1A), and the linear relationship was good (R^2=0.998).

The standard curve for caffeic acid was y = 544,660 x + 2,940 (Figure 3.2.1B), and the linear relationship was good (R^2=0.999). The standard curve for rutin was y = 110,639 x - 114 (Figure 3.2.1C), and the linear relationship was good (R^2=0.997).

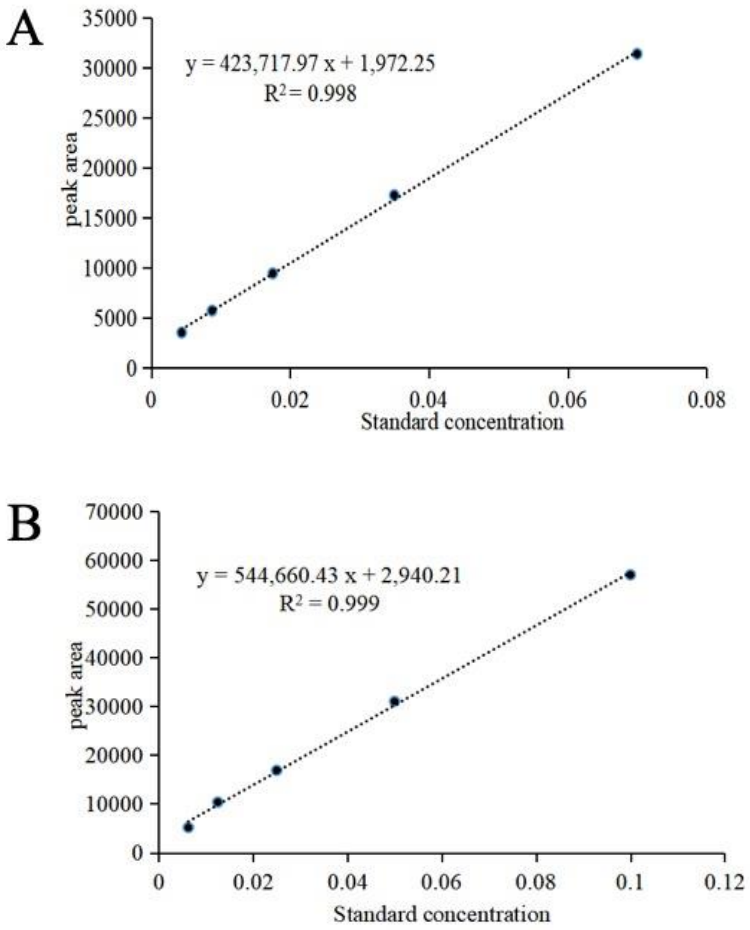
The standard curve for luteolin was y = 217,670 x - 753 (Figure 3.2.1D), and the linear relationship was good (R^2=0.998).

The standard curve for ferulic acid was y = 461,846 x + 2,689 (Figure 3.2.1E), and the linear relationship was good (R^2=0.999). The working stock solution (designated as the mixed standard solution) containing the five standard compounds

was formulated by mixing 1.0 mL of each standard compound stock solution in a 10 mL volumetric flask.

Finally, the linear relationship between the peak area and concentration of the five phenolic mixture reference solutions established by HPLC detection method were shown in Figure 3.2.2A. The results of HPLC detection of five phenolic acids in the mixed sample were shown in Figure 3.2.2B.

The peak areas were recorded. Through linear regression the linear equations were obtained. Calculate the content of each substance, the results showed that the highest concentration in the phenolic mixture was chlorogenic acid (1.34 mg/g), followed by luteolin (1.08 mg/g), caffeic acid (0.21 mg/g), rutin (0.19 mg/g) and ferulic acid (0.22 mg/g) (Table 3.2.1).



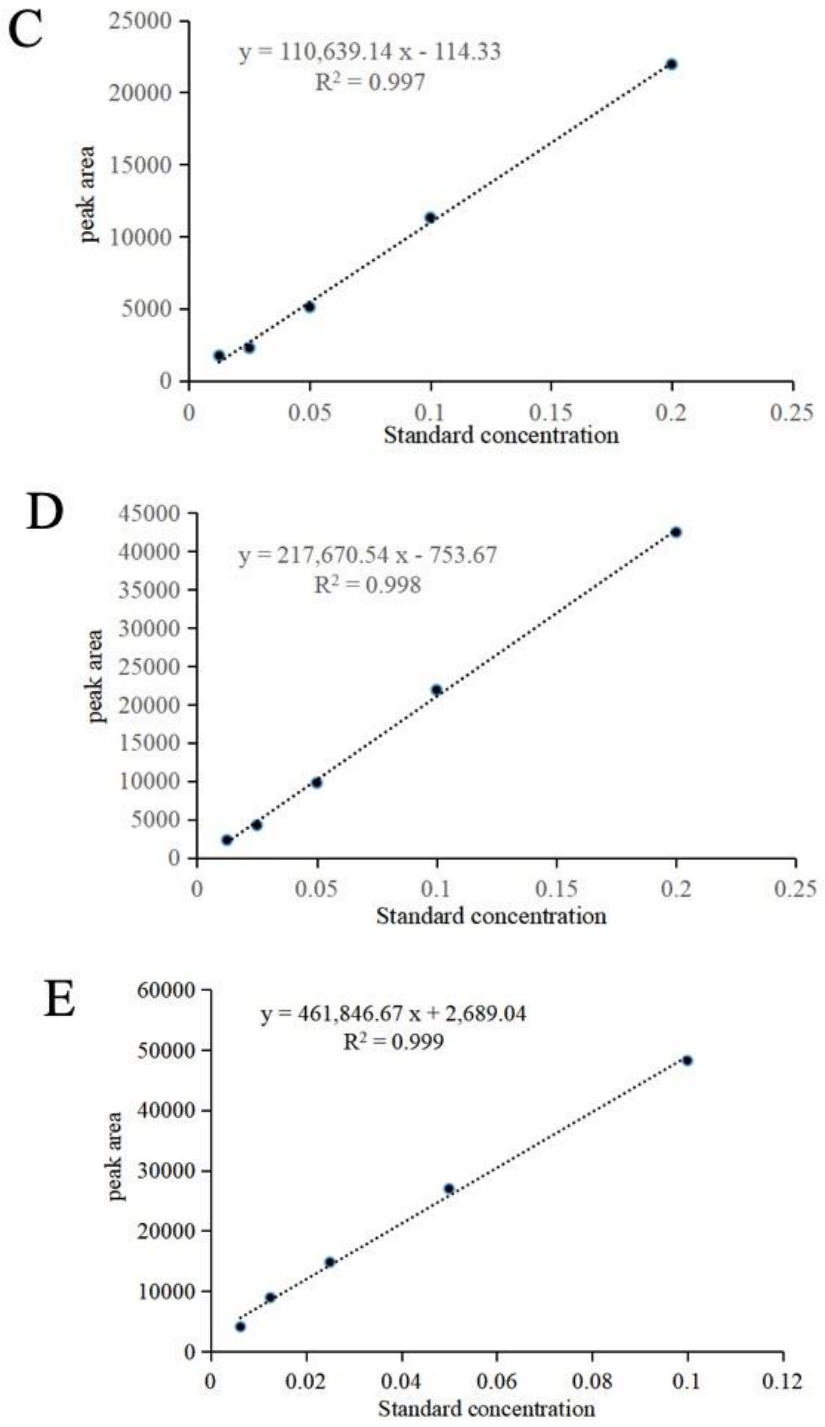


Fig. 3.2.1 Standard curve of five phenolic acids.

Note: A: Standard curve of chlorogenic acid; B: Standard curve of caffeic acid; C: Standard curve of rutin; D: Standard curve of luteolin; E: ferulic acid.

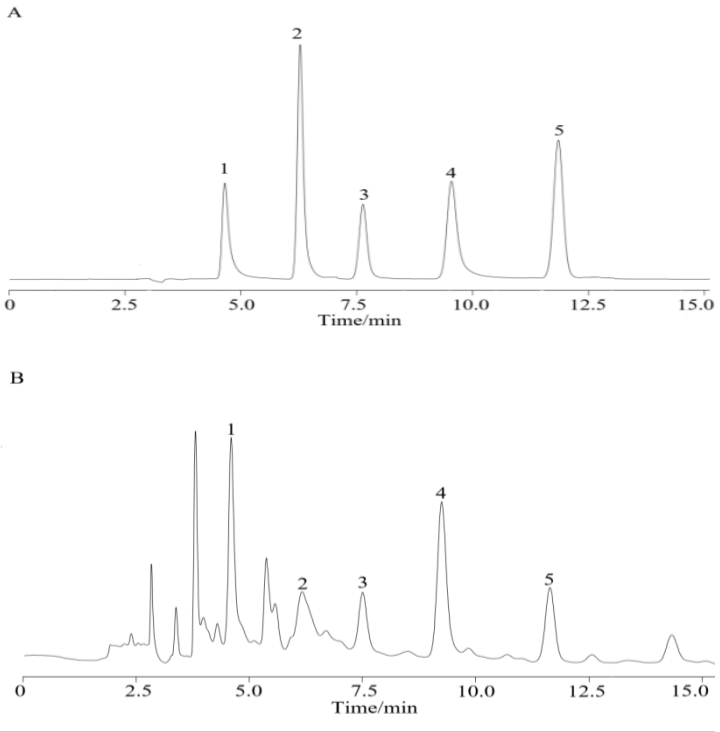


Fig. 3.2.2. HPLC chromatogram of five phenolic mixture reference solutions (A) and *T. Officinale* phenolic extract (B).

Note: 1: chlorogenic acid, 2: caffeic acid, 3: rutin, 4: luteolin, 5: ferulic acid.

Table 3.2.1.

The standard curve of five phenolic mixture reference solutions and the content in *T. Officinale* phenolic extract (average value ± SD)

Peak NO.	tR (min)	Compound	Standard curve	R^2	Content ($mg \cdot g^{-1}$)
1	4.6	Chlorogenic acid	y = 423,717 x + 1,972	0.998	1.3±0.09
2	6.3	Caffeic acid	y = 544,660 x + 2,940	0.999	0.2±0.02
3	7.5	Rutin	y = 110,639 x - 114	0.997	0.19±0.02
4	9.5	Luteolin	y = 217,670 x - 753	0.998	1.08±0.03
5	11.8	Ferulic acid	y = 461,846 x + 2,689	0.999	0.22±0.01

3.2.2 Inhibition Assay

The minimal inhibitory concentration (MIC) of *T. officinale* phenolic extract against *S.* aureus was determined by microbroth dilution technique with some modifications.

Tested pathogenic microorganisms were cultured in MHB at 37 ℃ for 24 h. The MIC was defined as the lowest concentration of *T. officinale* phenolic extract able to inhibit the microorganism's growth.

The results of minimum inhibitory concentration test showed that by the mass concentration of the extract was directly proportional to its inhibitory effect on *S.* aureus. The MIC value of TPE against *S. aureus* was 12.5 mg/mL.

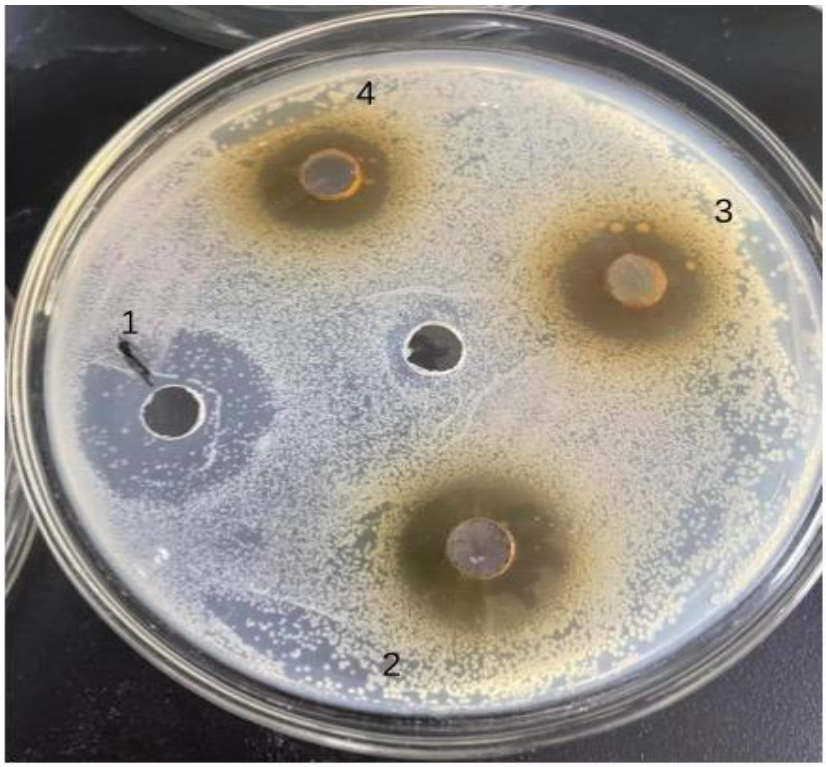


Fig. 3.2.3. Antibacterial activity of the plant extract.

Note: 1: positive control; 2-4: different concentrations of plant extract.

3.2.3 Microbial growth

The effect of TPE on the growth curve of *S. aureus* was determined by the absorbance value, and then the growth curve of *S. aureus* was drawed. The result was shown in Table 3.2.2 and Figure 3.2.4.

Compared with the blank control group, the sample group with 0.5MIC and 1MIC TPE showed a lower bacterial absorbance, indicating that the TPE had a certain inhibitory effect on the growth of *S. aureus*.

Table 3.2.2.

The absorbance value of *S. aureus*

Time (h)	OD_{600}		
	NC	0.5MIC	MIC
0	0	0.030	0.046
4	0.363	0.201	0.186
8	0.823	0.452	0.354
12	1.165	0.593	0.463
16	1.28	0.702	0.463
20	1.375	0.764	0.492
24	1.432	0.846	0.542
28	1.454	0.932	0.594
32	1.421	0.864	0.574

Within a certain range, the antibacterial effect was increased with the increase of TPE concentration.

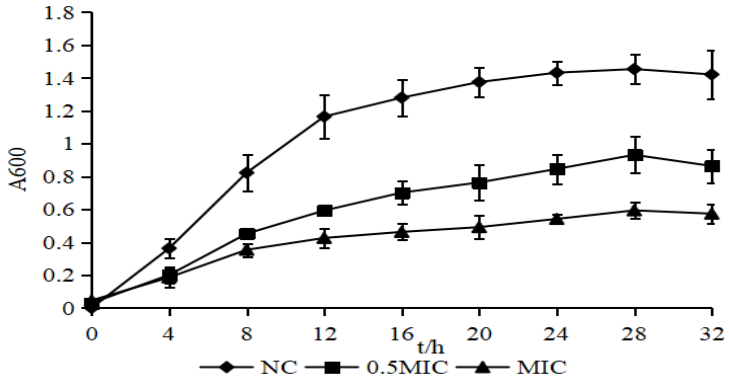


Fig. 3.2.4. Growth curves of S. aureus.

Note: NC: Normal control; 0.5MIC: TPE against *S. aureus* was 6.25 mg/mL; 1MIC: TPE against *S. aureus* was 12.5 mg/mL.

