



Extraction technology, component analysis, antioxidant, antibacterial, analgesic and anti-inflammatory activities of flavonoids fraction from *Tribulus terrestris* L. leaves



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ABSTRACT

Research on the extraction technology, component analysis, and antioxidant, antibacterial, analgesic and anti-inflammatory activities of flavonoids fraction from *Tribulus terrestris* L. leaves was carried out. The extraction process was optimized by response surface method, and the maximum yield 0.27% for flavonoids fraction was achieved with concentration of ethanol solution 25.87%, ratio of solvent to material 30:1 ml/g, and ultrasonic extraction time 27.93 min. Moreover, 14 compositions were identified separately from flavonoids fraction by HPLC-DAD-ESI-MS². In addition, flavonoids fraction exhibited a better antioxidant activity demonstrated by assays of ABTS, DPPH radical scavenging activity and ferric reducing antioxidant power activity. Furthermore, the antibacterial, analgesic and anti-inflammatory activities of flavonoids fraction were also proved to be stronger. Therefore, the present findings suggest that flavonoids fraction from *Tribulus terrestris* L. leaves can be a very interesting candidate for the research and development of natural and healthy herbal medicine for the pharmaceutical and food industries.

1. Introduction

In recent years, traditional Chinese medicine (TCM) has attracted more and more attention by the advantages of least toxicity, difficult to form drug resistance, safety and environmental protection, and applied widely in the agriculture, food and medicine industries.

Tribulus terrestris L. is usually used to treat the illness in eyes, cutaneous pruritus (Nam et al., 2016), chest tightness or pain (Nam et al., 2016), hypoimmunity, cerebral disease (Zhang et al., 2010; Phillips et al., 2006), and cancer (Kim et al., 2011). In addition, it exhibited better pharmacological activities, including antioxidant (Hammoda et al., 2013; Sailaja et al., 2013), antimicrobial (Zhang et al., 2006; Gopinath et al., 2012), anti-inflammatory (Hammoda et al., 2013; Mohammed et al., 2014), enhancement the level of hormones and gonadotropins (Martino-Andrade, 2010; Kam et al., 2012; Moghaddam et al., 2013; Fatima and Sultana, 2017), alleviating muscle damages (Ma et al., 2017),

improving mitochondrial dysfunction (Reshma et al., 2016), influence cytological and genetic effects of cultured human lymphocytes (Qari and El-Assouli, 2017), and regulation of the enzyme activity (Ercan and Nehir El, 2016).

Modern pharmacology studies have demonstrated that flavonoids constituent is an important category of natural compound with better antioxidant (Jiang et al., 2014), antibacterial, analgesic, cardiovascular protective effect (Jiang et al., 2014; Li et al., 2014) and anti-inflammatory activities. Guo et al. (2013), revealed that the flavonoids extract from mimenghua performed a stronger antioxidant activities *in vitro* demonstrated with scavenging capabilities of DPPH, hydroxyl and superoxide radicals, reducing power and total antioxidant capacity; the antibacterial activity of liquiritigenin and liquiritin against *E. coli* was measured with microcalorimetry and chemometrics methods (Kong et al., 2015); Chen et al. (2015) has reported that total flavonoids extract from *Sanguis draxonis* has shown potentially analgesic effect on

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spared nerve injury rat; flavonoids fraction of *E. lindleyanum* may attenuate lipopolysaccharide-induced acute lung injury in mice via reducing productions of pro-inflammatory mediators, and affecting the NO, SOD and MPO activity (Chu et al., 2016); linarins can down-regulate phagocytosis activity and the production of pro-inflammatory cytokine in RAW264.7 macrophages (Kim et al., 2016).

Tribulus terrestris L. is rich in saponins, flavonoids, alkaloids, fatty acids and amino acids (Bhutani et al., 1969; Kang et al., 2014; Ma et al., 2017; Qari and El-Assouli, 2017; Zheng et al., 2017). However, few studies have been reported on the flavonoids constituent of *Tribulus terrestris* L., especially the extraction technology, component analysis, and antioxidant, antibacterial, analgesic and anti-inflammatory activities of flavonoids fraction.

The aims of the present study were to optimize the extraction process of flavonoids fraction from *Tribulus terrestris* L. leaves by single factors test and response surface method, and to identify the chemical components by HPLC-DAD-ESI-MS², and then further to evaluate its antioxidant, antibacterial, analgesic and anti-inflammatory activities.

2. Materials and methods

2.1. Instruments and reagents

HPLC-DAD-ESI-MS² analysis was performed on an Agilent 1100 series HPLC (Palo Alto, CA, USA) and Agilent model Ion Trap mass spectrometer equipped with electrospray ionization ion source (ESI). The content and antioxidant activities of total flavonoids were assayed with T6 ultraviolet and visible spectrophotometer (Beijing Puxi Tongyong Inc., China).

Standard of rutin, dexamethasone (Dex) and gentamicin (HPLC purity >98.0%) were purchased from National Institutes for Food and Drug Control (Shenyang, China). DPPH, ABTS, TPTZ, LPS, dimethyl sulfoxide (DMSO) and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were bought from Sigma-Aldrich Chemical Company (St. Louis, MO). Other chemicals were of analytical grade.

Standard strains *Escherichia coli* (ATCC 25922), *Salmonella* (ATCC 51812), *Staphylococcus aureus* (ATCC 25923) and *Streptococcus* (ATCC 49619) were obtained from American Type Culture Collection.

RAW 264.7 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China).

2.2. Animals

Male Kunming mice (3–4 weeks, 18–22 g) were provided by Liaoning Changsheng biotechnology co. LTD and fed at room temperature of 24 ± 1 °C, humidity 60%–65% with a 12 h light/12 h dark cycle. Animal care and the experimental protocol followed the principles and guidelines suggested by Shenyang Agricultural University of Animal Experimentation (COBEA) and were approved by the local ethical committee.

2.3. Samples and processing

Tribulus terrestris L. leaves were gathered in Jilin province of China in September 2016 (No.1609), and were authenticated by professor Shaofan Du of Shenyang Agricultural University according to Compendium of Materia Medica. The voucher specimens KT/JL/CH/TTL/09/16 have been deposited College of Animal Husbandry and Veterinary, Shenyang Agricultural University.

0.5 g of *Tribulus terrestris* L. leaves powder was extracted twice by ultrasonic extraction method with a certain concentration of ethanol solution, ratio of solvent to material, and extraction time. The extraction solution was filtrated, combined and dried to yield a solid residue by vacuum rotary evaporation for further analysis.

2.4. Experimental design

2.4.1. Single factor experiments

According to the preliminary experiments and references, six levels of three major influence factors were set as follows: concentration of ethanol solution 0%, 20%, 40%, 60%, 80%, 100%, ratio of solvent to material 5, 10, 15, 20, 25, 30 ml/g, and extraction time 5, 10, 15, 20, 25, 30 min in single factor experiments, and the extraction yield of flavonoids fraction from *Tribulus terrestris* L. leaves was selected as the index. When evaluated any one of influence factors, the level of other two factors was the fourth one.

2.4.2. Optimization of extraction conditions by Box-Behnken design (BBD)

Based on the results of single factors experiments, a Box-Behnken design (Design-Expert software, Trial Version 8.0.6, State, Inc., Minneapolis, USA) of three variables and three levels was introduced to optimize the extraction parameters for flavonoids fraction from *Tribulus terrestris* L. leaves. Three independent variables including concentration of ethanol solution, ratio of solvent to material and extraction time, and their three levels were listed in Table 1. As shown in Table 2, the BBD was consisted of twelve factorial points and five replicates of central points in this experiment, and the yield of flavonoids fraction from *Tribulus terrestris* L. leaves was taken as dependent variable. Each experimental run was performed in triplicate except the five centre points.

