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Study on Extraction, Identification, and Biological Activity of Compounds in Leaves of *Corylus mandshurica*

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Abstract *Corylus mandshurica* (CM) was a shrub plant of the *Betulaceae*. It was not only edible but also could be used as medicine, widely used in traditional folk medicine. However, there were few studies on the medicinal value of *CM Leaves*(CML)the by-product of *CM*. In this article, a studied was carried out on hairy *CML*. The content of its compounds was determined and the extraction process was optimized. The optimum process conditions were obtained by single factor test and response surface optimization method was followed as ethanol concentration 70 %, liquid-solid ratio 20ml/g, extraction temperature 60 °C and extraction time 2 h. In addition, the structures of 25 compounds in *CML* were identified by HPLC-ESI-MS / MS, including 14 flavonoids, 6 phenolic acids, 1 tannin, and 4 other compounds. The activities of anti-inflammatory, anti-tumor, and antibacterial of *CML* were studied. The results displayed that when the concentration of total flavonoids was 0.4 μg/ml and 4 μg/ml, they significantly inhibited the production of TNF-α, IL-6, and IL-1β in RAW264.7 induced by Lipopolysaccharide (LPS). The inhibitory effect of an ethanol extract from CML on the proliferation of Caco-2 cells was more potent than that on the expansion of SGC-7901 cells. The total flavonoid extraction of *CML* had different degrees of inhibition on *Staphylococcus aureus* and *Escherichia coli*, and the inhibition of *Escherichia coli* was greater than that of *Staphylococcus aureus* at the same concentration. This study laid a theoretical basis for developing and utilizing the medicinal value of CML.

Keywords: *flavonoid compounds, phenolic acid, anti-inflammatory, anti-tumor, antibacterial*

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1. Introduction

Hazelnut was one of the most popular nuts in the world. It was native to the Mediterranean region and has a history spanning thousands of years. Hazelnuts were rich in wild resources, mainly *Corylus heterophylla*, *Corylus ferox*, and *Corylus mandshurica*. Currently, many wild CM are in the Changbai Mountain (CBM) area of China [1]. CM was rich in fat, protein, cellulose, and vitamins and was eaten in various forms due to its unique flavor and sensory characteristics [2].

Among them, the processing of *hazelnuts* will produce many by-products and waste, including hazelnut skin, hazelnut shell, green leaf cover, and *hazelnut* leaves. These by-products were rich in bioactive compounds, proteins, dietary fiber, vitamins, etc. However, only a tiny part of them was developed and utilized. The hard shell of *hazelnut* was often used as a heat source during combustion, and there was little commercial development. *Hazelnut powder* and *hazelnut peel* were used as excipients for animal feed. A small part of hazelnut leaves was used to produce hazelnut tree fertilizer, and most of the *hazelnut leaves* were treated with waste [3]. In fact, these by-products had great utilization value. Kaliora AC, Kogiannou DA [4], and others proposed that the products of hazelnut (*Corylus heterophylla Fisch*) contain a large number of phytochemicals, mainly phenolic compounds, with antioxidant, anti-inflammatory, and antibacterial biological activities. Amaral; Oliveira et al [5].found a variety of phenolic compounds in hazelnut leaves and leaf buds, including quercetin glycosides (quercetin-3-O-galactoside, quercetin-3-O-glucoside, and quercetin-3-O-rhamnoside), quercetin, myricetin derivatives, and kaempferol-3-O-rhamnoside. Several phenolic acids include gallic acid, caffeic acid, p-coumaric acid, ferulic acid, octanoic acid, coffee-soluble tartaric acid, and caffeoylquinic acid. Therefore, it was necessary to develop and utilize the by-products of *hazelnuts*. On the one hand, it avoids the waste of resources; on the other hand, it also drives local economic development.

Flavonoids are a class of natural phenolic compounds synthesized in plants. Studies have shown that flavonoids have immunomodulatory, anti-inflammatory [6], and anticancer activities [7,8,9]. Researchers have been extensively studying techniques and conditions specifically for the extraction of flavonoids from natural products and foods either for analytical, preparative, or industrial purposes. Various techniques have been

proposed, including maceration, percolation, hydro-distillation, boiling, reflux, soaking, and soxhlet [10].