3.2.4 Effect of plant extract on the permeability of *S. aureus* cell wall

The effect of plant extract on the permeability of *S. aureus* cell wall was tested by the alkaline phosphatase activity in accordance with the operating steps of alkaline phosphatase kit. Figure 3.2.5 showed the effect of TPE on the cell membrane permeability of *S. aureus* due to the change in alkaline phosphatase. Compared with little change in the control group, the TPE treatment on *S. aureus* showed that the activity of alkaline phosphatase outside the bacteria was increased significantly, which was positively correlated with its concentration. As the macromolecular substances such as alkaline phosphatase are located between the cell wall and the cell membrane, which was leaked out of the cell indicating that TPE caused changes in the permeability of *S. aureus* cell wall.

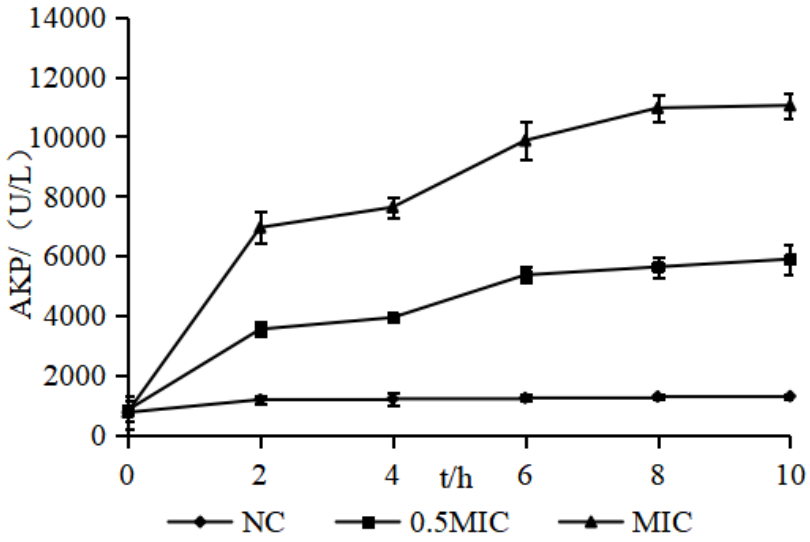


Fig. 3.2.5. The effect of the TPE on the cell wall permeability. Note: NC: Normal control; 0.5MIC: TPE against *S. aureus* was 6.25 mg/mL; 1MIC: TPE against *S. aureus* was 12.5 mg/mL.

3.2.5 Effect of plant extract on the integrity of *S. aureus* biofilms

The effect of TPE on the integrity of *S. aureus* biofilms was detectmined by electric conductivity assay. The result was showed in Figure 3.2.6. During the whole experiment period, there was a little change in the relative electric conductivity of control group. However, both 0.5MIC and 1MIC groups showed a significant increase in a dose-dependent manner after 2 h of TPE treatment. These results indicated that TPE treatment caused the leakage of intracellular electrolytes including K^+, Ca^{2+}, Na^+ and so on.

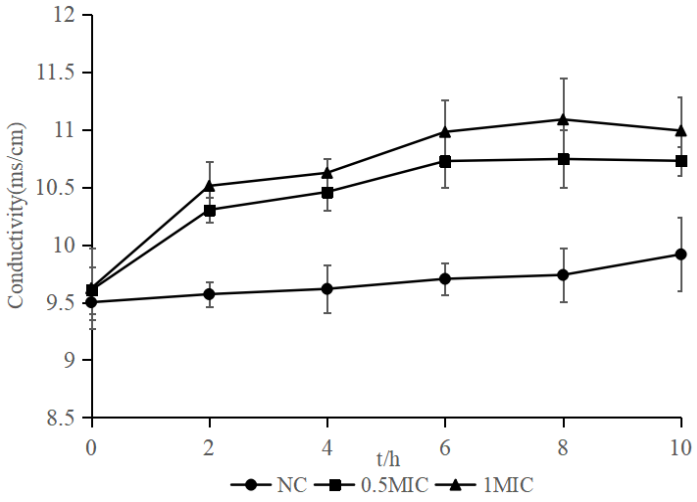


Fig. 3.2.6. The effect of the TPE on membrane permeability.

Note: NC: Normal control; 0.5MIC: TPE against *S. aureus* was 6.25 mg/mL; 1MIC: TPE against *S. aureus* was 12.5 mg/mL.

3.2.6 Effect of plant extract on extracellular protein content

The protein standard curve was drawn with bovine serum albumin as a standard solution (Figure 3.2.7).

The samples were measured at a wavelength of 595 nm of the absorbance, and then the protein content was drawed.

As shown in Figure 3.2.8 that after the action of TPE, the protein content in the *S.* aureus suspension increased, which was positively correlated with the drug concentration.

The protein contents changed significantly in the first 2 h. The change of the protein contents in the bacterial liquid also reflects the change of the permeability of the bacterial cell membrane.

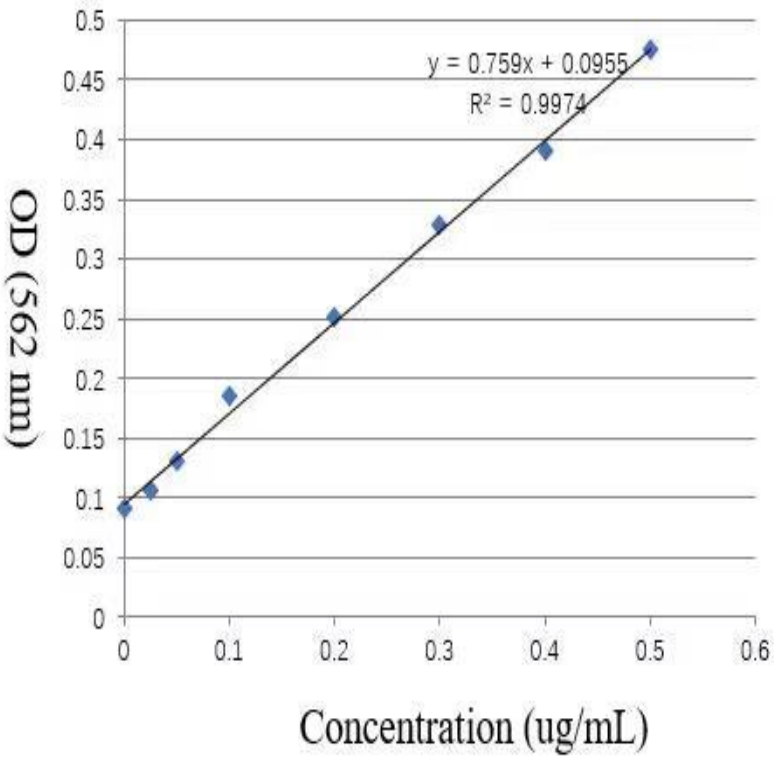


Fig. 3.2.7. Protein standard curve

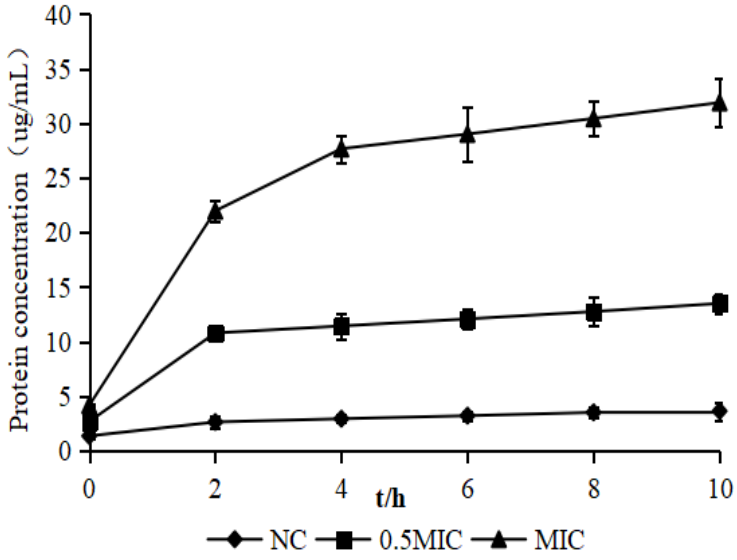


Fig. 3.2.8. The effect of the TPE on extracellular protein content.

Note: NC: Normal control; 0.5MIC: TPE against *S. aureus* was 6.25 mg/mL; 1MIC: TPE against *S. aureus* was 12.5 mg/mL.

3.2.7 Interaction between bacterial DNA and plant extract

The interaction of TPE with *S. aureus* DNA as shown in Figure 3.2.9. The plant extract with different concentrations (0~200 ng/μL) were co-culture with *S.* aureus for a period of time. As the concentration of the drug decreased, the brightness of the DNA band became brighter and brighter. When the concentration was 100 ng/μL, almost no brightness was observed. It showed that TPE may bind to the DNA of *S. aureus*, and the combination may affect its normal replication and transcription, and then affect the normal reproduction of *S. aureus*.

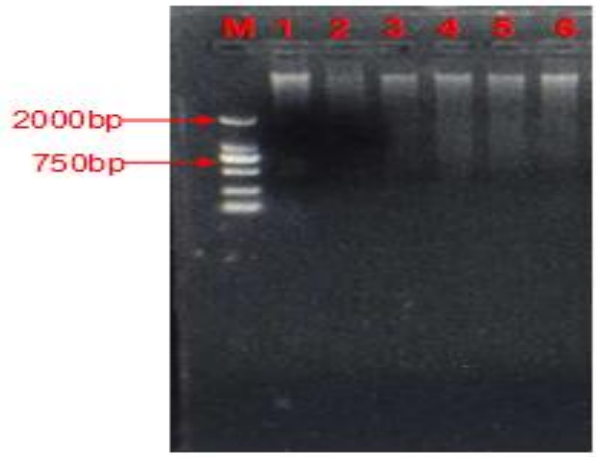


Fig. 3.2.9. Interaction of TPE with *S. aureus* DNA.

Note: M: DL2000 marker; 1: Control group; 2: 200 ng/μL; 3: 100 ng/μL; 4: 50 ng/μL; 5: 25 ng/μL; 6: 12.5 ng/μL.

In this part experiment, the results showed that chlorogenic acid (1.34 mg/g) was present in higher concentration, followed by luteolin (1.08 mg/g), ferulic acid (0.22 mg/g), caffeic acid (0.21 mg/g), and rutin (0.19 mg/g) in the dandelion phenolic extract. 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. After the action of dandelion phenolic extract, the growth of *S. aureus* was obviously inhibited entering into the decay phase early. Furthermore, after the action of dandelion, the

extracellular AKP contents of *S. aureus,* the electrical conductivity and the extracellular protein contents were all increased. The phenolic extract also affects the normal reproduction of *S. aureus*.

The results obtained in this work indicated that the mechanism of action of phenolic extract from *Taraxacum officinale* against *S. aureus* cause membrane depolarization and membrane permeabilization, affecting intracellular-enzyme activities, eventually leading to bacterial death. *Taraxacum 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.

3.3 In vitro culture and evaluation of bovine mammary epithelial cells from Ukraine dairy cows

Mammary epithelial cells (MECs) have been widely-used over the years as models to understand the physiological function of mammary disease. The first successful in vitro growth of BMECs in cell culture was done by Ebner et al. in 1961. Although the study of BMECs began many years ago, issues have remained that need to be addressed. It is not economical to slaughter a cow for the sole purpose of obtaining a mammary gland. Mammary gland tissue can be obtained from healthy cows via biopsy at any phase of reproductive life; however, the procedure is time-consuming and inconvenient. The Ukraine Holstein is the main breed of dairy cow in Ukraine, but studies of Ukraine Holstein mammary epithelial cells，or isolation of mammary epithelial cells from milk, have not yet been reported. With the rapid development of the dairy industry in Ukraine, mechanisms and/or factors that might affect milk synthesis and quality have garnered much attention. This part study aimed to establish a culture system and elucidate the unique characteristics of bovine mammary epithelial cells (BMECs) from the milk of Ukraine Holstein dairy cows in order to develop a general in vitro model. A primary objective of the current study was to isolate and establish an in vitro BMEC culture from milk and to

thoroughly characterize the BMECs via morphology, immunocytochemistry (ICC), and reverse transcription-polymerase chain reaction (RT-PCR) analysis. A culture system in which BMECs differentiate was developed to investigate the potential of BMECs for use as a model to study MEC function in this breed of cattle. The results were shown as following:

3.3.1 Establishment of BMECs

The whole growth process is different when culturing epithelial cells from tissue. The milk was obtained from several Ukrainian dairy cows in middle lactation (150-250 days after parturition). The nipples were scrubbed with sterile water, and the milk was squeezed by hand. Pellet in the milk were collected by centrifugation. And the culture dishes were incubated with 90% DMEM/F12 and 10% FBS at 37°C under 5% CO_2. The different culture stages are shown in Fig. 3.3.1, not including tissue culture. Single adherent cells appear on the culture dish after culturing for 3-4 days (Fig. 3.3.1A). Bovine mammary epithelial cells formed islands when cultured at low density after 5-7 days (Fig. 3.3.1B). Primary epithelial cells were elongated after culturing for 8-10 days (Fig. 3.3.1C). Mammary epithelial cells developed into different shapes, including oval, typical cobblestone, and irregular polygon. Most of the isolated cells that extended from the milk had an irregular polygon shape (Figs. 3.3.1D and E). The isolated cells had the morphological characteristics of mammary epithelial cells. Moreover, after multiple subcultures, the morphology of the cells has not been change. At the same time, the morphological changes of cells were observed by cryopreservation and resuscitation of cells. The cells obtained after freezing and thawing maintained normal morphology and growth characteristics (Fig. 3.3.1F). Thence, in the selection of frozen storage medium, the frozen solution containing 90% FBS and 10% DMSO was more conducive to the survival of cryopreserved BMECs than DMEM/F12: FBS: DMSO= 5:4:1. For subsequent research, the culture medium FBS containing 10% DMSO was used for preservation of frozen cells.

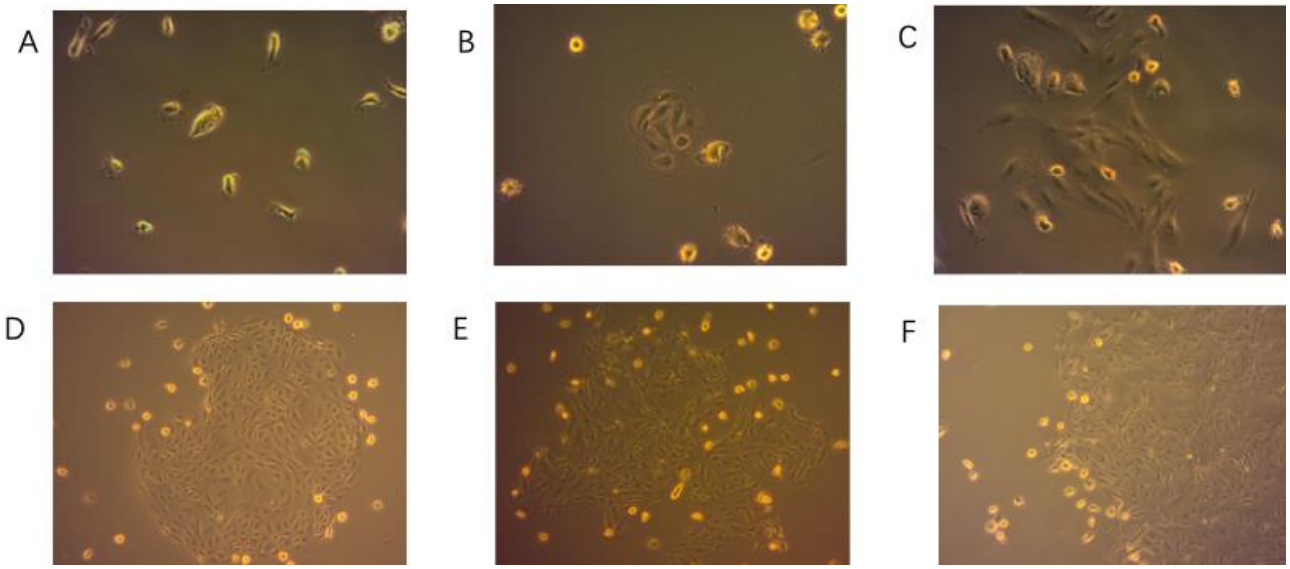


Fig. 3.3.1 Morphology of bovine mammary epithelial cells (BMECs). **A**: Cells from milk pellets on the 3rd day (×100), **B**: BMECs form islands when grown at low density between 5 and 7 days (×100), **C**: BMECs cultured between 8 and 10 days (×100), **D**: BMECs had typical cobblestone morphology between 13 and 14 days (×100), **E**: BMECs with irregular polygon shape (×100), and **F**: Morphology of resuscitated cells (×100).