2.4.3. Determination of total flavonoids content

Total flavonoids content (TFC) of *Tribulus terrestris* L. leaves was measured using aluminum nitrate-sodium nitrite-sodium hydroxide colorimetric method according to the previous reported (Tian et al., 2017). The absorbance value at 510 nm was used to determine TFC, which was expressed as percentage of rutin in *Tribulus terrestris* L. leaves.

Table 1

Independent variables and their levels in Box-Behnken design for flavonoids fraction of *Tribulus terrestris* L. leaves.

Levels	Factors		
	Ethanol concentration (X ₁)/(%)	Ratio of solvent to material (X ₂)/(ml/g)	Extraction time (X ₃)/(h)
-1	10	20	20
0	20	25	25
1	30	30	30

Table 2

Box-Behnken experimental design and the results for extraction yield of flavonoids fraction of *Tribulus terrestris* L. leaves (n = 3).

No.	Ethanol concentration (X ₁)/(%)	Ratio of solvent to material (X ₂)/(ml/g)	Extraction time (X ₃)/(min)	Yield of total flavonoids (%) ^a
1	30	30	25	0.25
2	20	25	25	0.24
3	20	20	20	0.22
4	20	30	20	0.23
5	30	25	30	0.25
6	20	25	25	0.24
7	20	25	25	0.24
8	30	20	25	0.25
9	10	20	25	0.20
10	30	25	20	0.23
11	20	20	30	0.23
12	20	30	30	0.25
13	20	25	25	0.24
14	20	25	25	0.25
15	10	25	30	0.21
16	10	30	25	0.23
17	10	25	20	0.20

^a Each value is the mean of triplicate measurements.

2.5. Component analysis

2.5.1. Sample preparation

0.5 g of *Tribulus terrestris* L. leaves powder was extracted with the optimized extraction conditions, and dried to yield a solid residue by vacuum rotary evaporation. The solid residue was dissolved with 10 ml of HPLC-grade methanol, and filtered by 0.22 µm microfiltration membrane prior to use in LC-MS analysis.

2.5.2. LC-MS² conditions and parameters

Flavonoids fraction was separated on a C₁₈ column (Waters platinum series, 250 mm × 4.6 mm, i.d. 5 µm) using acetonitrile (A) and 0.1% formic acid solution (B) as mobile phase. The procedure of gradient elution was 0%–45% of A for 0–45 min, and then 45%–0% of A for 45–50 min with a flow rate of 0.7 ml/min, column temperature 25 °C, and detection wavelength 350 nm. The operating conditions of HPLC-ESI-MS² were as follows: negative and positive ion modes, automatic secondary mass spectrum scan with the range 50–1000 m/z, drying gas 12 l/min and 350 °C, nebulizer pressure 30 psi, and capillary voltage 3500 V.

2.6. Antioxidant activity assay

2.6.1. Measurement of DPPH radical scavenging activity

The DPPH radical scavenging activity assay was carried out using the method described by Guo et al. (2013) with some modifications. The volume ratio was 1:9 for sample solution and 0.1 mmol/L DPPH working solution. The antioxidant activity was expressed with IC₅₀ (mg/ml), which is the sample concentration of inhibition 50% free radicals.

2.6.2. Measurement of ABTS radical scavenging activity

The ABTS radical scavenging activity was measured as the method of Lahouar et al. (2014) with minor modifications. The volume ratio of sample solution and ABTS working solution was the same with the method for measurement of DPPH radical scavenging activity.

2.6.3. Measurement of ferric-ion reducing antioxidant power

Ferric-ion reducing antioxidant power (FRAP) was measured according to the method of Griffin et al. (2004) with some modifications. An aliquot of samples solution was mixed with four times of FRAP reagent. FRAP was expressed as mmol Fe²⁺ per 100 µg/ml of sample.

2.7. Antibacterial activity assay

Antibacterial activity was evaluated with micro-well dilution method as described by Tian et al. (2017) with some modifications. Each well of 96-well plate was mixed with 100 µl of sample solution and bacterium solution, and then incubated at 37 °C for 24 h. Gentamicin and sterile water were used as positive and negative control, respectively, and MIC value was an evaluation index.

2.8. Acetic acid-induced writhing response

The analgesic activity of flavonoids fraction was determined by acetic acid-induced writhing response (Perazzo et al., 2013; Wang et al., 2013). Mice were randomly assigned to five groups as follows: (1) acetic acid model group: mice were intraperitoneal injection of 0.6% acetic acid (10 ml/kg). (2) positive group: 200 mg/kg of aspirin was intragastrically administered for three days before the injection of acetic acid. (3–5) flavonoids fraction of *Tribulus terrestris* L. leaves treatment groups: flavonoids fraction was administered by oral at a dose of 12.5, 25 and 50 g raw medicinal materials (RMM)/kg for five days before model establishment. One hour after the intraperitoneal injection of acetic acid, the writhing times was recorded in 20 min.

2.9. Xylene induced ear oedema

According to the method reported by Shabbir et al. (2018), the anti-inflammatory activity of flavonoids fraction was evaluated with xylene induced ear oedema model. Mice were intragastrically administered normal saline, aspirin (0.2 g/kg) and flavonoids fraction of *Tribulus terrestris* L. leaves with the concentration of 12.5, 25 and 50 g RMM/kg for five days. After 1 h of treatment, two sides of the right ear were smeared with 50 µl of xylene. The weight of two ears was measured after 40 min, and oedema was determined with the difference in right and left ear weight.

2.10. Anti-inflammatory activity against RAW 264.7 cells induced by LPS

2.10.1. Cell culture

RAW 264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) at 37 °C in a humidified atmosphere with supplemented 5%CO₂. The culture medium contained 10% fetal bovine serum and 100 U/ml of penicillin and streptomycin.

2.10.2. Samples solution preparation

Flavonoids fraction solution was prepared with the optimized extraction process, and dissolved completely with DMEM medium at a final concentration of 50, 100, and 200 µg/ml. Then, the mixture of flavonoids fraction solution and DMEM medium was filtered, packed, and preserved at -20 °C for later use.

2.10.3. Cell viability assay

According to the method reported by Kim et al. (2016) with some modifications, the cytotoxicity of flavonoids fraction against RAW 264.7 cells was evaluated using the MTT assay. Cells were cultured to a concentration of 1 × 10⁵ cells/well, and grouped into negative control group, Dex group (2 µmol/L), LPS model group and flavonoids fraction treatment group at concentrations of 50, 100 and 200 µg/ml. Then, 50 µl of LPS (5 µg/ml) was added to all the groups except control group with 50 µl of RPMI 1640 medium. After incubation for 18 h, 20 µl of MTT reagent (5 mg/ml) was supplemented and further culture for 4 h. Then, the supernatant was discarded and the residue was dissolved with 150 µl of DMSO, followed detected by microplate reader detection at 490 nm.

2.10.4. Phagocytic activity assay

According to the report by Tian et al. (2018), the phagocytic activity of RAW 264.7 cells was evaluated by neutral red. The cell inoculation density and pretreatment method were the same as cell viability assay. After incubated for 18 h, 100 µl of neutral red solution (100 mg/ml) was mixed and incubated continually for 30 min. Then, cells were sealed with 100 µl of cell lysis solution (ethanol: ice acetic acid = 1:1, v: v) at room temperature for overnight, and then measured at 540 nm with enzyme marker.