Because the content of flavonoids in *CML* was relatively high, the total flavonoids in *CML* were determined, and the extraction process was optimized. The structure of the compounds in the *CML* was identified and analyzed by HPLC-ESI-MS/MS. Finally, related biological activity experiments were carried out, including anti-inflammatory, anti-cancer, and antibacterial. The findings of this study provide a theoretical basis for further exploring the medicinal value of *CML*. To a certain extent, it has promoted developing and utilizing germplasm resources in CBM and enhanced local economic benefits.

2. Materials and Methods

2.1. Materials and Reagents

CML were provided by the CBM Ginseng Research Institute. HPLC-grade formic acid and acetonitrile were purchased from Shanghai Lingfeng Chemical Reagent Co., Ltd., methanol was purchased from Merck (analytically pure), and the remaining reagents were commercially available analytically pure. All ultrapure water was produced using the Milli-Q ultrapure water system (Millipore, France).

Standard epicatechin, gallic acid, kaempferol, rutin, and quercitrin were purchased from Shanghai Jingfeng Biotechnology Co., Ltd. Mouse mononuclear macrophage RAW264.7 was purchased from Guangzhou Saiku Biological Co., Ltd. RPMI-1640 medium purchased from Hyclone and lipopolysaccharide (LPS) were purchased from Shanghai Yuanye Biological Co., Ltd. The mouse TNF-α enzyme-linked immunosorbent assay kit, mouse IL-6 enzyme-linked immunosorbent assay kit and mouse IL-1β enzyme-linked immunosorbent assay kit were purchased from Wuhai Boster Biotechnology Co., Ltd., China. The human colorectal adenocarcinoma cell line Caco-2 and the human gastric cancer cell line SGC-7901 were purchased from Shanghai Chunmai Biotechnology Co., Ltd. PBS, DMSO, and MTT were purchased from Sigma Company, and dexamethasone acetate was purchased from the China Institute for Food and Drug Control. LB medium, *Escherichia coli*, and *Staphylococcus aureus* were made by our laboratory.

2.2. Sample Preparation

The *CML* of CBM were stored in a refrigerator at -80°C and pulverized to obtain hazelnut leaf powder. 10 g of CML powder was weighed and placed in a 50-mL centrifuge tube. 25 mL of 70% ethanol was added, mixed, extracted by ultrasound (250 W) for 20 min, centrifuged at 1500 r/min for 15 min, repeated extraction two times, and combined extract. The crude extract was removed from ethanol by a vacuum rotary evaporator, and the natural extract powder of CML was obtained by freeze-drying [11].

2.3. Determination of Total Flavonoid Content

The rutin standard was dried to a constant weight of 1.50 mg, and 100 ml was prepared with 70 % ethanol to obtain a 15 μg/ml standard solution. Precisely absorb rutin standard solution 0mL, 1mL, 2mL, 3mL, 4mL, and 5mL in a 25mL volumetric flask, add color reagent, and measure the absorbance at 510 nm with a visible spectrophotometer [12]. The standard curve was drawn with the absorbance (A) as the ordinate and the concentration of the standard solution (C) as the abscissa. The regression equation of rutin was $A = 0.011C + 0.0031$, $R = 0.9992$.

A certain amount of *CML* crude extract powder was weighed, the solvent was added, and ultrasonic extraction was performed. The obtained extract was extracted into a volumetric flask, and 1 % boric acid, 5 % $NaNO_2$, 10 % $Al(NO_3)_3$, 1 mol / L NaOH, and other chromogenic agents were added, respectively. After mixing evenly, add water to a constant volume of 100 ml. The above solution was placed at 20 °C for 2 h, and the absorbance value of each extract was determined at 510 nm [13]. The absorbance value was taken into the regression equation, and the yield of total flavonoids was yielded. The calculation formula is: The yield of total flavonoids = $(C \times V \times b) / m \times 100\%$, where C is the concentration of the measured solution, V is the volume after constant volume, b is the dilution multiple, and m is the quality of the hazelnut leaf crude extract powder.

2.4. Optimization of the Extraction Process of Total Flavonoids from CML

The crude extract powder of *CML* was weighed at 2.0 g and put into a 150-ml conical flask. The effects of liquid-solid ratio, extraction temperature, extraction time, and ethanol concentration on the yield of total flavonoids from CML were studied by a single-factor test, and the response surface optimization was carried out on this basis.