3.3.2 Growth characteristics of BMECs

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 BMECs was 1 × 104 cells (Fig. 3.3.2). The BMECs culture slowly grew within the first 3 days and cells entered the stable phase in the best seeding density.

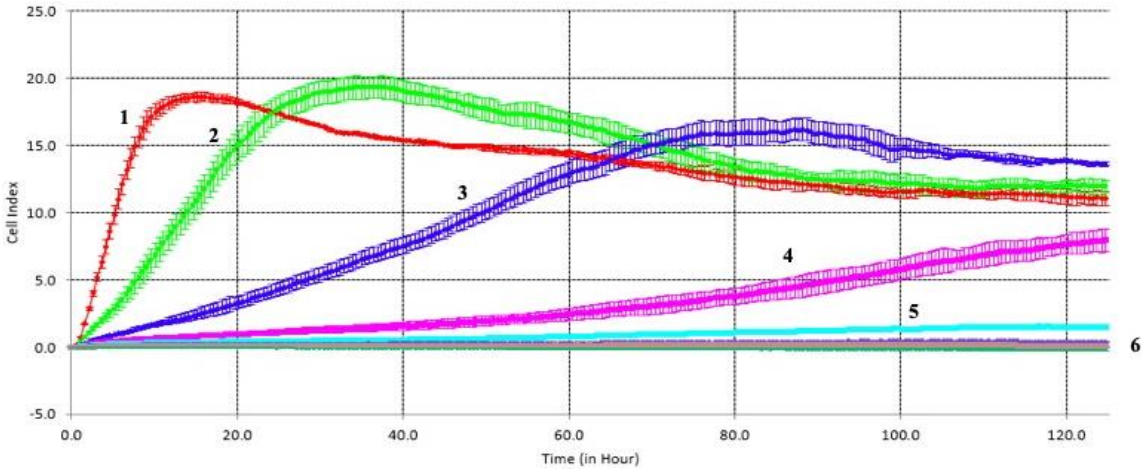


Fig. 3.3.2 Growth curves of bovine mammary epithelial cells (BMECs)

with different seeding densities.

Cells were plated in an E-plate at seeding densities of 4×10^4 cells (**1**), 2×10^4 cells (**2**), 1×10^4 cells (**3**), 5000 cells (**4**), 2500 cells (**5**), and 1250 cells (**6**).

3.3.3 Identification of cytokeratin 18 protein expression

Although the established cells appeared to have epithelial cell morphology, protein expression of cytokeratin 18, which is specific for epithelial cells, was examined. Cells exhibited strong positive staining for cytokeratin 18, indicating that the cultured cells possessed the properties of epithelial cells (Figs. 3.3.3A-C).

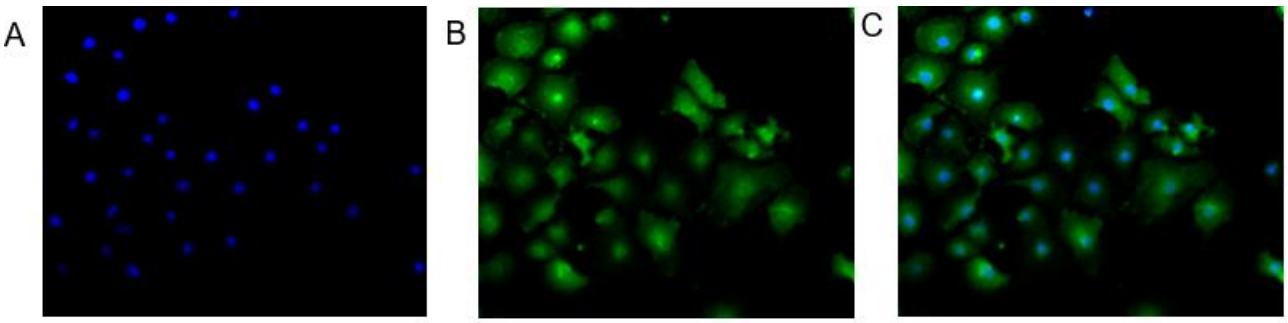


Fig. 3.3.3 Immunofluorescence identification of the isolated bovine mammary epithelial cells (BMECs) (×100).

A: 4,6-diamino-2-phenyl indole (DAPI) staining of nucleus,

B: 4,6-diamino-2-phenyl indole (FITC) staining of cytoplasm, and **C**: DAPI and FITC staining of BMECs.

3.3.4 Chromosomal analysis of BMECs

Chromosomal analysis demonstrated a non-transfromed normal mammary epithelial cell lineage (Figure.3.3.4). The isolated primary epithelial cells had a normal diploid configuration containing 60 chromosomes.

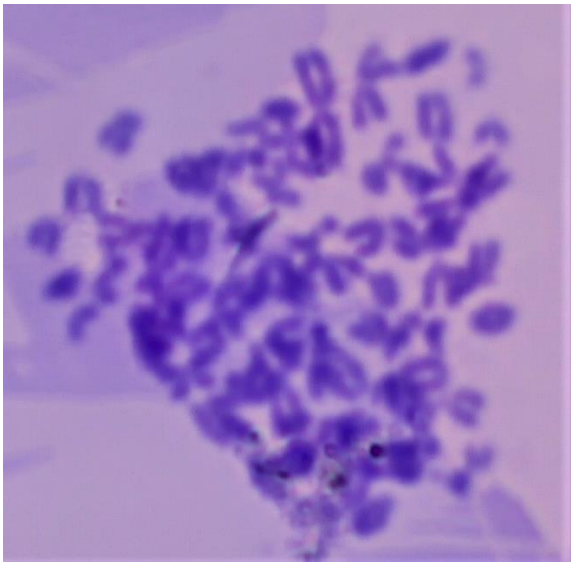


Fig. 3.3.4 Chromosome of the obtained bovine mammary epithelial cells.

3.3.5 RT-PCR analysis of BMECs

It is very important that MECs express milk proteins and can mimic the in vivo system. Total RNA was isolated from mammary tissue, MECs cultured in induction media, MECs cultured in basal media, and MAC-T cells. Reverse transcription-polymerase chain reaction was used to determine the mRNA expression of β-casein (CSN2) gene and the butyrophilin subfamily 1 member *A1* (BTN1A1) gene (Fig. 3.3.5). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the reference gene. The result showed that BMECs cultured in induction medium could expression CSN2 and BTN1A1 gene. The results confirmed the ability of the isolated cells to synthesize milk proteins.

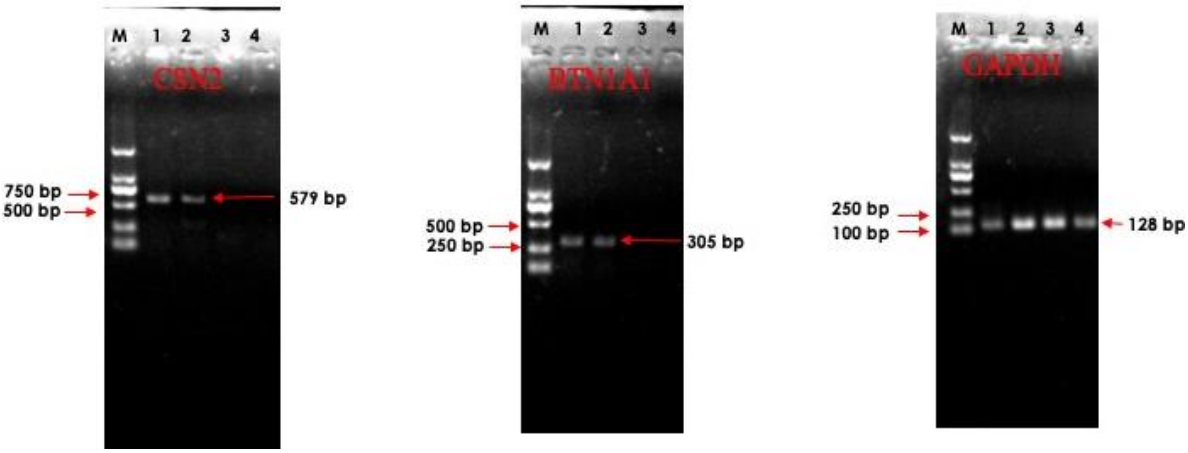


Fig. 3.3.5 Reverse transcription-polymerase chain reaction (RT-PCR)

analysis for *CSN2* and *BTN1A1* in bovine mammary epithelial cells (BMECs).

CSN2 is the β-casein gene, *BTN1A1* is the butyrophilin subfamily 1-member *A1* gene, and *GAPDH* was used as reference gene. M: DL2000 marker. Lane 1: Mammary tissues (the positive control), Lane 2: BMECs cultured in induction medium (100 ng/ml insulin like growth factor (IGF) + 10 ng/ml epithelial growth factor (EGF)), Lane 3: BMECs cultured in basal medium, and 4: Bovine mammary alveolar cell-T (MAC-T) cultured in basal medium.

3.3.6 WB analysis of BMECs

Not only at the molecular level, we also examined the lactation function of isolated cells at the protein level. WB was used to determine the milk proteins expression (Fig. 3.3.6). The result showed that BMECs cultured in induction medium could expression β-casein protein. The results also confirmed the ability of the isolated cells to synthesize milk proteins.

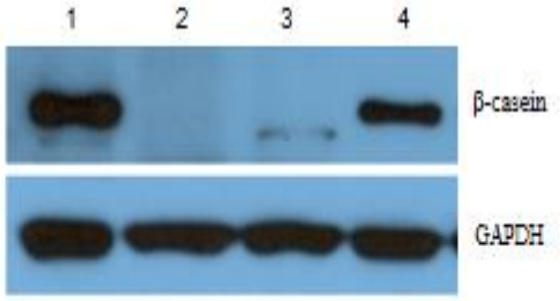


Fig. 3.3.6 Western blot analysis of β-casein in bovine mammary epithelial cells (BMECs).

1: Mammary tissues (the positive control), 2: Bovine mammary alveolar cell-T (MAC-T) cultured in basal medium, 3: BMECs cultured in basal medium, and 4: BMECs cultured in induction medium (100 ng/ml insulin like growth factor (IGF) + 10 ng/ml epithelial growth factor (EGF)).

In this part experiment, the results showed that BMECs can be recovered from milk, grown in culture, and exhibit the characteristic cobblestone morphology of

epithelial cells. The established BMECs retained MEC characteristics and secreted β-caseins even when grew on plastic substratum. Thus, the established cell line had normal morphology, growth characteristics, as well as secretory characteristics, and it could be considered as a model system and useful tool for understanding the biology of dairy cow mammary glands.

3.4 Establishment of inflammatory model of bovine mammary epithelial cells induced by lipoteichoic acid

Lipoteichoic acid (LTA) is the main component of *S. aureus* cell wall and the key cytotoxic factor causing inflammation. LTA has been shown to be an important pattern for immune recognition of *S. aureus*. One of the advantages of LTA as a tool to model inflammation, it is a defined bacterial PAMP which targets identified pattern recognition receptors (PRR) and increasingly defined accessory molecules for recognition and for the signaling cascade. LTA signals through toll like receptor 2 (TLR2) in the bovine mammary gland by bMEC. *S. aureus* by the mammary gland is not as well as known the recognition of *Escherichia* coli, another major pathogen for the mammary gland. The counterpart of *E.* coli outer membrane lipopolysaccharide (LPS) has been established, as a proinflammatory bacterial agonist of the mammary gland innate immune system. But, LTA as a proinflammatory bacterial agonist has not yet been established for *S.* aureus. In this study, we used a purified commercial preparation of *S.* aureus LTA to determine whether the bovine mammary gland responds to LTA, to determine the dose-response effects, and to begin to characterize the induced inflammatory response. The BMEC viability induced by LTA were evaluated by cell counting kit-8 (CCK-8) assay and real-time cell Assay (RTAC). The expressions of pro-inflammatory cytokines (TNF-α and IL-6) were measured by enzyme-linked immunosorbent assay (ELISA) and Real-time quantitative polymerase chain reaction (RT-qPCR). The establishment of the model will play an important role in

the screening of anti-inflammatory drugs and the study of the mechanism of action in the future. The results were shown as following:

3.4.1 Extraction and purification of total RNA

Sample RNA were extracted according to the instructions of the RNA extraction kit. The results showed that the bands were bright and intact and could be used for subsequent experiments.

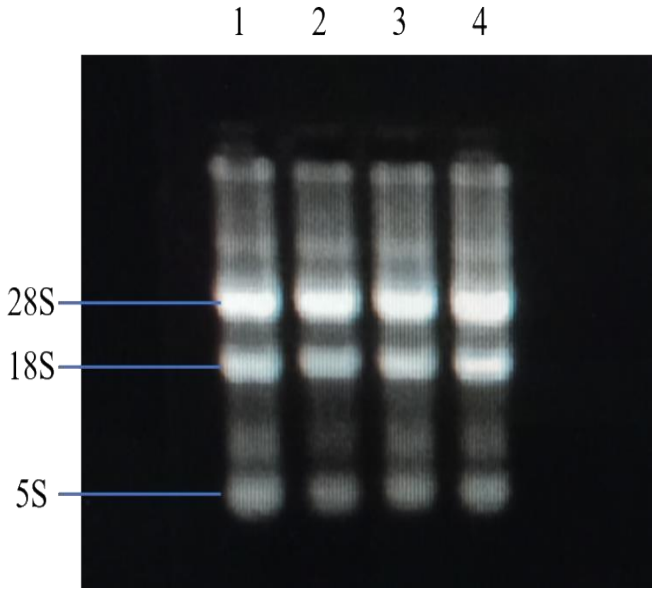


Fig. 3.4.1 The purity detection of total RNA. Note: 1~4: total RNA of samples.

3.4.2 CCK-8 and RTCA assay for cell viability

CCK-8 assay carried out to examine the viability of cells. The viability of BMECs infected with LTA were lower than that of the normal BMECs (Figure 3.4.2).

RTAC was used to detect the effect of different concentrations of LTA on the proliferation of BMECs, the results were shown in Figure 3.4.3.

With the increase of LTA concentration, the proliferation activity of BMEC cells was inhibited.

According to the change of cell index value and different proliferation curves, the dynamic detection after LTA treatment of cells was found.