2.10.5. NO measurement

NO concentration was analyzed by the Griess reagent method with modifications described by Su et al. (2014). The sodium nitrite standard diluents and the supernatant were reacted with isovolumetric of solvent A, which was consisted by 1.0 g of anhydrous sulfanilic acid, 6 ml of 85% phosphoric acid and 94 ml of deionized water, and the reaction solution was incubated at 37 °C for 10 min. Then, 50 ml of 0.1% n-naphthylethylenediamine solution was added, and incubated again at 37 °C for 10 min. After incubation, the absorbance was measured at 540 nm, and the concentration of NO was calculated from an established sodium nitrite standard curve.

2.11. Statistical analysis

Each experiment was performed in triplicate, and the results were expressed as means ± standard deviation (SD). The response surface

experiment data were analyzed by using Design Expert 8.0.6 software and the rest of data were analyzed statistically by SPSS 17.0 (SPSS 17.0 for WINDOWS; SPSS Inc., Chicago, IL). In all statistical analyses, *p* values ≤0.05 were regarded as statistically significant and *p* values ≤0.01 as very significance.

3. Results and discussion

3.1. The optimization of single factor experiments

In this part, the effects of the concentration of ethanol solution, ratio of solvent to material, and ultrasonic extraction time on content of flavonoids fraction were evaluated with the single factor experiments, and the results were listed in Fig. 1.

In order to optimize the extraction technology of flavonoids fraction, six levels of the three single factors were as follows: 0%, 20%, 40%, 60%, 80%, 100% for concentration of ethanol solution (*X*₁), 5, 10, 15, 20, 25, 30 ml/g for ratio of solvent to material (*X*₂), and 5, 10, 15, 20, 25, 30 min for ultrasonic extraction time (*X*₃). In the evaluation of single factor experiments, the influence of any factor was investigated on the yield of flavonoids fraction, and the other two factors were set to the fourth level.

As can be seen from *X*₁ line chart in Fig. 1, the yield of flavonoids fraction increased with increasing of the concentration of ethanol solution from 0% to 20%, and the yield reached the highest point at 20%, and then decreased from 20% to 100%. This phenomenon might be due to the polarity of flavonoids fraction in *Tribulus terrestris* L. leaves was much similar with low concentration of ethanol solvent, namely, the polarity of flavonoid components in is relatively large. This indicated that it was not a positive correlation relation between extraction yield and concentration of ethanol solution.

As described in Fig. 1, the yield of flavonoids fraction improved with the increase of ratio of solvent to material (*X*₂) and ultrasonic extraction time (*X*₃). *X*₂ and *X*₃ line charts all indicated that a higher level presented a better yield of flavonoids fraction. However, the growth speed of the yield of flavonoids fraction began to slow down with increasing of ratio of solvent to material (*X*₂) and ultrasonic extraction time (*X*₃), which can be explained that the competitive inhibition of the impurities was increased with the increasing of ratio of solvent to material (*X*₂) and ultrasonic extraction time (*X*₃).

According to the results of single factor experiments for extraction of flavonoids fraction from *Tribulus terrestris* L. leaves, three levels of the three factors were adopted for the RSM experiments as follows in Table 1: concentration of ethanol solution (10, 20, 30%), ratio of solvent to material (20, 25, 30 ml/g), and ultrasonic extraction time (20, 25, 30 min).

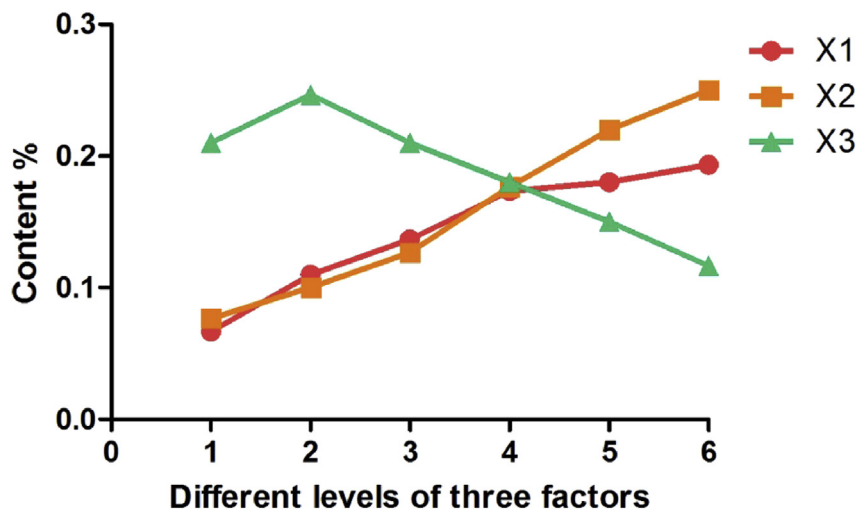


Fig. 1. Effect of ethanol concentration (*X*₁,%), ratio of solvent to material (*X*₂, ml/g) and extraction time (*X*₃, min) on the extraction yield of flavonoids fraction from *Tribulus terrestris* L leaves.

3.2. Optimization of extraction conditions by BBD

3.2.1. Model fitting and statistical analysis

As shown in Table 2, 17 experiments were performed for optimization the extraction conditions of flavonoids fraction from *Tribulus terrestris* L. leaves in the design matrix, and then the yield of flavonoids fraction was obtained as index.

In terms of coded factors, the final equation of flavonoids fraction was set up by multiple regression analysis with Design Expert software (version 8.0.6), as follows, $Y = 0.24 + 0.018X_1 + 0.0075X_2 + 0.0075X_3 - 0.0075X_1X_2 + 0.0025X_1X_3 + 0.0025X_2X_3 - 0.00975X_1^2 + 0.00025X_2^2 - 0.00975X_3^2$.

Table 3 described the analysis of variance for the experimental results of the BBD for flavonoids fraction from *Tribulus terrestris* L. leaves. The determined coefficients $R^2 = 0.9824$, which was presented by ANOVA of the quadratic regression model, indicated that only 1.76% of total variants cannot be explained by the model. The adjusted R^2 at 0.9598 suggested a better correlation between experimental values and predicted values. In addition, the lower *p*-value (*p* < 0.0001) indicated that the model can significantly represent the actual relationship between parameters and response. Meanwhile, a lower value of coefficient of the variation (C.V.) at 1.45 confirmed a higher degree of precision, accuracy,

Table 3 Analysis of variance of the experimental results of the BBD.

Variables	Sum of squares	DF	Mean square	F value	P value Prob > F
Model	0.004473	9	0.000497	43.49	<0.0001
<i>X</i> ₁	0.00245	1	0.00245	214.37	<0.0001
<i>X</i> ₂	0.00045	1	0.00045	39.37	0.0004
<i>X</i> ₃	0.00045	1	0.00045	39.37	0.0004
<i>X</i> ₁ * <i>X</i> ₂	0.000225	1	0.000225	19.69	0.0030
<i>X</i> ₁ * <i>X</i> ₃	0.000025	1	0.000025	2.19	0.1827
<i>X</i> ₂ * <i>X</i> ₃	0.000025	1	0.000025	2.19	0.1827
<i>X</i> ₁ * <i>X</i> ₁	0.0004003	1	0.0004003	35.02	0.0006
<i>X</i> ₂ * <i>X</i> ₂	0.000002632	1	0.000002632	0.023	0.8837
<i>X</i> ₃ * <i>X</i> ₃	0.0004003	1	0.0004003	35.02	0.0006
Residual	0.00008	7	0.00001143		
Lack of Fit	0.000	3	0.000	0.000	1.0000
Pure Error	0.00008	4	0.00002		
Cor total	0.004553	16			
<i>R</i> ²	0.9824				
Adj <i>R</i> ²	0.9598				
Pred <i>R</i> ²	0.9725				
Adeq Precision	19.284				
C.V.%	1.45				

reliability and reproducibility of the model.