2.4.1. Single-Factor Experiment

Under a liquid-solid ratio of 20 ml/g, an extraction temperature of 70 °C, and an extraction time of 2 h, different ethanol concentrations (50%, 70%, 90%) were tested to select the best ethanol concentration [14,15]. Under the conditions of an ethanol concentration of 70%, an extraction temperature of 70 °C, and an extraction time of 2 h, different liquid-to-solid ratios (10, 15, 20, 25, 30 ml/g) were detected to select the best liquid-solid ratio. To choose the best extraction temperature, the effects of different extraction temperatures (40, 50, 60, 70, 80 °C) on the extraction rate were studied under the conditions of a liquid-solid ratio of 20 ml/g, an ethanol concentration of 70%, and an extraction time of 2 h. To select the best extraction time, the effects of different extraction times (1 h, 2 h, 3 h) on the extraction rate were studied under a liquid-solid ratio of 20 ml/g, an ethanol concentration of 70%, and an extraction temperature of 70 °C.

2.4.2. Response Surface Optimization

Based on the single-factor experimental data, the four factors of ethanol concentration (A), liquid-solid ratio (B), extraction temperature (C), and extraction time (D) were selected as independent variables, and the yield of total flavonoids from *CML* (Y) was used as the response value. The Design-Expert software was used for the four-factor, three-level response surface test. The response surface test factors and levels are exhibited in Table 1.

Table 1. Response Surface Test Factor Level Design

Factor		Level	
	-1	0	1
A:ethanol concentration(%)	50	70	90
B:liquid to material ratio(mL/g)	15	20	25
C:extraction temperature(°C)	50	60	70
D:extraction time(h)	1	2	3

2.5. Structural Identification

2.5.1. Preparation of a Sample Solution

Take 2 g of the crude extract powder of *CML* was placed in a 100 mL round bottom flask, 30 mL of 70% methanol was added to weigh the fixed mass, and ultrasonic (250 W) extraction for 30 min was followed by cooling and weighing the fixed mass. Methanol was used to compensate for the loss of mass shake well. After filtration by a 0.22μm microporous membrane, the filtrate was taken for later use [16].

2.5.2. Preparation of a Standard Solution

The standard substances of epicatechin, gallic acid, kaempferol, rutin, and quercitrin were weighed at 2.00 mg each. Each sample was placed in a 10 mL volumetric flask and added with a certain amount of ethanol for ultrasonication. After dissolution, add ethanol to a constant volume to obtain the stock solution of each monomer standard. The standard solutions were diluted ten times with ethanol before injection and filtered with a 0.22 μm microporous membrane to obtain the filtrate for testing [17].

2.5.3. Chromatographic Condition

Thermo Hypersil C18 (250 mm × 4.6 mm, 5 μm); 0.1% formic acid-water solution (mobile phase A)-acetonitrile solution (mobile phase B); elution gradient 0 ~ 10min, 5% ~ 12 % B; 10 ~ 23 min, 12 % ~ 30 % B; 23 ~ 34 min, 30% ~ 50 % B; 34 ~ 40 min, 50 % ~ 100 % B; 40 ~ 41min, 100 % ~ 5 % B. The column temperature was 35 °C, the injection volume was 3 μL, and the flow rate was 0.3 mL/min [18].

2.5.4. Mass Spectrometer Conditions

An electrospray ionization (ESI) source was used in the negative ion mode. The scan range was 50 ~ 1500 m/z. The drying gas volume flow rate was 6L • min^{-1}, the drying gas temperature was 280 °C, and the atomization pressure was 80 kPa. The capillary voltage was 4.3 kV, and the fragmentation voltage was 200 kV.

2.5.5. Chemical Composition Database Analysis

With the help of CNKI and other databases, we can retrieve the relevant literature on *CML* [19,20], comprehensively collect the molecular formula, molecular weight, structural formula, related chemical names, and other related information about the compounds in *CML*, and analyze an information database. With the help of the ChemSpider network database, the mass-to-charge ratio of [M-H]- in negative ion mode was calculated. The original mass spectrometry data of CML was imported into Peak View 2.2 software. The first-order mass number extracted from the total ion current diagram was matched with the information corresponding to its mol file and compared with the fragment information of the standard. The mass error was less than 5 ppm, and the structural matching degree was more than 80%. The chemical composition was identified.