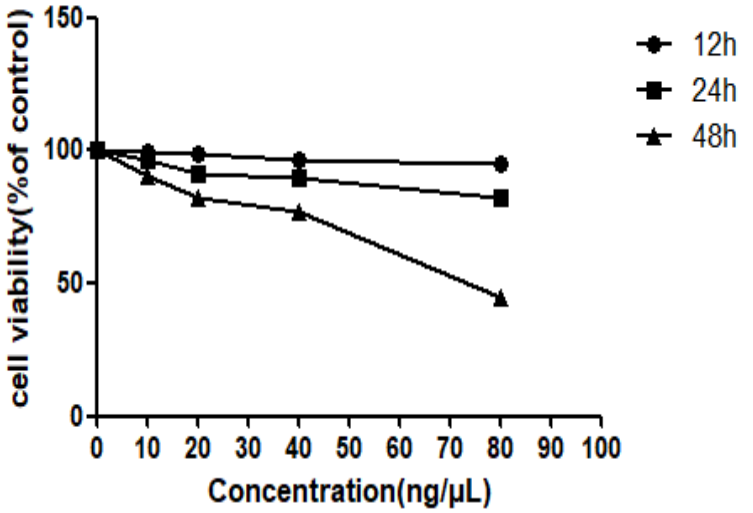


Fig. 3.4.2 The cell viability of LTA in BMEC

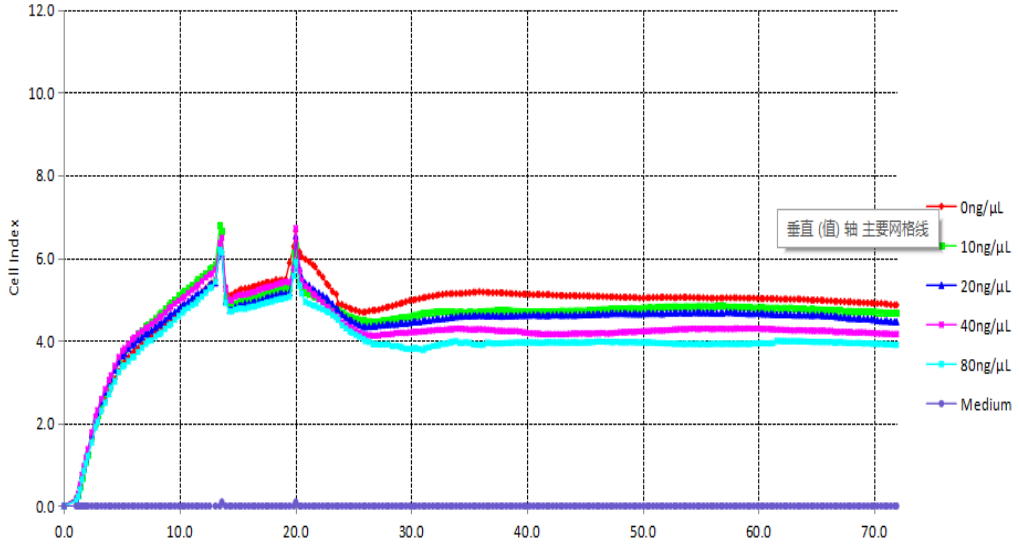


Fig. 3.4.3 Effect of LTA on the BMEC proliferation

3.4.3 Secretion of inflammatory cytokines by BMEC

It shown that the basal expression of TNF-α and IL-6 protein in the culture supernatant of BMECs in the blank control group was low (Figure 3.4.4). BMECs were stimulated by different mass concentrations of LTA for different time, the expression of TNF-α and IL-6 protein Significantly increased. When different mass concentrations of LTA acted on cells for 12h, 24h, and 48h, the expression of TNF-α and IL-6 protein reached its peak when the mass concentration of LTA was 20 ng/μL. At the LTA mass concentration was 40 and 80 ng/μL, the expression of TNF-α and IL-6 protein was lower than that at 20 ng/μL, but they were still

significantly higher than the control group.

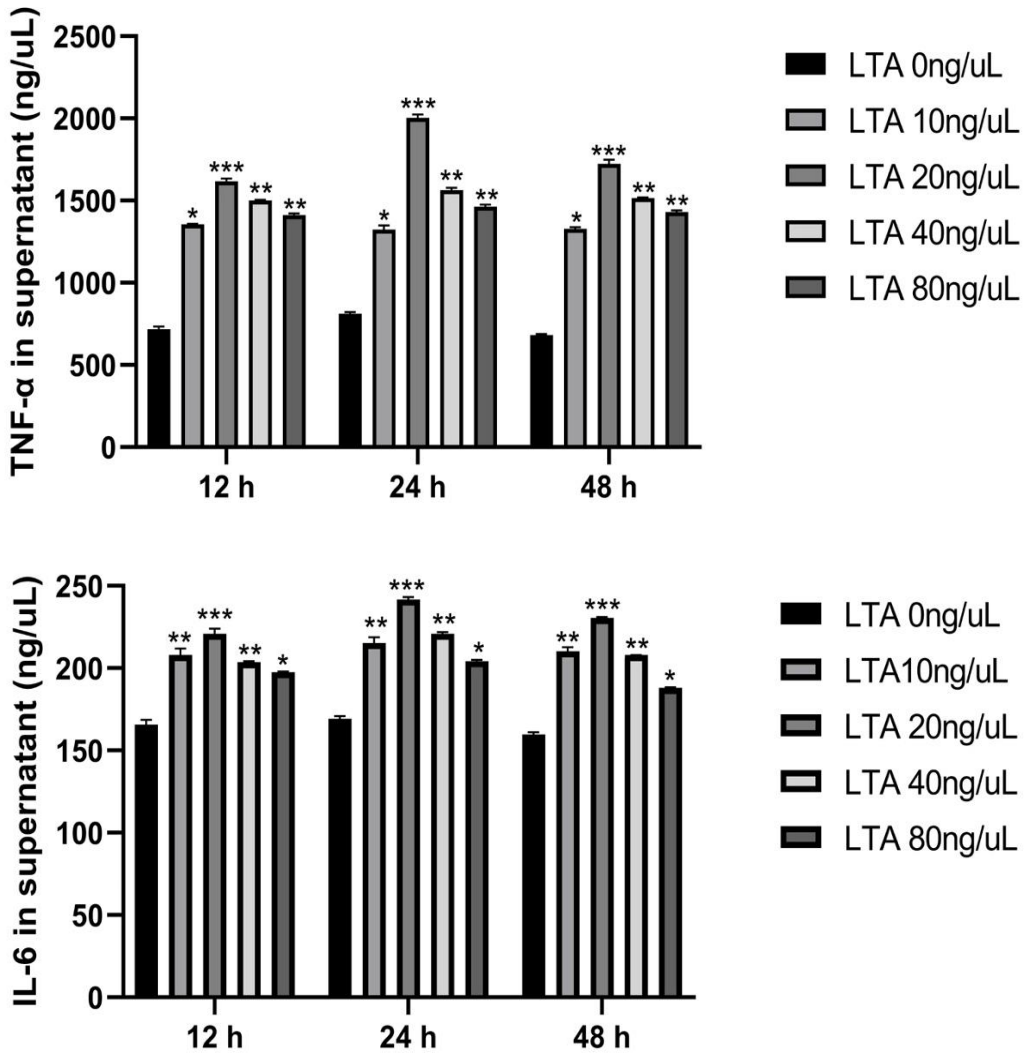


Fig. 3.4.4 Effect of LTA on the BMEC TNF-α and IL6 production.
Note: The values presented are the mean ± SE of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$vs. LTA 0 ng/uL group

3.4.4 Expression of inammatory cytokines by BMEC

It shown that the TNF-α and IL6 mRNA expression in the culture supernatant of BMECs in the blank control group was low (Figure 3.4.5). As the BMECs were stimulated by different mass concentrations of LTA for different time, the mRNA expression of TNF-α and IL-6 increased significantly. The LTA acted on the cells for 12h and 48h, the mRNA expression of IL-6 did not change significantly with the increase in mass concentration. However, the mRNA expression of TNF-α changes significantly. The mRNA expression of TNF-α and IL6 reached the maximum,when

the LTA concentration was 20 ng/μL with different times later. It can be seen that the LTA of 20 ng/μL treating BMECs for 24 h can induce a significant cellular immune response in BMECs.

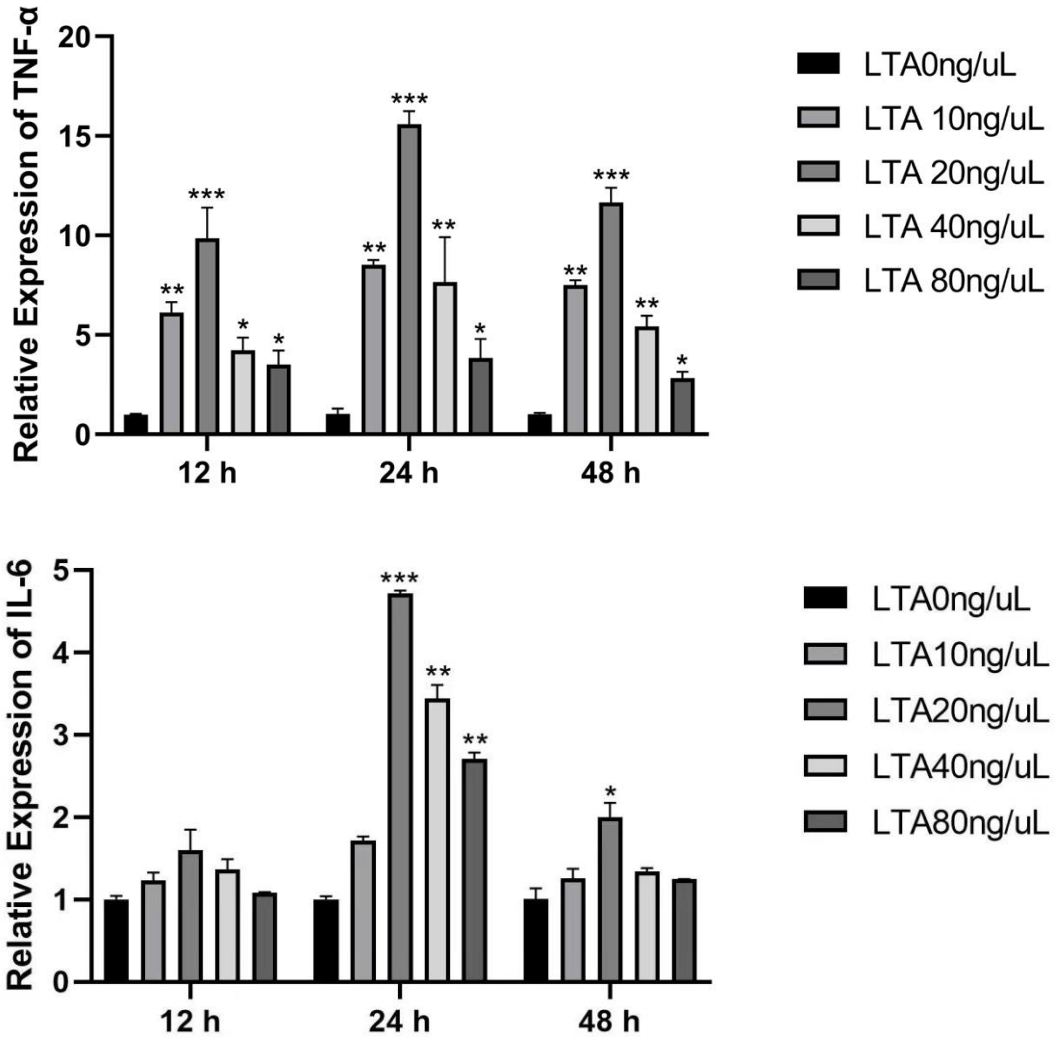


Fig. 3.4.5 Effect of LTA on the BMEC TNF-α and IL6 gene expression.

Note: The values presented are the mean ± SE of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$vs. LTA 0 ng/uL group

In this part experiment, the results showed that the treatment of BMECs with LTA at 20 ng/μL for 24 h obviously improved TNF-α and IL-6 protein and gene expression levels. The establishment of the model will play an important role in the screening of anti-inflammatory drugs and the study of the mechanism of action in the future.

3.5 Anti-Inflammatory Effects of Chlorogenic Acid from *Taraxacum officinale* on LTA-Stimulated Bovine Mammary Epithelial Cells via the TLR2/NF-κB Pathway

Mastitis is an inflammatory disease caused by microbial infection. The prevention and treatment of mastitis caused by *S.* aureus is very difficult, because *S.* aureus can evade the host immune system's attack, and has the ability to hide in the host cells, making it useless for antibacterial drugs. Lipoteichoic acid (LTA) is the main component of the cell wall of Gram-positive bacteria. Recent studies have shown that in addition to assisting *S.* aureus adhesion and colonization, LTA is also involved in stimulating inflammatory responses in host cells. Therefore, LTA is considered to play an important role in the pathogenesis of *S.* aureus. Chlorogenic acid (CGA), one of the major phenolic acids in *Taraxacum officinale*, has natural antioxidant, anti-inflammatory, antibacterial, and anti-viral effects in several studies. A previous study demonstrated that CGA alleviated the inflammatory response induced by *E.* coli in sheep endometrial epithelium cells via inhibiting activation of the TLR4/NF-κB signaling pathway.

Furthermore, CGA was found to be a potential therapeutic compound for LPS-induced bovine mastitis, via reduction of NF-κB. however, the effects of CGA on Lipoteichoic acid (LTA)-induced bovine mammary epithelial cells (BMECs) have not been investigated. In this study, the CGA content in *T. officinale* was determined by High-performance liquid chromatography (HPLC). BMECs were infected with LTA to induce the mastitis model. Different concentrations of CGA were administered after establishing the LTA infection.

Cell viability was determined by Real-Time Cell Assay (RTCA) and Cell counting kit-8 (CCK-8). Pro-inflammatory cytokine tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) levels were determined by enzyme-linked immunosorbent assay (ELISA) and quantitative real-time polymerase chain reaction (qRT-PCR). Nitric oxide (NO) production was measured

using a reagent kit. Toll-like receptor 2 (TLR2) expression and phosphorylation of related proteins in nuclear factor-kappa B (NF-κB) signaling pathways were detected by western blot. The results were shown as following:

3.5.1 Extraction and purification of total RNA

Sample RNA were extracted according to the instructions of the RNA extraction kit. The results showed that the bands were bright and intact and could be used for subsequent experiments.

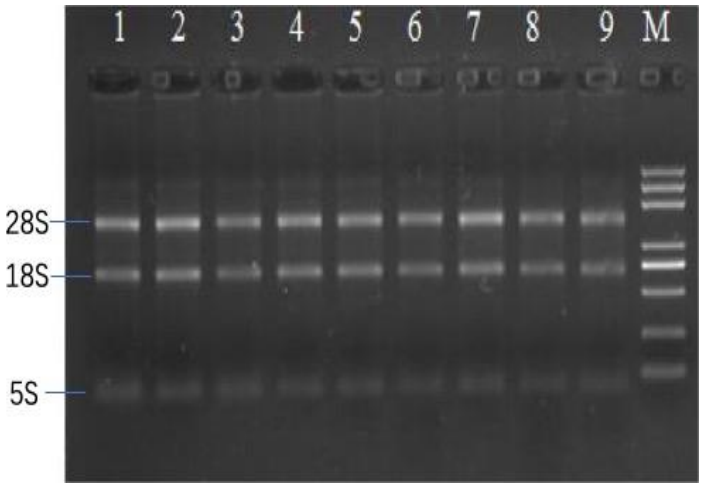


Fig. 3.5.1 The purity detection of total RNA. Note: M: Trans 2K Plus; 1~9: total RNA of samples.

