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Original article

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

P. Xu[1,2,3#], X.B. Xu[1#], A. Khan[4], T. Fotina[3], S.H. Wang[2]

[1] School of Life Science and Basic Medicine, Xinxiang University, Jinsui St. 191, 453003 Xinxiang, China
[2] College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Eastern Hua Lan Street, 453003 Xinxiang, China
[3] Department of Veterinary Medicine, Sumy National Agrarian University, Herasima Kondratieva St. 160, 40021 Sumy, Ukraine
[4] College of Animal Science and Veterinary Medicine, Shanxi Agricultural University, Taigu, Shanxi 030801, P.R. China

Abstract

Taraxacum Officinale, commonly called dandelion, is herbaceous perennial belonging to the family of Asteraceae, having good antibacterial effects which are related to its phenolic substances. In this study, 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 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 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 applying the dandelion phenolic extract, the growth of *S. aureus* was significantly 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 affected the normal reproduction of *S. aureus*. These results suggest that dandelion has an inhibitory effect on *S. aureus*, and the mechanism of its action was to destroy the integrity of the cell walls and cell membranes.

Key words: *Taraxacum Officinale*, phenolic, antibacterial mechanism, HPLC, *Staphylococcus aureus*

Correspondence to: S.H. Wang, e-mail: 121558423@qq.com.
[#] These authors contributed equally.

Introduction

Plants are the most important source of medicines. Large number of drugs are isolated and extracted from plants. Their clinical and pharmacological effects have been extensively studied from various viewpoints (Hudec et al. 2007). Besides effective and easy availability, the important advantage of medicinal plants as a therapeutic agent in various ailments and disorders is their safety. 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. Although, dandelion is considered a weedy species, but it has been used in many herbal medical systems, as has been mentioned particularly in Asia, Europe, and North America. Dandelion serves mainly as a diureticum, as well as a cleanser of the blood and liver. It is used to treat anemia, liver cirrhosis, hepatitis and rheumatism (Hu and Kitts 2005, Hudec et al. 2007) and has anti-inflammatory, anti-oxidative, anti-carcinogenic, analgesic, anti-hyperglycemic, anti-coagulatory, anticancer activities and prebiotic effects (Rudenskaya et al. 1998, Zhu et al. 1999, Schütz et al. 2005, Schütz et al. 2006, Shi et al. 2008a, Shi et al. 2008b, Shi et al. 2008c). It is also used to reduce serum cholesterol and triglycerides because it intensifies bile secretion (Hudec et al. 2007). Medicinal plants contain secondary metabolites which are responsible for the therapeutic activities of plants (Savithramma. 2011). Schütz et al. (2005) reported different phytochemical components of *T. officinale*. These secondary metabolites work synergistically to perform their beneficial antioxidant role in medicinal plants (Cortes et al. 2014). Dandelion contains numerous polyphenolic compounds which are classified as flavonoids (including luteolin, quercetin, chrysoeriol and their glycosides) and phenolic acids (caffeic, chlorogenic, coumaric, caftaric acid and others).

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 (Farnsworth et al. 2015, Ran et al. 2015). *S. aureus* can cause a wide range of infections and diseases including purulent, sepsis, endocarditis, meningitis and others (Oliveira et al. 2018). 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 (Adámez et al. 2012, Daglia. 2012, Chibane et al. 2018). 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* (Nostro et al. 2016, Brambilla et al. 2017). 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 purpose of this study was to investigate the antibacterial properties and mechanism of action of *T. officinale* phenolic extract against *S. aureus*.

Materials and Methods

Plant material and reagents

The whole plant of *Taraxacum Officinale* were collected from the campus of Sumy National Agrarian University, Sumy, Ukraine, in May 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. Five reference Phenolic standards, as chlorogenic acid, caffeic acid, rutin, ferulic acid and luteolin were purchased from Sigma (Sigma Aldrich; St. Louis, MO, USA). Acetonitrile (99.9%, HPLC grade) and methanol (99%, PA) were from Fisher Scientific (Lisbon, Portugal), and Alkaline Phosphatase Assay Kit (Colorimetric) from Abcam (Cambridge, UK, ab83369). All other chemicals and reagents used in the experiments were of analytical grade. The standard solutions were prepared by dissolving standards in methanol.

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 C_{18} (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(v/v). The flow rate was 1.0 mL/min. Detection wavelength was 320 nm and the column oven temperature was 35°C

Standard curve

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 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, 2, 4, 6, 8, and 10 μL of the working stock solution were injected and analyzed according to the above-mentioned high performance liquid chromatography conditions with 3 repetitions (Fig. 1A). The peak areas were recorded. Through linear regression the linear equations were obtained.

Preparation of phenolic extract

Phenolic extract was obtained from plant sample (1.0 g) with 30 mL of methanol-water (70:30, v/v) at 30°C for 30 min. Three times collected extracts were combined and concentrated under reduced pressure to a constant volume of 10 mL. The sample was passed through 0.45 **μm** membrane before the injection.