2.6. Cell Anti-inflammatory Activity

2.6.1. Separation and Purification of Total Flavonoids from *CML*

According to the Wang [21] method, take 50 g of dried *CML* powder was taken, and 400 mL of 70 % ethanol was added according to the ratio of liquid-solid of 1:8. The reflux extraction was carried out for 2 h. The extract was combined three times, filtered, and the filtrate was dried by rotary evaporation to obtain the extract. The extract was dissolved in distilled water and introduced into the macroporous adsorption resin chromatographic column. After 24 h, the crude extract of total flavonoids from *CML* was obtained by gradient elution, merging, and drying with 40 %, 60 %, and 80 % methanol aqueous solution of 5 times column volume.

2.6.2. The Effect of Total Flavonoids from *CML* on TNF-α, IL-6, and IL-1β Content in RAW264.7 Cells Induced by LPS

RAW264.7 cells in the logarithmic growth phase were taken, and the cell concentration was adjusted to 4×10^5/mL and inoculated into 96-well plates for culture, 200 μL per well. After 24 h of culture at 37 °C and 5% CO2, the drug concentrations in the total flavonoids group were 0.4 μg/ml, 4 μg/ml, and 40 μg/ml, respectively. The final administration concentration of the dexamethasone acetate group was 0.869 μg/ml (2 μmol/l), drug intervention for 4 h. The final concentration of LPS in each well was 1 μg/ml, and the supernatant was taken after 24 h of continuous culture at 37 °C and 5% CO2. The contents of TNF-α, IL-6, and IL-1β were detected according to the instructions of the ELISA kit [22].

2.7. Study on the Anti-Tumor Effect of *CML* in Vitro

Human colorectal adenocarcinoma cell Caco-2 and human gastric cancer cell SGC-7901 were cultured in 1640 medium (containing 10% fetal bovine serum) and placed in a CO2 incubator at 37 °C (CO2 volume fraction of 0.05, relative humidity of 95%). Once every two or

three days, the cells in the logarithmic growth phase were taken for experiments.

The logarithmic growth phase cells were washed with PBS and digested with trypsin to make a cell suspension. The cells were counted on the cell counting plate to make the concentration of the cells 5×10^5/ml. The cells were inoculated in 96-well plates, and 100 μl was added to each well. After 24 hours of culture in the incubator, different concentrations of ethanol extract of *CML* were given. Each concentration was repeated in 6 wells, and 100 μl was added to each well. The positive control group was added with 5-Fu, and the blank control group was added with the same volume of culture solution. After 72 hours of incubation, 0.5 mg/mL MTT 100μl was added to each hole, and the culture was continued for 4 hours. The liquid in the well was discarded, and 150 μl of DMSO was added to each hole. The RT-6100 microplate reader was used to shake the horizontal oscillator for 10 minutes, and the absorbance (OD) value was measured at the wavelength of 620 nm. The following formula was used to calculate the inhibition rate of drugs on tumor cells: the difference between groups was analyzed, and the IC50 was calculated [23].

Cell growth inhibition rate IR = (control group OD value-experimental group OD value) / control group OD value × 100%

IC50 = drug concentration with 50% cell growth inhibition rate; the IC50 value was obtained using GraphPad Prism 8 software.

2.8. Antibacterial Experiment

The LB solid medium and bacterial suspension were prepared, and the bacteriostatic solution was prepared using a doubling dilution, the bacteriostatic solution was the total flavonoid extract of *CML*. The double dilution concentrations were 1000 μg/ml, 500 μ g/ml, and 250 μg/ml, respectively. In sterile operation, 100 μL of bacterial liquid was drawn and coated into a plate, and three groups of parallel experiments were carried out. The sterilized Oxford cups were placed on the surface of the medium, four in each dish, one without bacteriostatic solution as the control group, and the other three were added with different concentrations of bacteriostatic liquid. After 24 hours of culture in a 37°C CO2 constant temperature incubator, the growth of colonies was observed, and the diameter of the bacteriostatic circle was measured to compare the bacteriostatic effect [24].