3.5.2 CCK-8 and RTCA assay for cell viability

In the CCK-8 assay using BMEC cells, different concentrations of CGA extract were prepared for the cytotoxicity evaluation. As calculated by Statistical Product and Graphpad Prism 5, the IC_{50} value was 320.4 μg/mL (Figure 3.5.2). Establishment of an inflammation model by treating BMECs with 20 ng/mL LTA for 24 hours. As shown in Figure 3.5.3, the results showed that CGA extract (25, 50, and 100 μg/mL) had no cytotoxic effects on BMECs. LTA has an inhibitory effect on cell viability compared to NC, and CGA could abrogate LTA-induced decrease of cell viability at the dose of 25, 50, and 100 μg/mL.

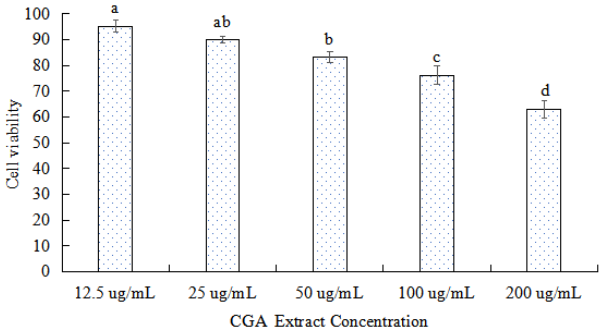


Fig. 3.5.2 Cell viability of BMEC induced by CGA extract after 24 h by CCK-8 assay.

Note: Different lowercase letters indicate significant differences at $P<0.05$.

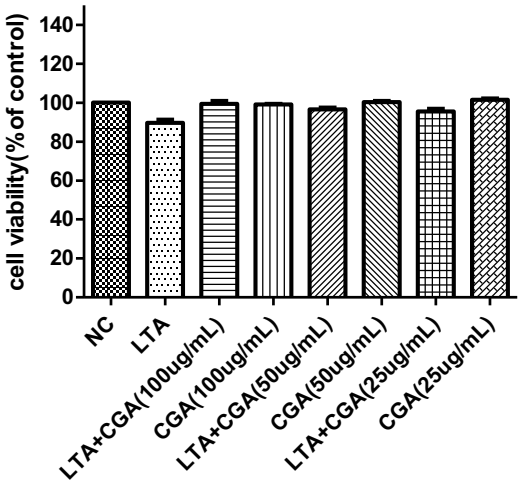


Fig. 3.5.3. The effect of chlorogenic acid (CGA) on the cell viability of bovine mammary epithelial cells (BMECs).

The values presented are the mean ± SE of three independent experiments. NC: normal control; LTA: lipoteichoic acid; LTA+CGA: lipoteichoic acid and chlorogenic acid.

In RTCA, CGA extract of diffffferent concentrations at 12.5, 25, 50, 100, and 200 μg/mL were chosen for recording the cell growth curves, and the IC_{50} value of 326.8 μg/mL was obtained, which is close to the result of the CCK-8 assay. The cells grew exponentially before the serum-free synchronization treatment, and that the growth rate of the cells was slower after the synchronization treatment, suggesting that the goal of synchronization treatment was achieved. The viability of BMECs decreased significantly with increasing CGA concentration, and at a concentration of 100 ug/mL, the cell index was only 3 (Figure 3.5.4).

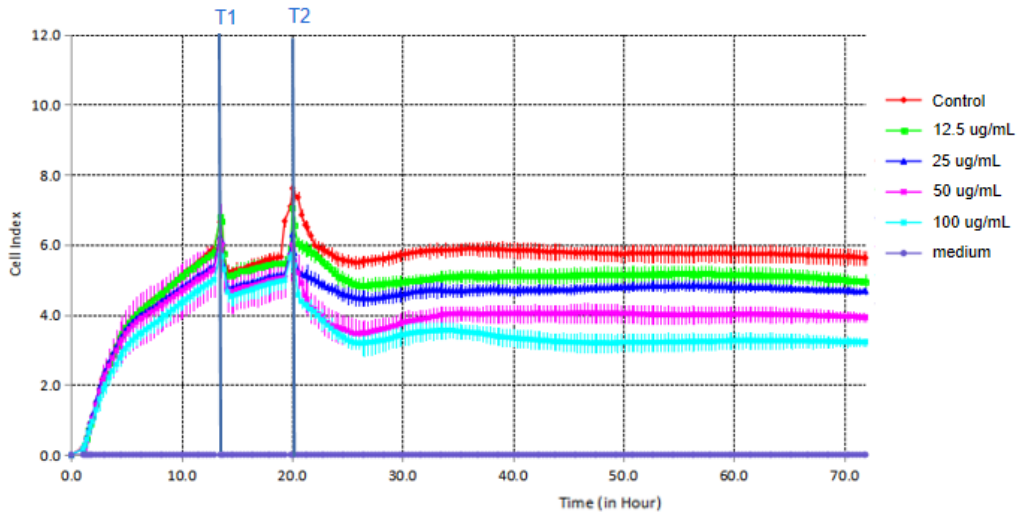


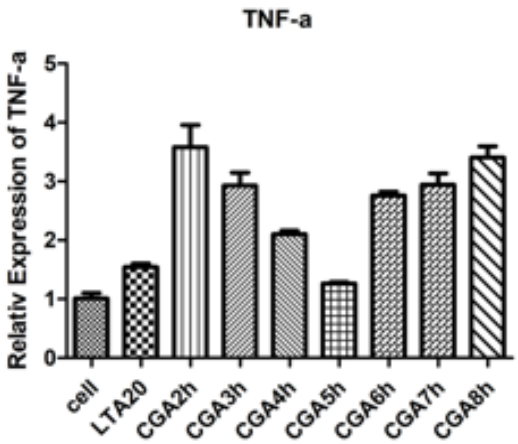
Fig. 3.5.4 Cell growth curve of bovine mammary epithelial cells (BMECs) induced by chlorogenic acid (CGA) extract in the Real-Time Cell Assay (RTCA).

Note: The T1-T2 time period represents the length of synchronization.

3.5.3 ELISA to detect the best time of CGA extract pretreatment BMEC

In order to detect the best time of CGA extract pretreatment BMEC, the expression of inflammatory factors in cell supernatants was measured by ELISA. The results showed that with the CGA extract pre-treat 5 h, IL-1β, IL-6, and TNF-α have the lowest expression.

Therefore, in the subsequent studies, the pretreatment time of CGA extract was selected to be 5 hours.



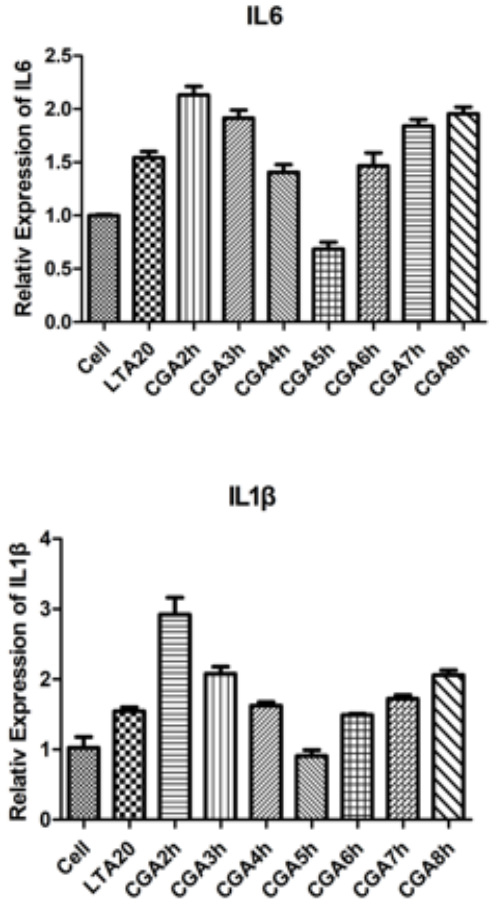


Fig. 3.5.5 The best time of CGA extract pretreatment BMEC

3.5.4 CCK-8 assay for BMEC cell viability with LTA infection

The cytotoxicity of CGA extract on cell viability was evaluated by CCK-8 assay after incubating BMEC for 24 h. As shown in Figure 3.5.6, the results showed that CGA extract (25, 50, 100 μg/mL) has no cytotoxic effects on BMEC. Therefore, in the subsequent studies, the doses of CGA extract were chosen as 25, 50, and 100 μg/mL.

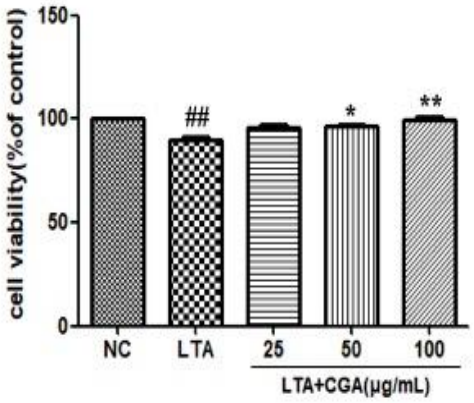


Fig. 3.5.6 Viability of BMEC after different CGA extract concentrations with LTA (20 ng/μL).

Note: The values presented are the mean ± SE of three independent experiments. ## $p < 0.01$ vs. control group; * $p < 0.05$, ** $p < 0.01$ vs. LTA group

3.5.5 Effects of CGA Extract from *Taraxacum Officinale* on Inflammatory Cytokines

TNF-α, IL-6, and IL-1β are important inflammatory mediators involved in the inflammatory response. To investigate the anti-inflammatory effects of CGA, the levels of pro-inflammatory cytokines were detected by qRT-PCR, and the expression of inflammatory factors in cell supernatants was measured by ELISA. As shown in Figures 3.5.7 and 3.5.8, LTA significantly upregulated TNF-α, IL-6, and IL-1β gene and protein expression compared to control. CGA increased TNF-α, IL-6, and IL-1β gene and protein expression. However, CGA suppressed TNF-α, IL-1β, and IL-6 gene and protein expression in LTA-stimulated BMECs in a concentration dependent manner.

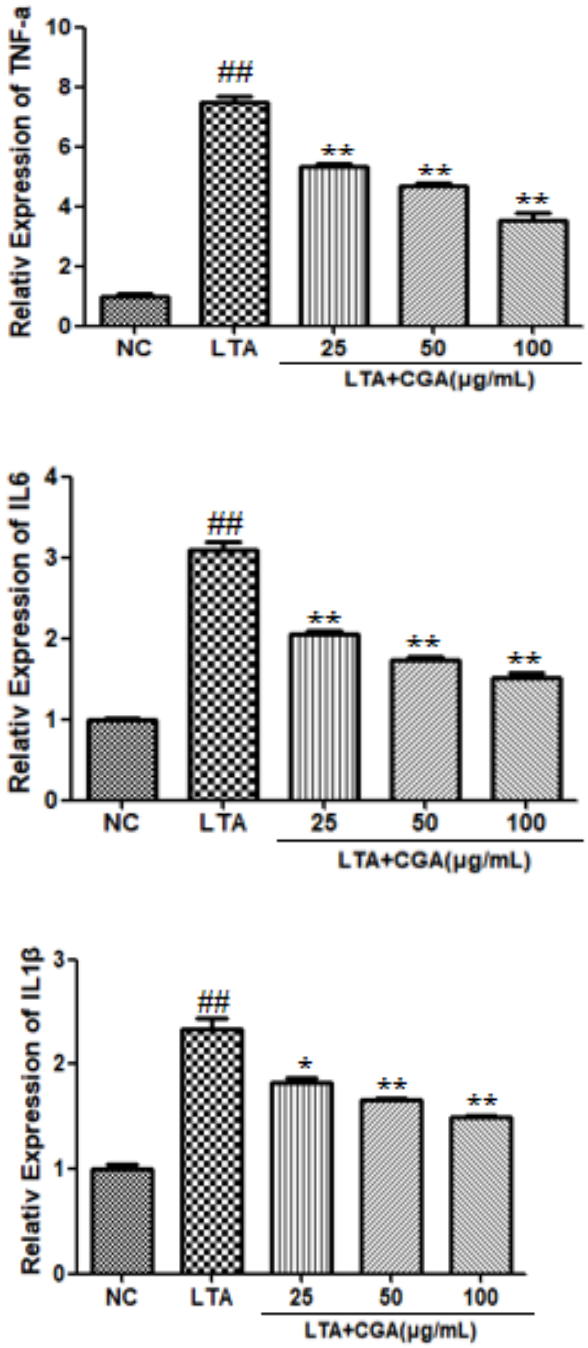


Fig. 3.5.7 Effect of chlorogenic acid (CGA) on lipoteichoic acid (LTA)-induced gene expression of inflammatory cytokines.

Note: The values presented are the mean ± SE of three independent experiments. ## $p < 0.01$ vs. control group; * $p < 0.05$, ** $p < 0.01$ vs. LTA group. NC: normal control; LTA: lipoteichoic acid; LTA+CGA: lipoteichoic acid and chlorogenic acid.

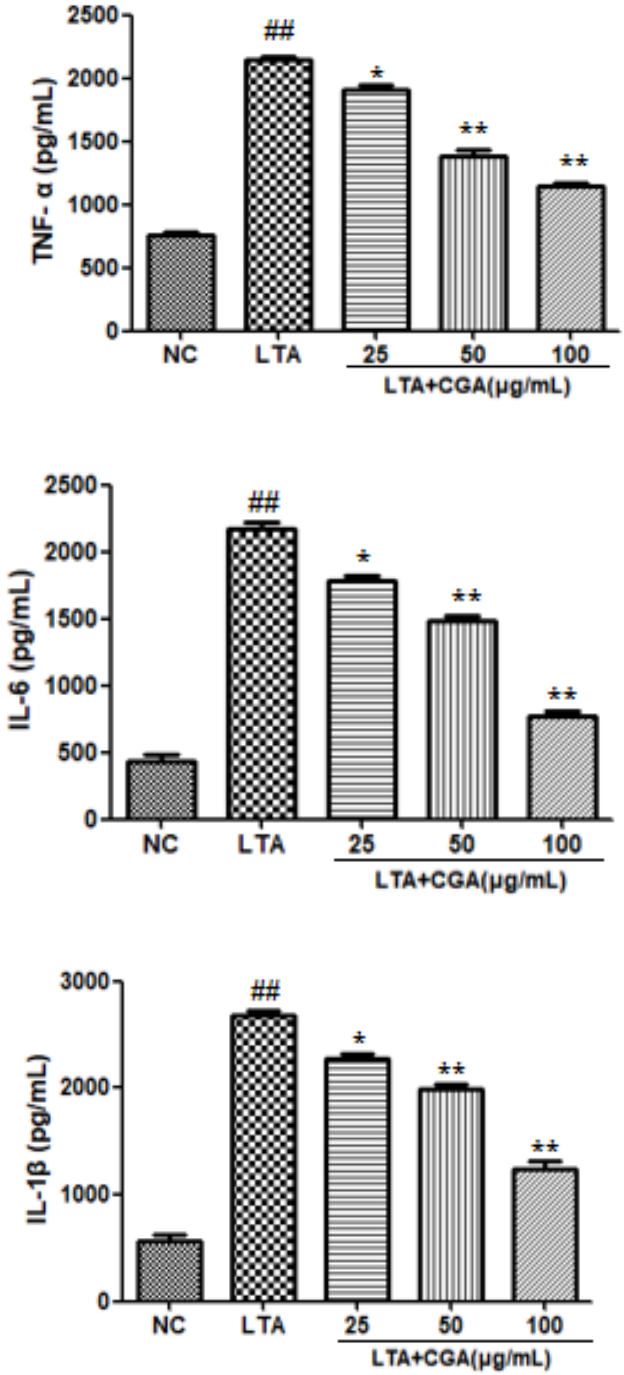


Fig. 3.5.8 Effect of chlorogenic acid (CGA) on lipoteichoic acid (LTA)-induced protein expression of inflammatory cytokines.