As listed in Table 3, *p*-value was used to evaluate the statistical significance of regression coefficients in equation. The linear coefficients of concentration of ethanol solution (X_1), ratio of solvent to material (X_2) and ultrasonic extraction time (X_3) were all very significant ($p < 0.01$) for the extraction of flavonoids fraction from *Tribulus terrestris* L. leaves. In addition, the quadratic coefficients were very significant in X_1^2 and X_3^2 with *p* value 0.0006, but there was no significant difference ($p > 0.05$) for ratio of solvent to material (X_2^2). The interaction factors were observed very significant between concentration of ethanol solution (X_1), ratio of solvent to material (X_2) ($p < 0.01$). However, there were no statistical significance between X_2 and X_3 , X_1 and X_3 ($p > 0.05$).

Each of the experimental values was compared to the predicted value calculated by this model. The results suggested that the model used in this study were able to identify the optimum extraction condition for flavonoids fraction from *Tribulus terrestris* L. leaves.

3.2.2. Graphical interpretation and optimization of procedure

Fig. 2 were the 3D response surface curves showing the effects of concentration of ethanol solution (X_1), ratio of solvent to material (X_2) and ultrasonic extraction time (X_3) on the yield of flavonoids fraction. The interactions among concentration of ethanol solution, ratio of solvent to material and ultrasonic extraction time, along with the effects of these three variables and their mutual interaction on the yield of flavonoids fraction were depicted in Fig. 2. From Fig. 2a to c, it can be seen that the yield of flavonoids fraction was improved with the increase of each of the three factors in a certain range, and the intensive contour and steeper surface of concentration of ethanol solution suggested that concentration of ethanol solution has a higher influence on the yield of flavonoids fraction than ratio of solvent to material and ultrasonic extraction time.

The final equation for flavonoids fraction obtained in this research, was applied for response surface optimization using Design-Expert software. The optimal extraction parameters for flavonoids fraction were list as follows: the concentration of ethanol solution 25.87%, ratio of solvent to material 30:1 ml/g, and ultrasonic extraction time 27.93 min. Triple experiments were performed under these optimized conditions, and the yield of flavonoids fraction was 0.27%, which was almost identical to predicted results at 0.26%, indicating a good correspondence between experimental and predicted values. The suitability of the model equation for predicting the optimum response values was verified using the optimal condition.

3.3. Chemical component analysis of flavonoids fraction

The chemical components present in the flavonoids fraction from *Tribulus terrestris* L. leaves, were separated and analyzed by HPLC-DAD-ESI-MS². As shown in Fig. 3, the chromatogram was recorded with a higher signal at 350 nm Table 4 described the data of exact mass for MS or MS² fragmentation obtained from their mass spectra. In this research, 14 compounds, including (1) quercetin-3,7 diglucoside + Glc, (2) quercetin-3-O-sophoroside-7-O-glucoside, (3) quercetin-3-O-arabinosyl galactoside, (4) isorhamnetin-3-glucoside + Xyl, (5) quercetin-3,7 diglucoside + Xyl, (6) quercetin-3-O-(2,6- α -L-dirhamnopyranosyl- β -D-glucopyranoside), (7) kaempferol-glucosyl-(1 \rightarrow 2)-rhamnoside, (8) quercetin-3-O- β -(2(G)-O- β -xylopyranosyl-6(G)-O- α -rhamnopyranosyl) glucopyranoside, (9) quercetin-3-gentiobioside, (10) kaempferol-3-gentiobioside, (11) isoquercitrin, (12) 26-O- β -D-glucopyranosyl-(25R)-5 α -furostan-12-one- β -22 α , 26-triol-3-O- β -D-glucopyranosyl-(1 \rightarrow 4)- β -D-galactopyranoside, (13) luteolin-7-O- β -D-glucoside and (14) apigenin-6,8 glucoside + Xyl, were characterized tentatively by literatures (Feng et al., 2016; Pereira et al., 2012; Zheng et al., 2017).

The derivative of disaccharide or monosaccharide could be inferred indirectly by the difference value of molecular weight for the two fragments in the MS² spectra and the related literature information (Feng et

al., 2016; Pereira et al., 2012; Zheng et al., 2017). Compounds 11, 12 and 16 with high content will be used as analysis objects for component identification and analysis. In ESI⁻ mode, compound 11, which was quercetin-3-gentiobioside with calculated mass 626, produced a deprotonated molecular ion at *m/z* 625 [M-H]⁻ and fragment ion at *m/z* 300. For compound 12 kaempferol-3-gentiobioside, the characteristic fragment ion at *m/z* 285 was observed because of losing of gentiobioside at *m/z* 325 from the parent ion 625 [M-H]⁻. Compound 13 produced a [M-H]⁻ ion at *m/z* 581, and then the major fragment ion was obtained at *m/z* 463, which was apigenin aglycone fallen the disaccharide from the parent ion apigenin-6,8 glucoside + Xyl. In addition, two unknown compounds with molecular weight 728 and 798, were not identified by HPLC-DAD-ESI-MS², and will be analyzed in future studies. This study focused on inference the major components in the extract, and selection the ingredients with better content for further structural confirmation and analysis. However, the exact structure of hexose or aglycon from disaccharides derivatives, will be analyzed, inferred and identified with structural fragments from LC-MSⁿ more than MS² in the future. These studies will provide a foundation for the research on the quality control and pharmacological mechanism for flavonoids fraction from *Tribulus terrestris* L. leaves.

3.4. Antioxidant activity

Table 5 exhibited the antioxidant activities of flavonoids fraction from *Tribulus terrestris* L. leaves against DPPH and ABTS free radicals, and ferric ion. Flavonoids can display a better antioxidant activity due to the multiple phenolic hydroxyl groups in the structure. Flavonoids extract from mimenghua exhibited a better antioxidant activities demonstrated by scavenging capabilities of DPPH, hydroxyl and superoxide radicals, reducing power and total antioxidant capacity (Guo et al., 2013).

The antioxidant activities of flavonoids fraction were evaluated with IC₅₀ DPPH 10.47 μ g/ml and IC₅₀ ABTS 7.99 μ g/ml, which were both lower than the control of BHT (IC₅₀ DPPH, 8.17 μ g/ml; IC₅₀ ABTS, 2.31 μ g/ml). However, FRAP of flavonoids fraction was 0.12 mmol Fe²⁺/100 μ g/ml almost the same as BHT with 0.14 mmol Fe²⁺/100 μ g/ml. Therefore, flavonoids fraction from *Tribulus terrestris* L. leaves shown a mild antioxidant activity and may be a very interesting candidate for the research and development of natural, environmental and healthy antioxidant for the pharmaceutical and food industries.

3.5. Antibacterial activity

Many studies have shown that flavonoids have antibacterial activity. Liquiritin expressed a stronger antibacterial effect with IC₅₀ of 198.6 μ g/ml than liquiritigenin with IC₅₀ of 337.8 μ g/ml against *E. coli*, and liquiritin might be a promising candidate (Kong et al., 2015).