Determination of minimal inhibitory concentration (MIC): The MIC of total flavonoid extract from CML was detected using *Staphylococcus aureus* and Escherichia coli as test bacteria. 50μL of the test bacterial suspension was diluted 100 times with an LB liquid medium. 50μl diluted bacterial solution was added into 2 ~ 6 holes of a sterile 96-well flat-bottomed micro-culture plate, and 100μl medium was added into the first hole as a negative control. Starting from the second hole, the total volume of the solution in each dilution hole was 100μl, and a series of dilution holes with decreasing drug concentrations were obtained by doubling the dilution method. Bacterial suspension and medium were added into the seventh hole as a positive control, and the results were observed after 24 h of culture in a 37°C constant temperature incubator [25]. Under the black background, the negative control hole solution should be clear and transparent, and the positive control hole bacteria should grow well; at this time, the drug concentration in the lowest concentration hole with a clear and transparent solution was the MIC.

2.9. Statistical Analyses

All tests were repeated three times, and the results are expressed as means ± standard deviations (X ± s.d.). SPSS19.0 statistical software was used for analysis of data variance; $P < 0.05$ was considered significant.

3. Results and Discussion

3.1. Single-Factor Test Results

As depicted in Figure 1a that the yield of total flavonoids in *CML* increases significantly with the addition of ethanol concentration, and the yield was the highest at 70%, which was 2.35%. Yields subsequently declined that this might be due to the higher the ethanol concentration, the easier it was to cause the dissolution of alcohol-soluble impurities, thus affecting the decomposition of flavonoid compounds [26]. The trend of Figure 1b was consistent with that of Figure 1a, which increases first and then decreases. This might be due to the saturation of the contact area with the rise in ethanol volume [27]. When the ratio of liquid-solid was 20 ml/g, the yield of total flavonoids was the highest, at 2.71%. Alessandro A. Casazza et al [28]. also reported similar results when studying the recovery of phenolic compounds in grape seeds: the effects of extraction time and solid-liquid ratio. Therefore, the optimal liquid-solid ratio was 20 mL/g.It was observed that from the Figure 1c that the yield of total flavonoids in *CML* began to increase slowly with the increase in temperature. This might be due to the decrease in solvent viscosity and the acceleration of molecular motion with the increase in temperature, which was beneficial to the release of bioactive substances from plant cells. However, higher temperatures promote the degradation of some thermosensitive compounds [29]. When the temperature was 70 °C, the yield of total flavonoids was the highest, at 2.62%. It was similar to the results Qian Xu et al.[30] reported on the effect of temperature on the extraction of flavonoids from Sophora flavescens. From the Figure 1d, that from 1h–2h, the yield of total flavonoids in *CML* increased with the increase of time, reaching a peak at 2h, and the extraction rate of total flavonoids was the highest, which was 2.33%. Then, it began to decrease significantly, which might be due to at the beginning of extraction, the release of flavonoid compounds from *CML* into solvent was promoted. The loss time of flavonoid compounds was too long, thus reducing the extraction rate [31]. Zun-Lai Sheng et al. [32] also reported similar results on the effect of time on the extraction of total flavonoids from poplar. Therefore, the best extraction time was 2 hours.

Table 2. Experimental Design and Results for Response-surface Analysis

Run	Factor 1 *A*:Ethanol concentration %	Factor 2 *B*: Liquid -solid ratio ml/g	Factor 3 *C*: Extraction Temperature ℃	Factor 4 *D*: Extraction Time h	Y: Total flavonoid yield %
1	70	25	60	1	2.29
2	70	20	50	1	2.39
3	70	20	60	2	2.78
4	50	15	60	2	2.23
5	70	20	70	3	2.58
6	90	20	70	2	2.43
7	70	20	70	1	2.367
8	70	15	50	2	2.33
9	70	15	60	1	2.16
10	70	25	50	2	2.35
11	70	20	60	2	2.79
12	50	20	70	2	2.5
13	50	20	60	1	2.347
14	50	25	60	2	2.45
15	70	15	60	3	2.44
16	70	20	60	2	2.78
17	70	25	60	3	2.42
18	50	20	60	3	2.4961
19	70	25	70	2	2.4
20	70	15	70	2	2.27
21	70	20	60	2	2.81
22	50	20	50	2	2.51
23	70	20	50	3	2.51
24	90	20	60	3	2.52
25	70	20	60	2	2.79
26	90	15	60	2	2.3
27	90	20	60	1	2.26
28	90	20	50	2	2.431
29	90	25	60	2	2.27