Note: The values presented are the mean ± SE of three independent experiments. ## $p < 0.01$ vs. control group; * $p < 0.05$, ** $p < 0.01$ vs. LTA group. NC: normal control; LTA: lipoteichoic acid; LTA+CGA: lipoteichoic acid and chlorogenic acid.

3.5.6 Effects of CGA Extract on LTA-Induced NO Production

The persistent expression of iNOS could lead to the production of NO. In this study, we measured the effects of CGA on the NO production induced by LTA (Figure 3.5.9). The results showed that stimulation of BMEC with LTA significantly increased that the production of NO. However, the increases of NO production was concentration -dependently suppressed by CGA extract.

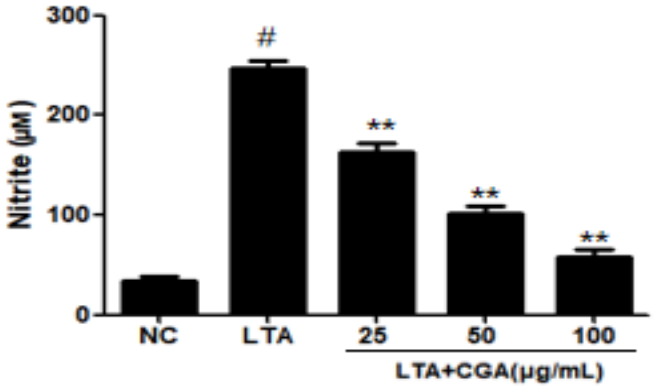


Figure 3.5.9 Effect of chlorogenic acid (CGA) on Nitric oxide (NO) production. Note: The values presented are the mean ± SE of three independent experiments. # $p < 0.05$ vs. control group; ** $p < 0.01$ vs. LTA group. NC: normal control; LTA: lipoteichoic acid; LTA+CGA: lipoteichoic acid and chlorogenic acid.

3.5.7 Effects of CGA Extract on LTA-Induced TLR2 Expression

The anti-inflammatory mechanism of CGA was further studied by detecting the expression of TLR2 by Western blot. The results showed that the expression of TLR2 was significantly up-regulated with LTA treatment. Meanwhile, pretreatment with CGA significantly inhibited LTA-induced TLR2 expression by a dose-dependent manner (Figure 3.5.10).

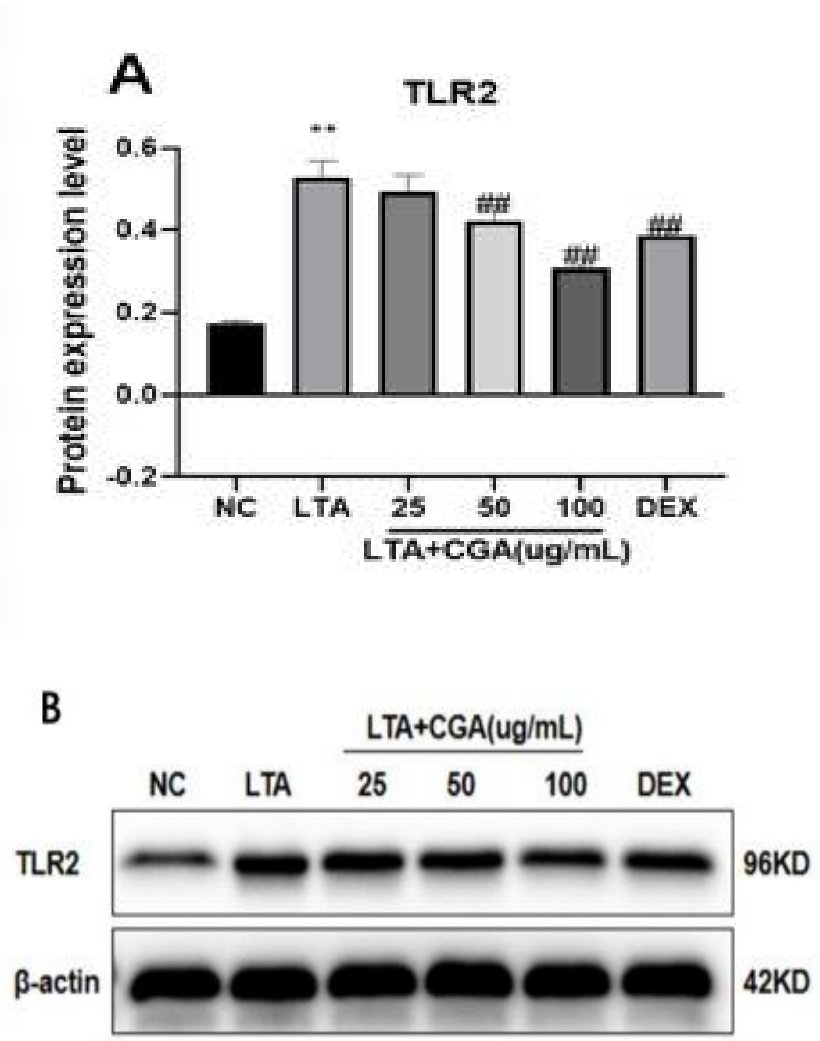
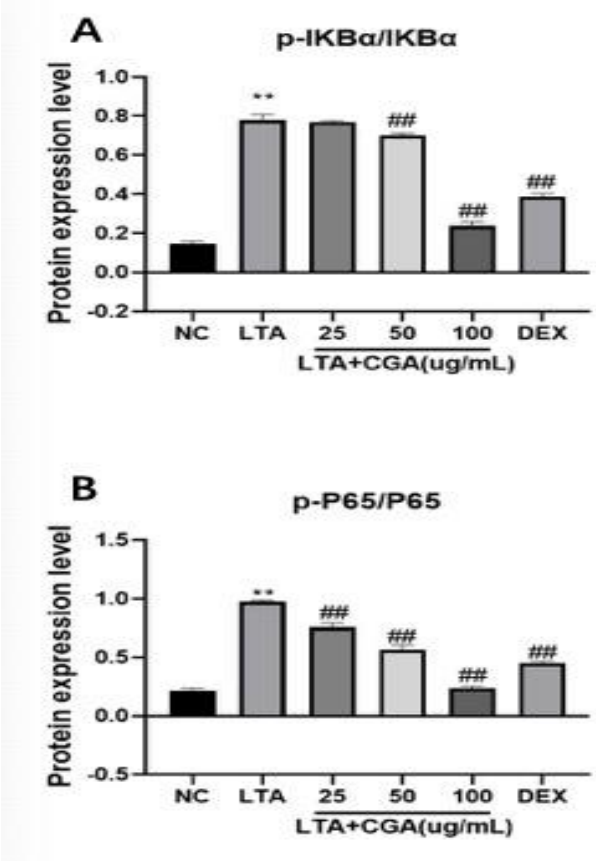


Fig. 3.5.10 Effect of chlorogenic acid (CGA) extract on toll-like receptor 2 (TLR2) expression. The expression of TLR2 in BMECs was analyzed via Western blot. β-actin was used as a reference control. Dexamethasone (DEX) was used as a positive control. (A) The quantification histogram of TLR2 protein expression normalized by β-actin.

Note: The values presented are the mean ± SE of three independent experiments. ** $p < 0.01$ vs. control group; ## $p < 0.01$ vs. LTA group. (B) The expression levels TLR2. Original blots are presented in Supplementary Figure 1,2. NC: normal control; LTA: lipoteichoic acid; LTA+CGA: lipoteichoic acid and chlorogenic acid; DEX: Dexamethasone.

3.5.8 Effects of CGA Extract from *Taraxacum Officinale* on NF-κB pathway

Whether NF-κB is involved in the mechanism of CGA regulating inflammatory response was investigated by assessing the phosphorylation status of IkBα and p65. The results showed that the NF-κB signaling pathway was significantly up-regulated after LTA treatment. However, with CGA pretreatment, the phosphorylation of IkBα and p65 in LTA-stimulated BMECs (Figure 3.5.11) was significantly inhibited. Meanwhile, the results also indicated that CGA inhibited LTA-induced NF-κB activation in a dose-dependent manner.



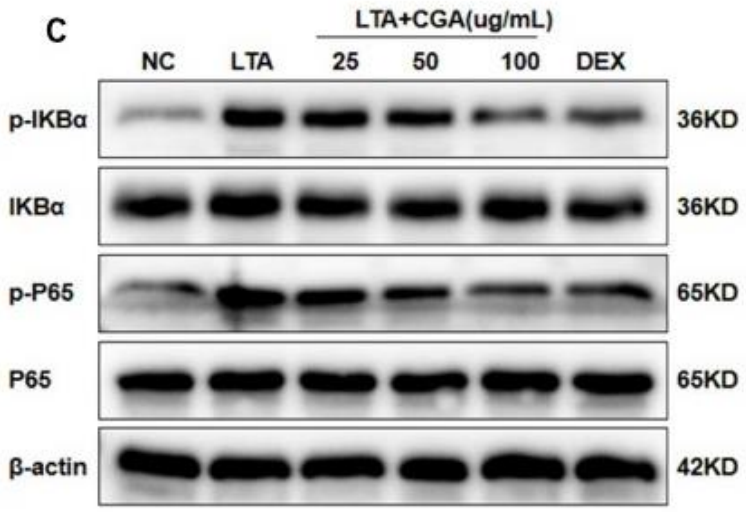


Fig. 3.5.11 Effect of chlorogenic acid (CGA) on nuclear factor-κB (NF-κB) activation. The NF-κB pathway was analyzed in BMECs via Western blot. β-actin was used as a reference control. Dexamethasone (DEX) was used as a positive control. (A) The quantification histogram of p-IκBα protein expression normalized by β-actin; and (B) the quantification histogram of p-p65 protein expression normalized by β-actin. Note: The values presented are the mean ± SE of three independent experiments. **p < 0.01 vs. control group; ##p < 0.01 vs. LTA group. (C) The expression levels of p-IκBα, IκBα, p-p65, and p65. Original blots are presented in Supplementary Figure 3,4,5,6,7. NC: normal control; LTA: lipoteichoic acid; LTA+CGA: lipoteichoic acid and chlorogenic acid; DEX: Dexamethasone. In this part experiment, the results showed that CGA significantly reduced the pro-inflammatory gene and protein expression of TNF-α, IL-6, and IL-1β. In addition, CGA downregulated the NO, TLR2, and NF-κB signaling pathways in LTA-infected bovine mammary epithelial cells. Our results indicate that CGA reduced the expression of TNF-α, IL-6, IL-1β, and TLR2 by inhibiting the phosphorylation of proteins in the NF-κB signaling pathways in a dose-dependent manner. This finding suggests that CGA could be used for the prevention or management of mastitis in dairy cows.

CHAPTER 4

SUMMARY AND ANALYSIS OF RESULTS

Worldwidely, there is an increasing effort to investigate the antibacterial efficacy of traditional medicinal plants. Dandelion root has been reported to possess antibacterial activity which is linked to the presence of phenolic based compounds in it [19, 54]. In spite of this, very few evidences are available which can clearly identify the specific phenolic compounds responsible for the antibacterial activity within the extract of dandelion. In order to better evaluate the antibacterial effect of the dandelion extract, not only the comption but also the contents of the phenolic extract need to elucidate. This study determined the contents of chlorogenic acid, caffeic acid, rutin, luteolin and ferulic acid in the phenolic extract of the whole dandelion plant by HPLC, which were 1.34 mg/g, 0.21 mg/g, 0.19 mg/g, 1.08 mg/g, and 0.22 mg/g, respectively. Compared with Kenny et al [57] results, luteolin and rutin were higher while others like ferulic acid, chlorogenic acid and caffeic acid were lowered than dandelion roots. It is speculated that organic acids are unstable or transported to the roots in the later stage of leaf growth, while flavonoids components are synthesized and stored in the leaves.

Solvent polarity has a huge influence on the biological activity of extract. Previous study showed that methanol is most effective in extracting flavonoids and polyphenols from plants [165]. Wojdylo et al. [153] identified caffeic acid (0.726 ±0.001 mg/g) from a methanol (80% v/v) dandelion root extract by HPLC-DAD phenolic quantification analysis. Williams et al. [152] identified three caffeic acid esters from a methanolic (80% v/v) dandelion root extract, including chlorogenic acid, chicoric acid and caftaric acid. The study also highlighted the absence of flavonoid glycosides in root extracts compared to leaf, bract and flower. However, they both were unable to detect either luteolin or ferulic acid in the methanol root extract which were both detected and quantified in our study (luteolin 1.08 mg/g

and ferulic acid 0.22 mg/g). The reason was that we used the whole dandelion plant for the extraction purpose. This may be also due to the increased detection sensitivity obtained due to the difference in concentration of methanol. We optimized the concentration of methanol to 70% (v/v).

Chlorogenic acid, caffeic acid and ferulic acid belongs to the C6-C3 type, cinnamic acid type phenolic acid. Chlorogenic acid has antibacterial activity against major pathogenic bacteria, especially *S. aureus* [61]. Up till now, the antibacterial mechanism of phenolic acid compounds are not yet completely investigated, which can be roughly divided into four aspects: (1) By destroying the barrier function of the cell wall and cell membrane, increasing the permeability of the membrane, causing the leakage of microbial intracellular components and affecting the stability of cell structure [66]; (2) Through the energy metabolism system, the metabolism is blocked, causing death of the bacteria; (3) Phenolic acid compounds can combine with the genetic material of microorganisms and change the physiology of microorganisms so that cells lose the basis for growth and reproduction, thereby inhibiting their growth; (4) Phenolic acid compounds cause protein denaturation through the acidification of cytoplasm, produce antimicrobial activity and achieve antibacterial effect [76]. In our study, the changes of extracellular alkaline phosphatase (AKP) contents, electrical conductivity, intracellular protein contents, and DNA as well as the permeability of *S. aureus* cell wall and cell membrane were investigated after the action of dandelion phenolic extract. The results showed that dandelion extract increased the extracellular AKP contents of *S. aureus*, electrical conductivity and extracellular protein, and affect the normal reproduction of *S. aureus*. Cell membrane is a kind of supramolecular structure, composed of protein, peptidoglycan, etc. It is closely related to energy metabolism and information transmission [27]. The cell membrane is an effective barrier to protect the entrance of bacteria. After the bacteria interact with drugs having antibacterial activity, the cell membrane is destroyed and the protective barrier is broken, causing the macromolecular proteins

in the bacterial cytoplasm to leak into the culture solution, and the protein in the culture solution contains. Therefore, change in the protein content in the bacterial liquid reflects change in the permeability of the bacterial cell membrane. When the permeability of the cell membrane of pathogenic bacteria is destroyed, the electrolyte in the bacteria will infiltrate into the culture medium. According to the conductivity of supernatant of *S. aureus* culture medium, it can indirectly reflect the change of cell membrane permeability. Therefore, we thought that the antibacterial mechanism of dandelion phenolic extract against *S. aureus* was to destroy the barrier function of the cell wall and cell membrane.