The antibacterial activities of flavonoids fraction from *Tribulus terrestris* L. leaves against *Escherichia coli* (ATCC 25922), *Salmonella* (ATCC 51812), *Staphylococcus aureus* (ATCC 25923) and *Streptococcus* (ATCC 49619), were listed in Table 6, and gentamicin was used as a positive control for ensuring the accuracy and reliability of assay method. As shown in Table 6, the inhibition effect of gentamicin was measured with MIC values ranged from 0.5 μ g/ml to 2.0 μ g/ml against *Escherichia coli*, *Salmonella*, *Staphylococcus aureus* and *Streptococcus*, which was in accordance with the related requirements of Clinical and Laboratory Standards Institute. In addition, flavonoids fraction exhibited a stranger antibacterial effect on gram-positive bacteria (MIC, 0.25 g RMM/ml) than gram-negative bacteria (MIC, 1.0 g RMM/ml). The results suggested that flavonoids fraction from *Tribulus terrestris* L. leaves was a candidate drugs for replacing or decreasing of antibiotic application in diseases caused by gram-positive bacteria. In the further research, we will study the antibacterial activity of main flavonoid compounds from flavonoids fraction, which will be an important foundation for the screening of new antibacterial drugs.

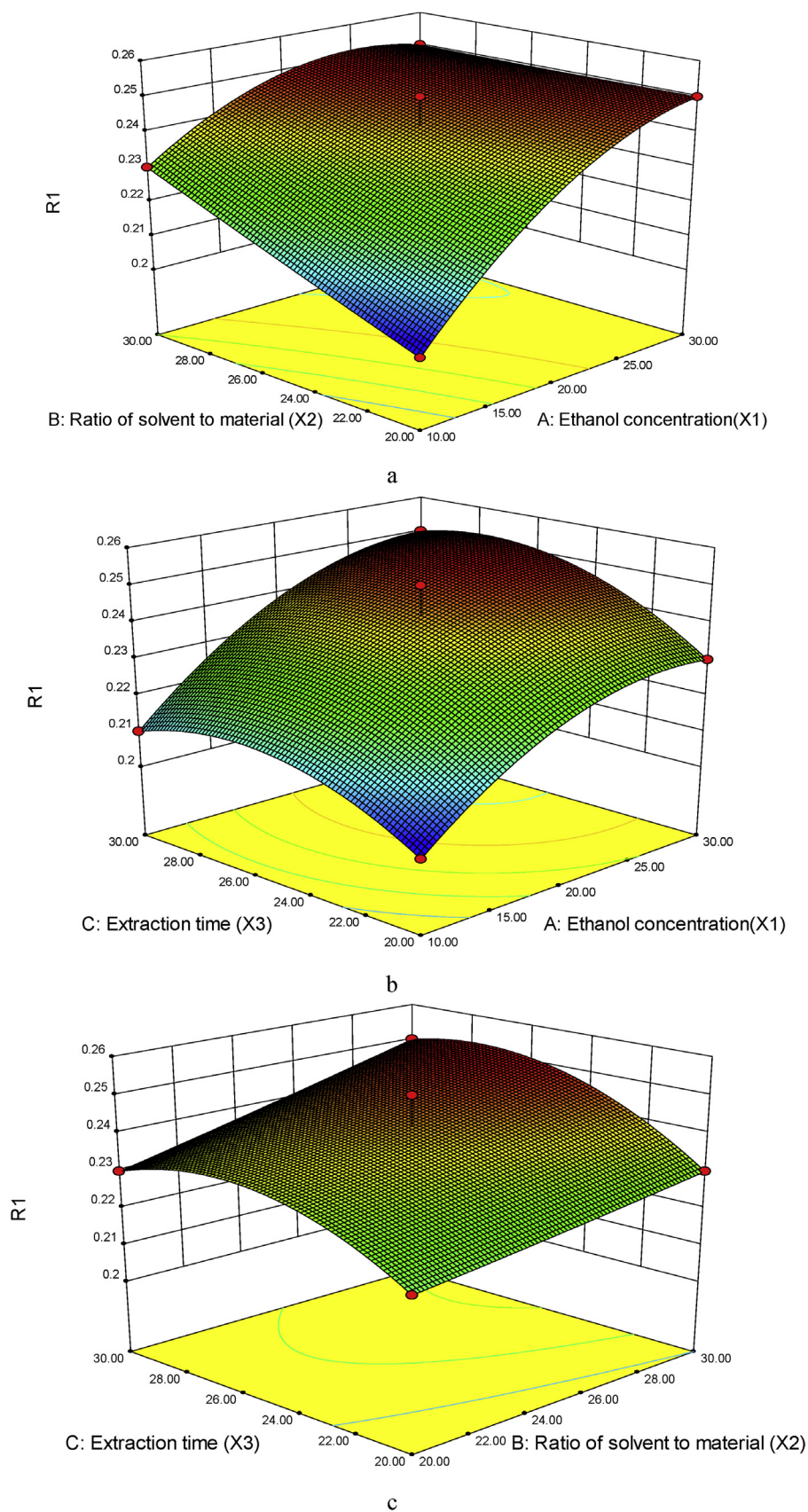


Fig. 2. (a–c) Response surface plots of ethanol concentration (X_1 ,%), ratio of solvent to material (X_2 , ml/g) and extraction time (X_3 , min) on the extraction yield of flavonoids fraction from *Tribulus terrestris* L leaves.

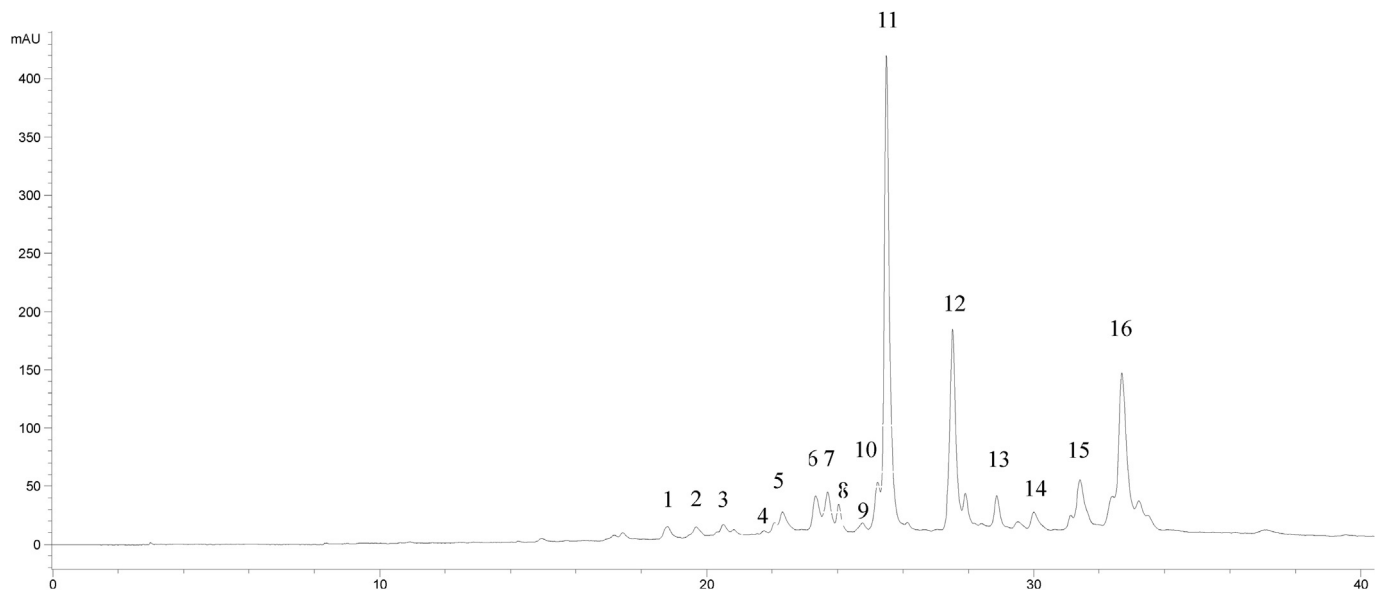


Fig. 3. Chromatographic profile of HPLC-MS at 350 nm for flavonoids fraction from *Tribulus terrestris* L. leaves.