Bacterial strains and growth condition

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

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 concentrations of 25, 20, 15, 10, 5 mg/mL. Tested pathogenic microorganisms were cultured in MHB at 37°C 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. Aliquots of bacterial suspensions (50 μL) were added to a sterile 96-well plate containing 100 μL of MHB and 100 μL of dandelion phenolic extract dilutions. A positive control (without dandelion phenolic extract) was added on each microplate. The plates were incubated in aerobic conditions at 37°C 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.

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. At the same time, a blank was set as control group. All the samples were grown under aerobic conditions at 37°C in a shaking incubator at 120 rpm/min. The samples were selected at different times to determine the absorbance value, and to draw the growth curve of *S. aureus*.

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 the control group. All the samples were 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 extract on the integrity of *S. aureus* biofilms

Samples collected at different time intervals were centrifuged at 4500 rpm/min for 10 minutes. The super-

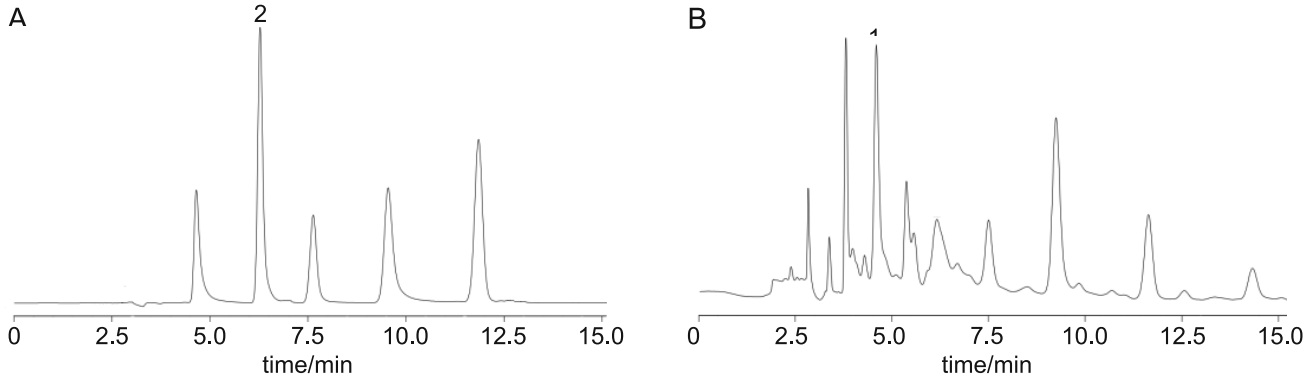

Fig. 1. HPLC chromatogram of five phenolic mixture reference solutions (A) and *T. Officinale* phenolic extract. 1: chlorogenic acid, 2: caffeic acid, 3: rutin, 4: luteolin, 5: ferulic acid.

Table 1. The standard curve of five phenolic mixture reference solutions and the content in *T. Officinale* phenolic extract (average value±SD).

Peak NO	t_R (min)	Compound	Standard curve	R^2	Content (mg/g)
1	4.6	chlorogenic acid	y = 423,717 x + 1,972	0.998	1.34 ± 0.09
2	6.3	caffeic acid	y = 544,660 x + 2,940	0.999	0.21 ± 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

natant 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. All the samples were 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 was 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.

Results

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

Generally, not only the total phenolic acid content but also its composition has the impact on its bioactivities. From *T. Officinale,* the phenolic acid composition was analyzed by HPLC. The linear relationship between the peak area and concentration of the five phenolic mixture reference solutions established by HPLC detection method were shown in Fig. 1 and Table 1. 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).

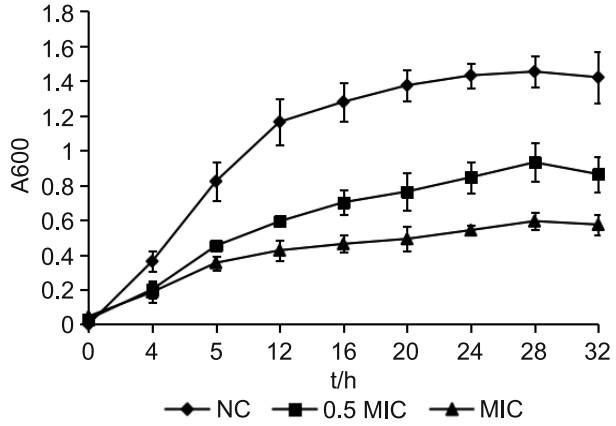

Fig. 2. Growth curves of *S. aureus.* NC: Normal control; 0.5MIC: TPE against *S. aureus* was 6.25 mg/mL; 1MIC: TPE against *S. aureus* was 12.5 mg/mL.