Like other epithelial cells, MECs exfoliate and are shed into secretions. The somatic cells described in dairy manuals as part of typical milk sediment are actually leukocytes and exfoliated epithelial cells [65]. Isolating and cultivating BMECs from milk by manual milking is not only simple to do but also low cost, harmless to cows, and can be carried out in most laboratories. In this study, the milk of healthy, high-producing Ukrainian Holstein dairy cows was mixed with antibody-containing PBS at a ratio of 1:1. After centrifugation, the supernatant was discarded and the pellet was cultured from which BMECs were successfully separated. Compared with the supernatant, the pellet contained a larger number of cells and fewer impurities, such as milk fat. Previous studies have reported that BMECs can be obtained by centrifugation [11, 21, 22, 24], however, few studies reported that using antibiotics in BMECs culture could effectively reduce the pollution. Data from the present study suggest that BMECs can be obtained from milk after culturing for 10-12 days, which is earlier than with the explant culture method. Studies have shown that BMECs were elongated from the breast tissue in 8-10 days when using the tissue adherence method, though further purification with fibroblasts is required for another 25-30 days [48, 115]. Therefore, although milk can be easily contaminated when using the milk separation method [59, 176] due to factors such as recessive mastitis in cows or centrifugation defect to remove milk fat, it has the advantage of being fast. The main disadvantage of using milk as

a source of BMECs is the relatively small number of mammary cells obtained compared with explant cultures. However, in this study, the breast epithelial cells in milk were generally from the breast tissue and the cell cycles were relatively long, so the isolated primary cells grew very slowly. The 3-8 generation cells grew faster than the 1-2 generation cells, however, the cells then appeared to grow slowly again, and after forming islands in isolation, they no longer covered the whole culture dish. The cause of this phenomenon needs further investigation.

The cells were cultured overnight in DMEM/F12 medium containing 10% FBS and 10% EGF, the cells were distributed across 80% of the bottom. In previous studies, only 10% FBS were used [37, 93, 124], the cells needed to cultured over 48 h, when they were distributed across 80% of the bottom. However, the use of EGF in this study significantly increased the cell doubling time.

Dairy cow MECs are a type of adherent culture cell that is not easily digested from a culture dish. Compared with other cells, BMECs took longer to digest (usually 6-7 min). In order to achieve complete digestion during passaging, the density of the cultured cells and cell digestion time needed to be controlled. According to the RTAC analysis, the optimal seeding density was 1×10^4 cells/0.32 cm^2. At the optimal seeding density, the logarithmic growth period of the cells was longer and various experimental treatments could be performed. However, when the cell density was high, the cells entered quickly to a stationary phase without sufficient time for other treatments. When the cell density was low, there was no stable phase.

Keratins are unique to epithelial cells of mammalian species. Cytokeratin 18 is one of the most common members of the intermediate filament gene family [30, 118]. It is expressed in single layer epithelial tissues of the body and is specific for epithelial cells [7, 103, 119, 133, 138]. The purified BMECs showed positive staining to cytokeratin 18, thereby provided direct evidence of their epithelial nature.

Milk protein secretion is an important mammary-specific feature [108].

Generally, insulin, hydrocortisone, and prolactin are used in culture media to induce milk protein expression [2, 110, 132]. Casein secretion is the hallmark of BMECs [1, 4]. In the present study, variations in marker gene expression between cells cultured in basal and induction media were observed, indicating that the difference in the composition of culture media may influence functional gene expression. The expression of the CSN2, and BTN1A1 genes was determined using RT-PCR. This finding suggests that BMECs exhibited normal secretory function. This study established a method for the isolation and culture of cow MECs from the milk of Ukrainian

Inflammatory response refers to the multi-cytokine involved in the occurrence and development of inflammation by regulating the balance between pro-inflammatory factors and anti-inflammatory systems [23, 156]. TNF-α is the earliest and most important inflammatory mediator in the process of inflammation. IL6 can induce B cells to differentiate and produce antibodies, and is a promoter of inflammatory response. LTA is an important component of the cell wall of Staphylococcus aureus and can activate inflammatory cells to cause inflammation. In the process of inflammation, LTA activates macrophages through the TLR2 receptor, which leads to the production of inflammatory cytokines TNF-α and IL6. The secretion of cytokines can induce further activation of inflammatory cells, leading to excessive or uncontrolled inflammatory response, and ultimately causing inflammatory case damage to host tissues and organs [39]. Due to the immune characteristics of BMECs, specific inflammatory substances can be selected to induce BMECs to produce an immune response. The most used in the experiment is LPS and bacterial culture filtrate. However, there are few reports on the establishment of inflammation models by LTA on BMECs.In this study, LTA was used as a proinflammatory mediator. The two classic acute early cytokines, IL-6 and TNF-α, were selected as the criteria for measuring the success of the model. CCK-8, RTAC, ELISA and qRT-PCR were used to test the method, the results showed that treatment of bovine mammary epithelial cells with 20 ng/μL LTA for 24 h can

significantly increase the protein and gene expression levels of TNF-α and IL-6. The establishment of this model could play an important role in screening anti-inflammatory drugs and studying the mechanism of action in the future.

Nowadays, with the fast development and technical breakthrough in the pharmaceutical industry, there is an urgent need for the drug screening and toxicity tests. According to the analyzed signals, cell-based assays, in principle, can be classified into colorimetric assays [28,30], luminogenic assays [26], electrochemical methods [181], cell counting methods [9], and so on. Among these methods, two conventional assays are usually applied for in vitro cellular cytotoxicity evaluation because of their easy operation and standardized readout: electrochemical methods as typified by the real-time cell analysis (RTCA) and cell counting kit-8 (CCK-8) assay, respectively.

CCK-8 assay was established for the cytotoxic evaluation of drugs on the basis of dye labels, it has the remarkable sensitivity and operational convenience [75, 84]. In the CCK-8 measurement, the dye of WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4disulfophenyl)-2H-tetrazolium, monosodium salt] was reduced by dehydrogenase in cells to form a water-soluble orange-colored product (formazan). The amount of the produced formazan dye by cellular dehydrogenases is correlated with the number of living cells. Therefore, the cell viability can be simply estimated by recording the optical density (OD) of formazan at 450 nm using a microplate reader. Although CCK-8 assay as a representative end point method allows convenient colorimetric readout, it limits in that each measurement can be conducted at a single time point.

Recently, RTCA holds promising potential in cellular assays because of the advantages of recording signals in a dynamic and label-free mode. In a typical run, upon cells' incubation on the arrayed gold microchips, the produced electrical impendence reflecting the physiological status of cells such as cell proliferation and viability was continuously monitored [158, 159, 160]. Without the use of labeled dyes, RTCA permits a direct and continuous measurement of cells under

physiological conditions [99]. Notably, as electrochemical signals were obtained, the cell number and morphology are the only determining factors in the RTCA assay, whereas other physicochemical properties like the spectroscopic absorbance of cellular components exert no influences on the analysis. Nevertheless, in the cytotoxic assay by RTCA, attention should be paid to the drug formulations that may contain electrically conductive additives.

From the perspective of the entire experimental process, RTCA can monitor the growth status of cells at different times in real time. From the cell index curve, you can intuitively see the growth of BMEC cells in the entire experimental process. The node selection for processing time can be based on the growth of cells. The basis for judging the situation is more sufficient and reliable. These results were consistent with those of both the CCK-8 and RTCA assays, which also accord well with those reported previously [79, 114, 131]. Taken together, the data obtained by manual cell counting supported the comparison results between the CCK-8 and RTCA assays, which suggested that the applicability of these two methods should be taken into consideration in terms of cytotoxicity evaluation of the drugs.

we have compared the cytotoxicity evaluation results of CGA extract between the CCK-8 and RTCA assays. The IC_{50} values obtained by the two methods are 326.8 and 320.4 μg/mL. The results indicated that the CCK-8 assay as an end point method measured the optical intensity of dyes in the cell at a specific time point, of which the readout interpretation can be interfered by the colored drugs used, especially those with the absorbance peak close to 450 nm of dyes used for the assay. By comparison, RTCA measured the drug-added cells on the basis of electrochemical impedance and hence can give more reliable cytotoxicity evaluation results. With the fast development of new drug formulation, our studies may indicate that the preselection of methods is necessary for the drug cytotoxicity evaluation and screening to avoid the misleading results, which will be meaningful for later clinical practice.

Mastitis, an inflammation of the mammary gland, is a serious infectious disease

triggered in cattle by infection with bacteria, and results in decreased milk production and quality [5]. The inflammatory response to bovine mastitis caused by *S. aureus* infection is often slow, and the infection causes both recessive mastitis and clinical mastitis [121]. Lipoteichoic acid (LTA) is the main component of the cell wall of Gram-positive bacteria [175,58]. Recent studies reported that LTA palys an essential role in the pathogenesis of *S. aureus* by participating in its adhesion and colonization, and by stimulating the inflammatory response of the host cells [151,64]. When *S. aureus* LTA invades the mammary gland, it could induce TLR2 activation, and the signaling of TLR2 leads to the significant secretion of inflammatory cytokines by mediating nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) signaling pathways [97,174].

For a long time, plants have been a rich source of antibacterial, antiviral, and immunomodulatory metabolites. In this sense, *Taraxacum Officinale* is a famous traditional Chinese herb, and commonly used for the treatment of inflammatory and infectious diseases, such as respiratory tract infection, hepatitis, bronchitis, mastopathy and pneumonia [92,122,25]. Some plant extracts have been used in treating clinical mastitis on organic dairy farm and demonstrated with good therapeutic effect [107]. *Taraxacum Officinale* has been long used for treat human diseases including inflammation [135]. Such anti-inflammation effect of dandelion may be via its effect on macrophage by reducing nitric oxide (NO), prostaglandin (PG) E2, TNF-α, IL-1β, IL-6, and cycloxygenase (COX)-2 [60,104,170]. Diverse reports have shown that chlorogenic acid (CGA) was isolated from the *Taraxacum Officinale* and has been known to have various activities, such as anti-inflammatory [74], antibacterial [85], anti-viral [105] and anti-oxidative [116] effects. A previous study demonstrated that CGA has alleviate the inflammatory response induced by *E. coli* in the sheep endometrial epithelium cells via inhibiting the activation of the TLR4/NF- κB signaling pathway [50]. Furthermore, CGA could be used as a potential therapentic compound for bovine mastitis induced by LPS, through reduction of NF- κB [33].

TLR2 is the major receptor of LTA. Activation of TLR2 leads to activation of NF-κB, which regulates the expression of inflammatory cytokines [78]. In this study, our results showed that CGA significantly inhibited *S. aureus* LTA-induced TLR2 expression, suggesting that CGA inhibited *S. aureus* LTA-induced inflammation by suppressing the TLR2 signaling pathway. The transcription factor NF-κB is a key regulator of inflammation and immune responses [38]. In normal cells, NF-κB is localized to the cytoplasm and bound to an inhibitory protein called IκBα. Once cells are treated with various inducers, IκBα is degraded, and phosphorylated NF-κB p65 is transferred from the cytoplasm to the nucleus and promotes the transcription of inflammatory cytokines [91]. A previous study showed that *S. aureus* internalization was associated with the active state of NF-κB, and inhibition of NF-κB activation could attenuate the internalization of *S. aureus* into bMECs [100]. In the present study, *S. aureus* LTA caused phosphorylation of NF-κB p65 and IκBα in BMEC cells. Subsequently, we found that CGA could inhibit LTA-induced NF-κB activation in a dose-dependent manner. These results suggest that CGA inhibits NF-κB activation and reduces bacterial toxin-induced inflammation responses.

CONCLUSIONS

In the dissertation work, based on research, it is explored the anti-inflammatory effect of Chlorogenic acid on LTA-induced Bovine Mammary Epithelial Cells (BMEC) and its mechanism, especially on the pathway. We provided 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. Based on the results of the research, the following conclusions are justified:

1. The best parameters of the extractiont echnology of chlorogenic acid were obtained that the ultrasonic temperature was 80 ℃, 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%.

2. The phenolic extract content in *Taraxacum officinale* were chlorogenic acid (1.34 mg/g), luteolin (1.08 mg/g), ferulic acid (0.22 mg/g), caffeic acid (0.21 mg/g), and rutin (0.19 mg/g).

3. *Taraxacum officinale* phenolic extract 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.

4. Bovine mammary epithelial cells can be recovered from milk, grown in culture, and exhibit the characteristic cobblestone morphology of epithelial cells.

5. The treatment of BMECs with LTA at 20 ng/μL for 24 h obviously improved TNF-α and IL-6 protein and gene expression levels, it is a good inflammatory model induced by LTA.

6. The real time cell analysis method and the CCK-8 method showed that different concentrations of chlorogenic acid extract reduced the viability of dairy cow mammary epithelial cells, and the decrease was most obvious at 400 ug/mL. The IC50 of the two analysis methods were 326.8 and 320.4 ug/mL.

7. The chlorogenic acid extract from *Taraxacum officinale* is a modulator of innate immune response in bovine mammary epithelial cells during *S. aureus* infection. These data suggest that chlorogenic acid extract could be useful for mastitis control.

PRODUCTION PROPOSALS

1. On the basis of our recearsh Methodological Recommendations of Modern methods of cow mastitis diagnostic and prevention, for laboratory, practical classes and independent work for master's students of veterinary department from disciplines "Veterinary Microbiology"and "Veterinary Zoohygiene", specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise" was development. (approved by the Scientific Council of SNAU, protocol No. 5, dated December 29, 2021).

2. We 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 (HIST).

3. We can recommend that at farm level, dandelion extract may use as a new type of feed additive in production. And new therapies with CGA as a drug should be developed in future.