3.6. Analgesic activity

Intragastric administration of 100, 200, 400 mg/kg of total flavonoids from *Sanguis draxonis* can alleviate significantly spared nerve injury-induced mechanical hypersensitivity by reducing the level of NO, NOS, TNF- α and IL-1 β , upregulation the level of IL-10, and inhibition the expression of FGFR3, GFAP and p-CREB in the spinal dorsal horn (Chen et al., 2015).

The analgesic activity of flavonoids fraction (12.5, 25 and 50 g RMM/

kg) from *Tribulus terrestris* L. leaves was evaluated with writhing response of mice induced by 0.6% acetic acid, and the writhing times were evaluation index. As described in Fig. 4, the writhing times decreased very significantly ($p \leq 0.01$) in the aspirin group compared to the control group, which was indicating that the pain model was established successfully and can be used for evaluation the analgesic effect of flavonoids fraction. In addition, 12.5, 25 and 50 g RMM/kg of flavonoids fraction decreased writhing times with a dose-dependent manner. Compared with the aspirin group, 25 g RMM/kg of flavonoids fraction exhibited similar

Table 4

Chemical compositions of flavonoids fraction from *Tribulus terrestris* L. leaves.

No.	Rt	[M-H] ⁺	MS ² [M-H] ⁺	Calculated mass	Formula	Proposed molecule	References ^a
1 ^b	18.79	787	625,301	788	C ₃₃ H ₄₀ O ₂₂	Quercetin-3,7 diglucoside + Glc	Zheng et al., 2017
2	19.66	727	709	728	-	Unknown ^c	-
3	20.49	771	609	772	C ₃₃ H ₄₀ O ₂₁	Quercetin-3-O-sophoroside-7-O-glucoside	Zheng et al., 2017
4	21.74	595	-	596	C ₂₆ H ₂₈ O ₁₆	Quercetin-3-O-arabinosyl galactoside	Zheng et al., 2017
5	22.07	609	447	610	C ₃₃ H ₅₄ O ₁₀	Isorhamnetin-3-glucoside + Xyl	Zheng et al., 2017
6	23.32	797	387	798	-	Unknown	-
7	23.69	757	625,300	758	C ₃₂ H ₃₈ O ₂₁	Quercetin-3,7 diglucoside + Xyl	Zheng et al., 2017
8	24.02	755	591	756	C ₃₃ H ₄₀ O ₂₀	Quercetin-3-O-(2,6- α -L-dirhamnopyranosyl- β -D-glucopyranoside)	Zheng et al., 2017
9	24.75	593	473	594	C ₂₇ H ₃₀ O ₁₅	Kaempferol-glucosyl-(1 \rightarrow 2)-rhamnoside	Zheng et al., 2017
10	25.22	741	609	742	C ₃₂ H ₃₈ O ₂₀	Quercetin-3-O- β -(2(G)-O- β -xylopyranosyl-6(G)-O- α -rhamnopyranosyl) glucopyranoside	Zheng et al., 2017
11	25.48	625	300	626	C ₂₇ H ₃₀ O ₁₇	Quercetin-3-gentiobioside	Zheng et al., 2017
12	27.51	609	285	610	C ₂₇ H ₃₀ O ₁₆	Kaempferol-3-gentiobioside	Zheng et al., 2017
13	28.86	463	301	464	C ₂₁ H ₂₀ O ₁₂	Isoquercitrin	Zheng et al., 2017
14	29.99	933	771	934	C ₄₅ H ₇₄ O ₂₀	26-O- β -D-glucopyranosyl-(25R)-5 α -furostan-12-one-3 β ,22 α ,26-triol-3-O- β -D-glucopyranosyl-(1 \rightarrow 4)- β -D-galactopyranoside	Zheng et al., 2017
15	31.40	447	-	448	C ₃₀ H ₁₈ O ₁₁	Luteolin-7-O- β -D-glucoside	Pereira et al., 2012
16	32.68	581	463	564	C ₂₆ H ₂₈ O ₁₄	Apigenin-6,8 glucoside + Xyl	Feng et al., 2016

^a The reference column relates previous reports on metabolites in different plants.

^b Peak number as in Fig. 4.

^c The compound was not identified in this research.

Table 5

IC₅₀ and FRAP values for evaluated antioxidant assays of flavonoids fraction from *Tribulus terrestris* L. leaves.

Sample	IC ₅₀ DPPH (μg/ml)	IC ₅₀ ABTS (μg/ml)	FRAP (mmol Fe ²⁺ /100 μg/ml)
Flavonoids fraction	10.47	7.99	0.12
BHT	8.17	2.31	0.14

IC₅₀, 50% of inhibition concentration.

Table 6

MIC values of flavonoids fraction from *Tribulus terrestris* L. leaves against pathogenic bacteria (Mean ± SD; n = 3).

Sample	<i>Escherichia coli</i> (ATCC 25922)	<i>Salmonella</i> (ATCC 51812)	<i>Staphylococcus aureus</i> (ATCC 25923)	<i>Streptococcus</i> (ATCC 49619)
Gentamicin (μg/ml)	2.00 ± 0.02	0.50 ± 0.01	0.50 ± 0.01	1.00 ± 0.01
Flavonoids fraction (g RMM/ml)	1.00 ± 0.03	1.00 ± 0.03	0.25 ± 0.04	0.25 ± 0.04

analgesic effect. The analgesic intensity of 50 g RMM/kg of flavonoids fraction was stronger than aspirin group ($p \leq 0.05$). However, the

analgesic action of 12.5 g RMM/kg of flavonoids fraction shown very significantly ($p \leq 0.01$) comparing to the aspirin group, namely, 12.5 g RMM/kg of flavonoids fraction exhibited a weaker analgesic action. Moreover, the effects of different concentrations of flavonoids fraction from *Tribulus terrestris* L. leaves on inhibition ratio (%) were the same with the writhing times in Fig. 5. The above result indicated that flavonoids fraction can reveal a better analgesic activity at a certain concentration, which was a foundation for the research and development of analgesic candidate drugs in clinic.

3.7. Anti-inflammatory activity in vivo

Rutin can treat vascular inflammatory diseases by suppressing the production of TNF- α and activation of NF- κ B (Lee et al., 2016). Moreover, rutin also can attenuate inflammation induced by cyclophosphamide through down regulate the content of inflammatory markers, such as TNF- α , IL-6 and expressions of p38-mitogen-activated protein kinase (MAPK), NF- κ B, i-NOS and COX-2 (Nafees et al., 2015).