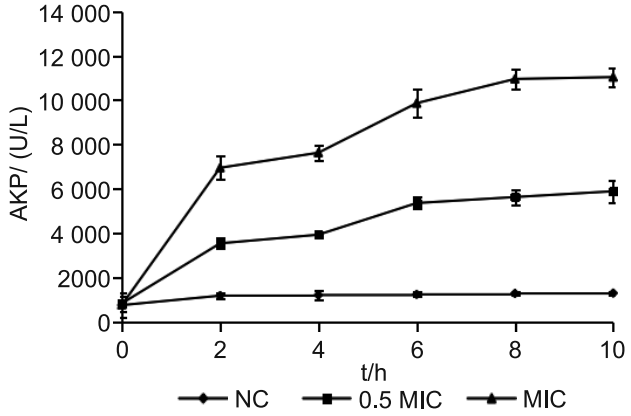

Fig. 3. The effect of the TPE on the cell wall permeability. NC: Normal control; 0.5MIC: TPE against *S. aureus* was 6.25 mg/mL; 1MIC: TPE against *S. aureus* was 12.5 mg/mL.

Inhibition Assay

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.

Microbial growth

The effect of TPE on the growth curve of *S. aureus* was shown in Fig. 2. 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*. Within a certain range, the antibacterial effect was increased with the increase of TPE concentration.

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

Figure 3 shows 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.

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

Figure 4 shows the effect of TPE on the integrity of *S. aureus* biofilms by electric conductivity assay. 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.

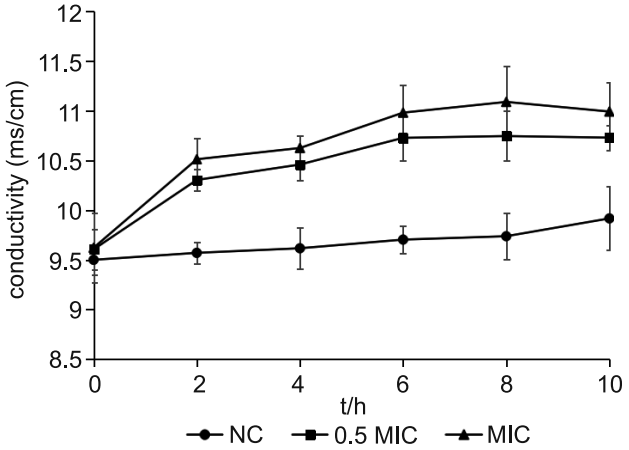

Fig. 4. The effect of the TPE on membrane permeability. NC: Normal control; 0.5MIC: TPE against *S. aureus* was 6.25 mg/mL; 1MIC: TPE against *S. aureus* was 12.5 mg/mL.

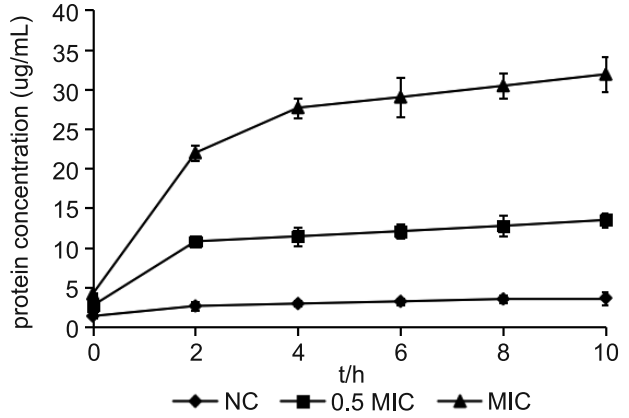

Fig. 5. The effect of the TPE on extracellular protein content. NC: Normal control; 0.5MIC: TPE against *S. aureus* was 6.25 mg/mL; 1MIC: TPE against *S. aureus* was 12.5 mg/mL.

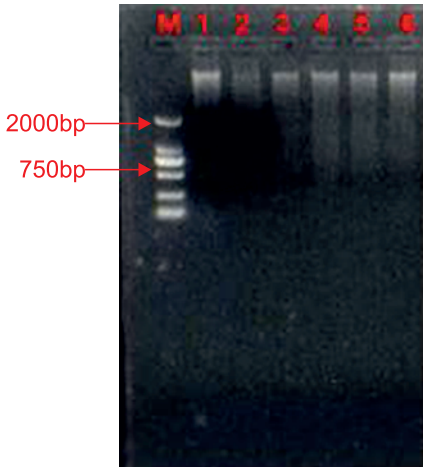

Fig. 6. Interaction of TPE with *S. aureus* DNA. M: DL2000 marker; 1: Control group; 2: 200 ng/μL; 3: 100 ng/μL; 4: 50 ng/μL; 5: 25 ng/μL; 6: 12.5 ng/μL.

Effect of plant extract on extracellular protein content

As shown in Fig. 5 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.

Interaction between bacterial DNA and plant extract

The interaction of TPE with *S. aureus* DNA as shown in Fig. 6, 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*.

Discussion

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 (Cho et al. 2002, Jeon et al. 2008). 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 needs 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 (2013) 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 (Zhang et al. 2020). Wojdylo et al. (2007) 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. (1995) 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* (Kumar and Goel. 2019). 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 (Li C et al. 2020); (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 (Lima et al. 2019). 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 (Farha et al. 2015). 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 dande-

lion phenolic extract against *S. aureus* was to destroy the barrier function of the cell wall and cell membrane.

Conclusions

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.

Acknowledgements

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