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APPLICATIONS

Appendix A

List of works published on the topic of the dissertation

LIST OF WORKS PUBLISHED ON THE THEME OF THE DISSERTATION

Articles in scientific professional publications of Ukraine:

1. **Ping Xu**. (2021). Research progress on the dairy cow mastitis. *Biol. Tvarin.*, 23 (1), 44-46. https://doi.org/10.15407/animbiol23.01.044 http://aminbiol.com.ua/index.php/174-archive/bt-23-1-2021/1931-research-progress-on-the-dairy-cow-mastitis

2. **Ping Xu**. (2021). Comparison of cytotoxicity evaluation of chlorogenic acid extract between Real-time cell analysis and CCK-8 method. *Ukrainian Journal of Veterinary and Agricultural Sciences,* 4 (2), 58-61. **DOI:** https://doi.org/10.32718/ujvas4-2.10 https://ujvas.com.ua/index.php/journal/article/view/92

3. **Ping Xu**, Hanna Fotina, Tetiana Fotina, Sanhu Wang. **(**2020). Establishment of inflammatory model of bovine mammary epithelial cells induced by Lipoteichoic acid. Вісник Сумського національного аграрного університету, Серія "Ветеринарна медицина" Випуск 3 (50), 31-37. DOI: 10.32845/bsnau.vet.2020.3.5 https://snaubulletin.com.ua/index.php/vm/article/view/276 *(The applicant participated in research, analysis of the results and writing the article).*

4. **Ping Xu**, Hanna Fotina, Tetiana Fotina, Sanhu Wang. **(**2021). Use of plant-derived drugs in the prevention and treatment of dairy cow mastitis. *Ukrainian Journal of Veterinary and Agricultural Sciences,* 4 (1), 24-28. **DOI:** https://doi.org/10.32718/ujvas4-1.05 https://ujvas.com.ua/index.php/journal/article/view/77 *(The applicant participated in research, analysis of the results and writing the article).*

Scopus publication:

5.Ping Xu, Tetiana Fotina, Sanhu Wang. **(**2021). Detertion and Extraction Process of Chlorogenic Acid from *Taraxacum officinale*. Journal of Hygienic Engineering and Design, 35(96), 67-72. JHED | Volume 35 | FPP (5) - https://keypublishing.org/jhed/jhed-volumes/jhed-volume-35-fpp-5-ping-xu-tetiana-fotina-2021-detection-and-extraction-process-of-chlorogenic-acid-from-taraxacum-officinale/ (*The applicant participated in research, analysis of the results and writing the article).*

6. **Xu, P.**, Fotina, H., Fotina, T. and Wang, S. **(**2021). *In vitro* culture and evaluation of bovine mammary epithelial cells from Ukraine dairy cows. Iranian Journal of Veterinary Research, 22, 1(74), 65-71. DOI: 10.22099/ijvr.2020.37714.5508. https://pubmed.ncbi.nlm.nih.gov/34149858/ *(The applicant participated in research, analysis of the results and writing the article).*

7. **Ping Xu**, Xiaobo Xu, Ajab Khan, Tetiana Fotina, Sanhu Wang. **(**2021). Antibiofilm activity against *Staphylococcus aureus* and content analysis of *Taraxacum Officinale* Phenolic extracts. Polish Journal of Veterinary Sciences, 24 (2), 243-251. DOI: 10.24425/pjvs.2021.137659. PMID: 34250777. https://pubmed.ncbi.nlm.nih.gov/34250777/ *(The applicant participated in research, analysis of the results and writing the article).*

China publication：

8. **Xu Ping**, Zhao Jian, Yao Jingjie, Fotina Tetiana, Wang Sanhu, Zhang Xiaojian. (2021). Cloning and bioinformatics analysis of the CDS region and 3'UTR of *klf4* gene in dairy cow. Journal of Fujian Agriculture and Forestry University (Natural Science Edition), 50 (03): 383-389. https://caod.oriprobe.com/articles/61587373/Cloning_and_bioinformatics_analysis

_of_the_CDS_reg.htm *(The applicant participated in research, analysis of the results and writing the article).*

9. **Xu Ping**, Xu Xiaobo, Feng Yuxiang, Fotina Tetiana, Wang Sanhu, Zhao Kun. (2021). Amplification of promoter and exon of dairy cow CCL11 gene and analysis of exon polymorphism. Heilongjiang Animal Science and Veterinary Medicine, 2021(21): 81-85+154.

DOI: http://www.10.13881/j.cnki.hljxmsy.2021.03.0211*)*.https://kns.cnki.net/kcms/detail/detail.aspx?dbcode=CJFD&dbname=CJFDLAST2021&filename=HLJX202121015&uniplatform=NZKPT&v=zRgQrmgkgLC0MrMKTDrEGRgmzmYb_eDJIu_iFuXjx8axURQzV0sfu1GVrMUKnBKn. *(The applicant participated in research, analysis of the results and writing the article).*

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Conference papers*:*

11. **Ping Xu,** Fotina Hanna. **(**2018). Identification of LncRNAs and its regulatory mechanism in the development of *Staphylococcus aureus* mastitis. Матеріали Всеукраїнської студентської наукової конференції, присвяченої міжнародному дню студента (12-16 листопада 2018 р.) *(PhD participant in*

carrying out of experimental researches, processing of results, preparation of article for printing).

12. **Ping Xu,** Fotina Tetiana. **(**2019). In Vitro Culture and Evaluation of a Bovine Mammary Epithelial Cell Line from Ukraine Dairy Cow. Sumy National Agrarian University of the All-Ukrainian Student Scientific Conference, 697. (November 11-15, 2019, Sumy) (PhD participant in carrying out of experimental research, processing of results, and writing the article).

13. **Ping Xu,** Fotina Hanna, Fotina Tetiana. **(**2019). The Taraxasterol and Chlorogenic acid content, antioxidant and anti-inflammatory activity in *Taraxacum officinale.* Food Quality and Safety, Health and Nutrition Congress (June 12-14, 2019. Ohrid, Macedonia) *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

14. **Ping Xu,** Fotina Hanna, Fotina Tetiana. **(**2019). The taraxasterol and chlorogenic acid content of *Taraxacum officinale* and its antioxidant and anti-inflammatory activity. BTRP Ukraine Regional One Health Research Symposium (20-24 May 2019, Kyiv, Ukraine) *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

15. **Ping Xu,** Zhang Xiaojian, Wang Sanhu, Fotina Hanna, Fotina Tetiana. (2019). Anti-Inflammatory Effects of Taraxacum officinale Chlorogenic acid on LTA- Stimulated Bovine Mammary Epithelial Cells. Twentieth Chinese National Conference on Animal Genetics & Breeding (5-8 December 2019, Guangzhou, China) *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

16. Jingjie Yao, **Ping Xu,** Jian Zhao, Yanan Lv, Liya Guo, Sanhu Wang, Xiaojian Zhang. (2019). MicroRNA-145 participates in the depolymerization of microfilaments of bovine mammary gland epithelial cell by targeting PXN in mastitis. The 6th International Symposium on Dairy Cow Nutrition and Milk Quality (3-5 May 2019, Beijing, China) *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing)*.

17. Yanan Lv, **Ping Xu,** Jian Zhao, Zhixing An, Liya Guo, Xiaojian Zhang. (2019). Establishment of Inflammatory Model of Bovine Mammary Epithelial Cells Induced by LPS and LTA in vitro. The 6th International Symposium on Dairy Cow Nutrition and Milk Quality (3-5 May 2019, Beijing, China) *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

18. **Ping Xu,** Fotina Tetiana. **(**2020). Establishment of Inflammatory Model of Bovine Mammary Epithelial Cells Induced by Lipotelchoic acid. Матеріали Всеукраїнської студентської наукової конференції, присвяченої міжнародному дню студента (16-18 листопада 2020 р.) *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

19. **Ping Xu,** Fotina Tetiana, Sanhu Wang. **(**2020). Detection and Extraction Process of Chlorogenic acid from Taraxacum officinale. Food Quality and Safety, Health and Nutrition Congress (September 2-4, 2020. Ohrid, Macedonia) *(PhD participant in carrying out of experimental researches, processing of results, preparation of article for printing).*

Methodological recommendations:

20. **Ping Xu,** Fotina T. I, Fotina H.A. Methodical guidelines “Modern methods of cow mastitis diagnostic and prevention”, for laboratory, practical classes and independent work for master’s students of veterinary department from disciplines "Veterinary Microbiology"and “Veterinary Zoohygiene”, specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise". (approved by the Scientific Council of SNAU, protocol No. 5, dated December 29, 2021). (*The applicant analyzed the research results, prepared and issued materials for methodical recommendations).*

Appendix B

Methodical Guidelines

Methodical guidelines “Modern methods of cow mastitis diagnostic and prevention” 2021, 35 pp. (approved by the Scientific Council of SNAU, protocol No. 5, dated December 29, 2021).

MINISTRY OF EDUCATION AND SCIENCE OF UKRAINE
SUMY NATIONAL AGRARIAN UNIVERSITY

Methodical guidelines

"Modern methods of cow mastitis diagnostic and prevention"

for laboratory, practical classes and independent work for master's students of veterinary department from disciplines "Veterinary Microbiology"and "Veterinary Zoohygiene", specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise".

Sumy- 2021

Compilers:

Ping Xu, postgraduate student of Department Of Veterinary Examination, Microbiology, Zoohygiene And Safety And Quality Of Livestock Products

Fotina T.I., doctor of veterinary science, professor, head of the Department of Veterinary Expertise, Microbiology, Zoohygiene and Safety and Quality of Animal Husbandry Products

Fotina H.A., doctor of veterinary science, professor, professor of Department of Veterinary Examination, Microbiology, Zoohygiene and Safety and Quality of Livestock Products

Methodical guidelines "Modern methods of cow mastitis diagnostic and prevention", for laboratory, practical classes and independent work for master's students of veterinary department from disciplines "Veterinary Microbiology"and "Veterinary Zoohygiene", specialties: 211 "Veterinary Medicine", 212 "Veterinary Hygiene, Sanitation and Expertise". 35p

Reviewers:

R.V. Petrov, Doctor of Veterinary Medicine, Professor, Head of the Department of Department Of Virusology, Patanatomy And Bird Diseases Named After Professor I.I.Panicar of Sumy NAU;

O.I. Shkromada, Doctor of Veterinary Medicine, Professor, Head of the Department of Obstetrics and Surgery of the Sumy National University of Science.

Recommended for publication by the educational and methodical council of the Faculty of Veterinary Medicine of SNAU, protocol No.5 dated "29" 12. 2021.

Appendix C

Conclusion of the commission on bioethics in Chines

伦理审查批件号：**XYLL—22—006**

新乡学院生物与医学伦理委员会

伦 理 审 查 报 告

项目名称： 蒲公英绿原酸对 LTA 诱导的奶牛乳腺炎的作用机制研究

项目负责人：徐 萍

伦理审查意见：

本研究探讨蒲公英绿原酸对 LTA 诱导的奶牛乳腺炎的作用机制。该研究将开展采集牛乳腺组织样本的动物实验，课题主要实验人员均已接受了相关培训，实验方案中说明了使动物痛苦降至最低的方法(实验设计中使用动物数量合理)及其实验结束后合理处置动物的方式等内容，经审查课题的执行与运行符合《实验动物管理条例》及国家有关法律法规规定。

经新乡学院伦理委员会审查，该项目研究内容和过程遵循国际及国家颁布的有关生物医学研究的伦理要求，同意申报 2022 年国家自然科学基金项目，并开展相关研究。

新乡学院生物与医学伦理委员会

2022 年 3 月 2 日

Appendix D

Conclusion of the commission on bioethics in English

Laboratory Animal Welfare and Ethical review of Henan Institute of Science and Technology

Application Number: 201909-027 Application Date: September 6, 2019 Issue No.

<table>
<tr><td colspan="3">Program and No.: Anti-inflammatory effect and mechanism of Chlorogenic acid extract from Taraxacum officinale on LTA-induced mastitis in dairy cows</td></tr>
<tr><td rowspan="2">Applicant</td><td>Name: Ping Xu</td><td>Organization: College of animal science and veterinary medicine</td></tr>
<tr><td>E-mail: afraxp1986 @126.com</td><td>Telephone Number: 15937160122</td></tr>
<tr><td rowspan="2">Animals</td><td colspan="2">Animal Source: Henan Animal Experiment Center</td></tr>
<tr><td colspan="2">Animal Grade（Normal、SPF or Others）: Normal</td></tr>
<tr><td colspan="3">（Experimental objective, necessity and significance and how the program has been designed to achieve the objectives of the research）:
Objective: To explore the anti-inflammatory effect of Chlorogenic acid on LTA-induced Bovine Mammary Epithelial Cells (BMEC) and its mechanism, especially on the pathway.
Necessity and significance: This study can 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.
experimental steps:
1. Obtaining raw milk from lactating cows and isolating mammary epithelial cells.</td></tr>
<tr><td>Animal Care</td><td colspan="2">The nursing standards of ordinary piglets were adopted</td></tr>
<tr><td rowspan="2">Animal Disposition</td><td colspan="2">Death conduct:
□ CO_2 suffocated □Exsanguinations with anesthesia
□ Cervical dislocation □ Anesthesia overdose
■Others, detailed description</td></tr>
<tr><td colspan="2">Not for the death of the animal disposition:
■ Continue to use □ Save in the agency
□ Release to the wild
□ Others, detailed description:</td></tr>
<tr><td colspan="3">Poisonous (harmful) material (infection, radiate, chemical poison and other) being used
□ Yes ■ no
Declare:</td></tr>
<tr><td colspan="3">Supplementary instruction for investigate
No</td></tr>
<tr><td colspan="3">Declaration for the information disclosure and confidentiality requirements, declaring the information need to be kept secret, the information can be disclosed
No</td></tr>
<tr><td colspan="3">Claiming jurors for being debarb
No</td></tr>
</table>

Applicant's Declaration:

1. I will abide by the law and regulation stipulation, and accept the supervision and inspection by the committee and laboratory animal department.
2. The information I have given is accurate, detailed and comprehensive.

Applicant Signature (Seal): Sanhu. Wang

September 6, 2019

Approval opinions:

■ Approval □ Not approve

Authorized Personnel Signature（Stamp）:

September 13, 2019

Appendix E

Laboratory Biosafety Certificate

实验室生物安全证书

我校博士生徐萍，在开展研究：蒲公英绿原酸对 LTA 诱导的奶牛乳腺炎的作用机制研究过程中，涉及病原微生物的实验。该生在实验过程中，严格遵守国家及学校生物安全相关规定，并根据《人间传染的病原微生物名录》（卫科教发：2006]15 号） 规定的生物安全防护和标准操作规程开展实验研究，并确保实验室生物安全。

Laboratory Biosafety Certificate

Ping Xu, PhD student who involved in the use of pathogenic microorganisms in the research on Anti-inflammatory effect and mechanism of Chlorogenic acid extract from Taraxacum officinale on LTA-induced mastitis in dairy cows. During the experiment, she strictly abide by the relevant national and school biosafety regulations, and carry out experimental activities in accordance with the standard operating procedures in the biosafety protection level laboratory stipulated in the "List of Pathogenic Microorganisms Infected Between Humans" (Wei Ke Jiao Fa: 2006] No. 15) and ensure laboratory biosafety.

Henan institute of Science and Technology

Date: September 6, 2019

河南省教育厅

教科外〔2009〕80号

河南省教育厅
关于公布2008年度河南省高校工程技术研究中心建设项目的通知

各高等学校：

根据《河南省教育厅关于开展2008年度河南省高校工程技术研究中心立项建设的通知》（教科外〔2008〕737号）精神，经学校申报、中心负责人答辩、专家评审、现场考察，教育厅审核并公示，决定依托河南科技大学等学校建设12个河南省高校工程技术研究中心（以下简称工程中心）。现将工程中心及依托单位名单予以公布（附件1），有关事宜通知如下：

附件1

2008年度河南省高校工程技术研究中心建设项目名单

序号	中心名称	依托学校	技术领域	类别
1	轴承工程技术	河南科技大学	制造业	认定
2	动物疫病和残留物防控	河南科技学院	农业与生物	认定
3	生态化工	郑州大学	资源与环境	立项
4	作物抗逆改良	河南大学	农业与生物	立项
5	农业资源与环境	河南农业大学	资源与环境	立项
6	道地中药材保育及利用	河南师范大学	农业与生物	立项
7	矿物加工与矿用材料	河南理工大学	新材料	立项
8	粮食信息与检测技术	河南工业大学	现代服务业	立项
9	高性能土木工程材料与环境	郑州航空工业管理学院	新材料	立项
10	生物质降解与气化	商丘师范学院	资源与环境	立项
11	信阳毛尖茶产业	信阳农业高专	农业与生物	立项
12	怀药提取分离	焦作大学	化学化工	立项