To investigate the anti-inflammatory activities of flavonoids fraction from *Tribulus terrestris* L. leaves *in vivo*, the ear swelling was selected as inflammatory model induced by xylene in mice. According to the results shown in Fig. 6, the ear swelling degree (mg) decreased very significantly ($p \leq 0.01$) in the aspirin group compared to the control group. Moreover, 12.5, 25 and 50 g RMM/kg of flavonoids fraction can reduce the swelling degree in a dose-dependent manner. The anti-inflammatory activities of 12.5 and 50 g RMM/kg of flavonoids fraction exhibited very significantly

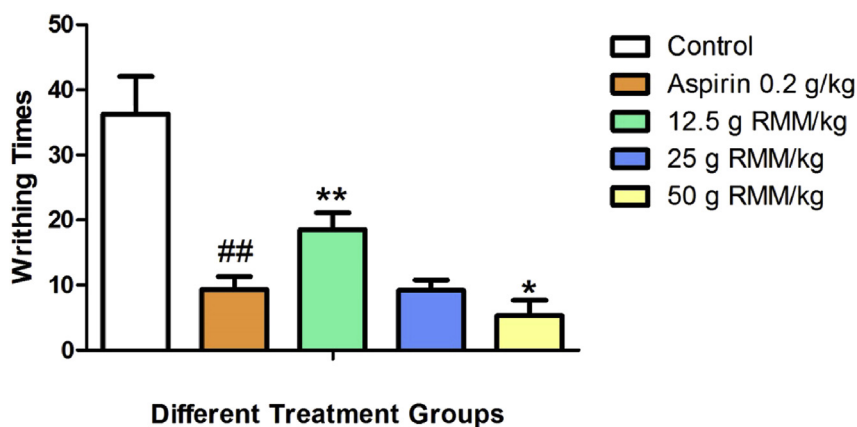


Fig. 4. Effects of different concentrations of flavonoids fraction from *Tribulus terrestris* L. leaves on writhing times induced by 0.6% acetic acid in mice. Mice were pretreated with flavonoids (12.5, 25 and 50 g RMM/kg) for five days, followed by 0.6% acetic acid stimulation. Values represent the mean ± SD of the three independent experiments (*compared with the control group, *compared with Aspirin treatment group, */# $p < 0.05$, **/# $p < 0.01$).

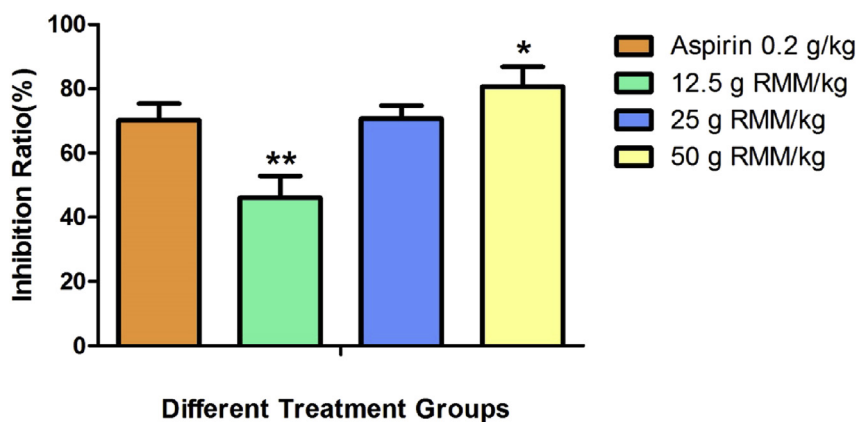


Fig. 5. Effects of different concentrations of flavonoids fraction from *Tribulus terrestris* L. leaves on inhibition ratio (%) induced by 0.6% acetic acid in mice. Mice were pretreated with flavonoids (12.5, 25 and 50 g RMM/kg) for five days, followed by 0.6% acetic acid stimulation. Values represent the mean ± SD of the three independent experiments (*compared with Aspirin treatment group, * $p < 0.05$, ** $p < 0.01$).

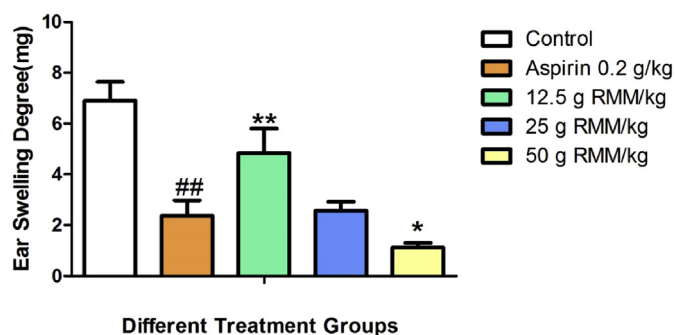


Fig. 6. Effects of different concentrations of flavonoids fraction from *Tribulus terrestris* L. leaves on ear swelling degree induced by xylene in mice. Mice were pretreated with flavonoids (12.5, 25 and 50 g RMM/kg) for five days, followed by xylene stimulation. Values represent the mean \pm SD of the three independent experiments (*compared with Aspirin treatment group, * $p < 0.05$, ** $p < 0.01$).

($p \leq 0.01$) and significantly ($p \leq 0.05$) comparing to the aspirin group, and there was no significant difference between 25 g RMM/kg of flavonoids fraction and significant aspirin group ($p > 0.05$). 25 g RMM/kg of flavonoids fraction exhibited similar anti-inflammatory effect to aspirin, and 50 g RMM/kg of flavonoids fraction revealed stronger anti-inflammatory activity. In addition, there was a similar trends in inhibition ratio (%), which was illustrated in Fig. 7. The research has demonstrated that the flavonoids fraction from *Tribulus terrestris* L. leaves exhibits a good anti-inflammatory activity *in vivo*, which will provide an important basis for the research and development of anti-inflammatory

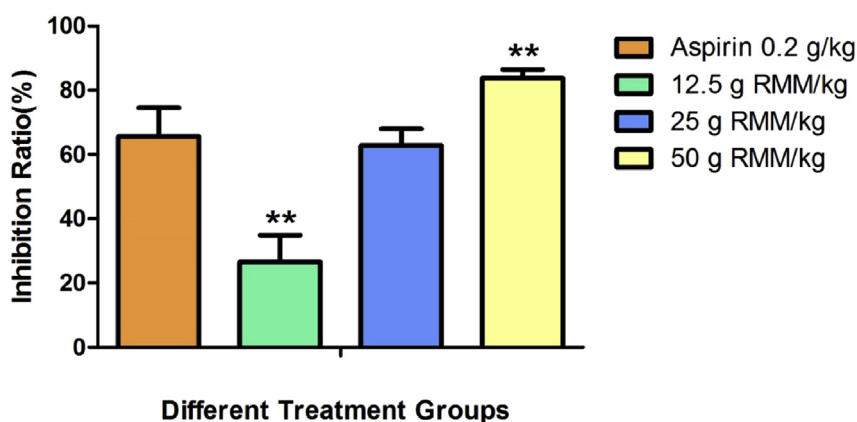


Fig. 7. Effects of different concentrations of flavonoids fraction from *Tribulus terrestris* L. leaves on anti-inflammatory activity induced by xylene in mice. Mice were pretreated with flavonoids (12.5, 25 and 50 g RMM/kg) for five days, followed by xylene stimulation. Values represent the mean \pm SD of the three independent experiments (# compared with the control group, *compared with Aspirin treatment group, */# $p < 0.05$, **/# $p < 0.01$).

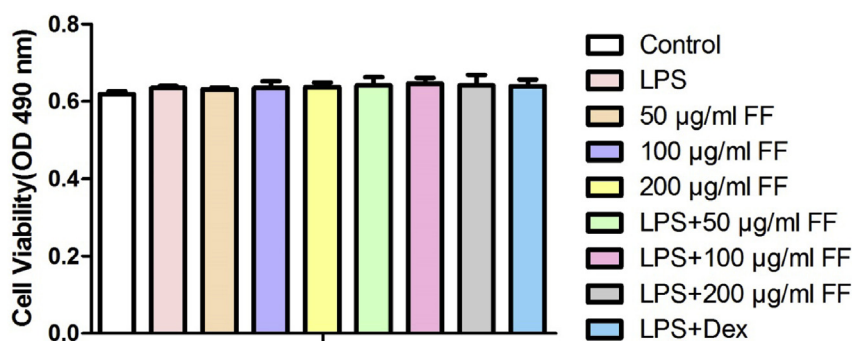


Fig. 8. Effect of flavonoids fraction (FF) on the viability of RAW 264.7 cells. Cells were cultured with FF (50, 100 and 200 µg/ml) in the absence or presence of 1 µg/ml LPS for 18 h; then, cell viability was measured by MTT assay. Data are presented as means \pm SD of three independent experiments.

Chinese medicine in clinic.

3.8. Anti-inflammatory activity *in vitro*

3.8.1. Effect of TFE on the cell viability

Cytotoxic effect of flavonoids fraction from *Tribulus terrestris* L. leaves on RAW 264.7 cells was measured by MTT assay. Cells were incubated with flavonoids fraction at the concentrations of 50, 100 and 200 µg/ml after pretreated with LPS or medium. As described in Fig. 8, the results indicated that cell viability was not significantly ($p > 0.05$) affected by flavonoids fraction in the experimental concentration range from 50 to 200 µg/ml, namely, the experimental concentration of flavonoids fraction is safe for RAW 264.7 cells.

3.8.2. Effect of TFE on the phagocytic activity

Flavonoids can play biological roles by binding to a variety of receptors on the surface of macrophages and activation different signaling pathways, and then enhance the phagocytic activity, promote the secretion of related cytokines, and stimulate the secretion of related inflammatory cytokines by macrophages. Compared with LPS model group, 20, 50 and 100 µM of rutin can reduce the phagocytic activity very significantly ($p < 0.01$), and there was no significant ($p > 0.05$) for 20 µM of rutin and Dex (Tian et al., 2019).

Cells were pretreated with 50, 100 and 200 µg/ml of flavonoids fraction for 1 h, followed by LPS (1 µg/ml) stimulation for 18 h, and then incubated with 100 µl of neutral red solution (100 mg/ml) for 30 min. As shown in Fig. 9, it was found that phagocytosis increased significantly ($p < 0.01$) after the LPS-induced comparing to the control group, which was revealed that the experiment model was established successfully. Moreover, flavonoids fraction can reduce phagocytosis significantly ($p <$

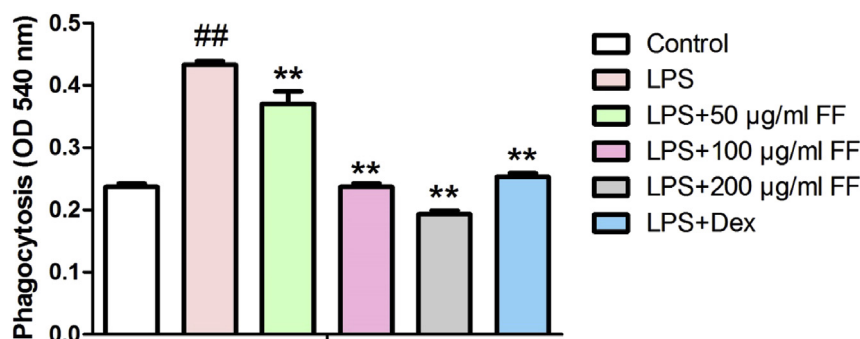


Fig. 9. Effects of different concentrations of flavonoids fraction (FF) on LPS induced phagocytic activity in RAW 264.7 cells. Cells were pretreated with FF (50, 100 and 200 µg/ml) for 1 h, followed by LPS (1 µg/ml) stimulation for 18 h. Values represent the mean \pm SD of the three independent experiments ([#]compared with the control, *compared with LPS, ^{*}/[#] $p < 0.05$, ^{**}/^{##} $p < 0.01$).

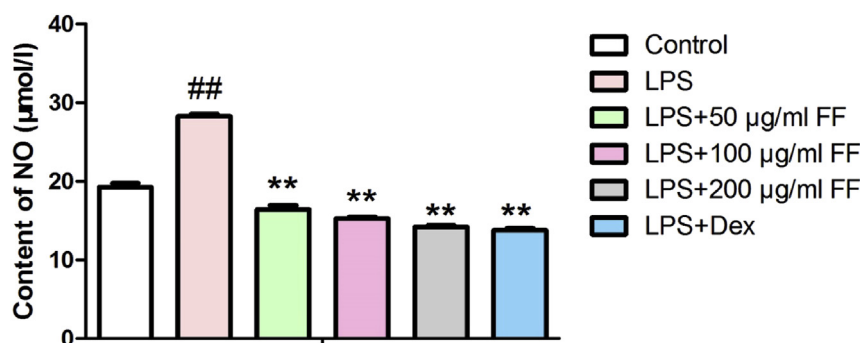


Fig. 10. Effects of different concentrations of flavonoids fraction (FF) on LPS induced NO production in RAW 264.7 cells. Cells were pretreated with FF (50, 100 and 200 µg/ml) for 1 h, followed by LPS (1 µg/ml) stimulation for 18 h. Values represent the mean \pm SD of the three independent experiments ([#]compared with the control, *compared with LPS, ^{*}/[#] $p < 0.05$, ^{**}/^{##} $p < 0.01$).

0.01) compared with the LPS treatment group in a dose-dependent manner. In addition, it was exciting that the 100 and 200 µg/ml of flavonoids fraction exhibited much better inhibition of phagocytic activity than Dex, which will be an important enlightening effect on the follow-up study.

3.8.3. Effect of TFE on the content of NO

As a major inflammatory molecule, NO plays an essential role in the regulation of inflammation. 5, 10, 20, 50 and 100 µM of rutin can decrease ($p < 0.01$) the content of NO with a dose-dependent manner compared with LPS model group, and the inhibitory effect of 100 µM of rutin was stronger than Dex ($p < 0.01$), and 5–50 µM of rutin was equivalent to Dex ($p > 0.05$), which suggested that low concentration of rutin can play the same role as Dex in reducing the content of inflammatory medium NO (Tian et al., 2019).

According to the results in Fig. 10, the content of NO was improved very significantly ($p < 0.01$) in LPS group comparing to the control group; and compared with the LPS group, flavonoids fraction groups were all very significantly ($p < 0.01$) decreased with a dose-dependent manner in the experimental groups pretreated by the concentration of 50, 100 and 200 µg/ml. Compared with dexamethasone, the inhibition effect of flavonoids fraction was a little weaker. However, there was more attention to the characteristics of low toxicity, less residue and no resistance of Traditional Chinese medicine.

4. Conclusion

This study focused on the extraction technology, component analysis, and antioxidant, antibacterial, analgesic and anti-inflammatory activities of flavonoids fraction from *Tribulus terrestris* L. leaves. The extraction process of flavonoids fraction was optimized by single factor experiment and response surface method, and the maximum extraction yield was

achieved with the concentration of ethanol solution 25.87%, ratio of solvent to material 30:1 ml/g, and ultrasonic extraction time 27.93 min. Moreover, 14 compositions were preliminarily identified from flavonoids fraction by HPLC-DAD-ESI-MS². In addition, flavonoids fraction exhibited a better antioxidant, antibacterial, analgesic and anti-inflammatory activities, and will be a potential candidate drug for further research and development of new, healthy and nature herbal medicine for food and pharmaceutical industries.

Declarations

Author contribution statement

Chunlian Tian: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yu Chang: Performed the experiments; Analyzed and interpreted the data.

Zehui Zhang: Performed the experiments.

Hong Wang: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Shibo Xiao, Cancan Cui: Contributed reagents, materials, analysis tools or data.

Mingchun Liu: Conceived and designed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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