Table 3. Analysis of Variance (ANOVA) for the Quadratic Polynomial Mode

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.94	14	0.067	142.11	< 0.0001	significant
A-Ethanol concentration	8.65E-03	1	8.65E-03	18.37	0.0008	
B-Material liquid ratio	0.017	1	0.017	35.86	< 0.0001	
C-Extraction temperature	5.63E-05	1	5.63E-05	0.12	0.7345	
D-Extraction time	0.11	1	0.11	235.08	< 0.0001	
AB	0.016	1	0.016	33.21	< 0.0001	
AC	2.03E-05	1	2.03E-05	0.043	0.8386	
AD	3.08E-03	1	3.08E-03	6.53	0.0228	
BC	3.03E-03	1	3.03E-03	6.43	0.0238	
BD	5.63E-03	1	5.63E-03	11.95	0.0038	
CD	2.16E-03	1	2.16E-03	4.6	0.0501	
A^2	0.23	1	0.23	484.39	< 0.0001	
B^2	0.55	1	0.55	1173.09	< 0.0001	
C^2	0.14	1	0.14	297.74	< 0.0001	
D^2	0.22	1	0.22	461.41	< 0.0001	
Residual	6.59E-03	14	4.71E-04			
Lack of Fit	5.99E-03	10	5.99E-04	3.99	0.0972	Not significant
Pure Error	6.00E-04	4	1.50E-04			
Cor Total	0.94	28				

3.2. Response Surface Analysis

According to the existing experimental results, using Design Expert software for statistical regression analysis, the following second-order polynomial equation was obtained:

$$Y=2.79-0.027A+0.038B+0.002167C+0.096D-0.063AB+0.00225AC+0.028AD+0.027BC+0.037BD+0.023CD-0.19A^2-0.29B^2-0.15C^2-0.18D^2$$

A, *B*, *C*, and *D* were

ethanol concentration, liquid-solid ratio, extraction temperature, and extraction time, respectively. *Y* was the yield of total flavonoids.

From the analysis of the variance of the second-order polynomial regression model in Table 3, the F value was 142.11 ($p < 0.0001$), indicating that the regression model was highly significant. Due to noise, there was only a 0.01% chance of such a large model F value. The determination coefficient $R^2 = 0.993$ was close to 1, indicating that the actual value was consistent with the predicted value [33,34,35]. $R^2_{adj} = 0.986$, indicating that the model could predict most changes in total flavonoid content in *CML*; only 1.4% cannot be predicted. The model lack of fit $F = 3.99$, $P = 0.0972 > 0.05$, indicating that the mismatch was not significant, so the fitting degree of the model was good. According to the size of the F value, the order of influence of various factors on the yield of total flavonoids from *CML* was D > B > A > C.

As disclosed in Figure 2, the response surface and contour map are obtained by processing the regression analysis results. The three-dimensional (3D) response surface image displayed the interaction of any two independent variables on the dependent variable, and the shape of the three-dimensional response surface provided information on the degree of influence. The more significant the response value, the steeper the response surface; the contour lines between the corresponding parameters were elliptical, indicating that the relationship between the two was more significant. Figure 2a demonstrated the effects of ethanol concentration (A) and liquid-solid ratio (B) on the yield of total flavonoids. Figure 2b shows the effects of ethanol concentration (A) and extraction time (D) on the yield of total flavonoids. Figure 2c shows the impact of liquid-solid ratio (B) and extraction temperature (C) on the yield of total flavonoids. Figure 2d exhibited the effects of liquid-solid ratio (B) and extraction time (D) on the yield of total flavonoids. The yield of total flavonoids increased first and then decreased with the increase in ethanol concentration, liquid-solid ratio, extraction temperature, and extraction time. The results displayed that the optimum extraction conditions of total flavonoids from *CML* were within the designed experimental range, consistent with Ghafoor et al.'s research results [36].

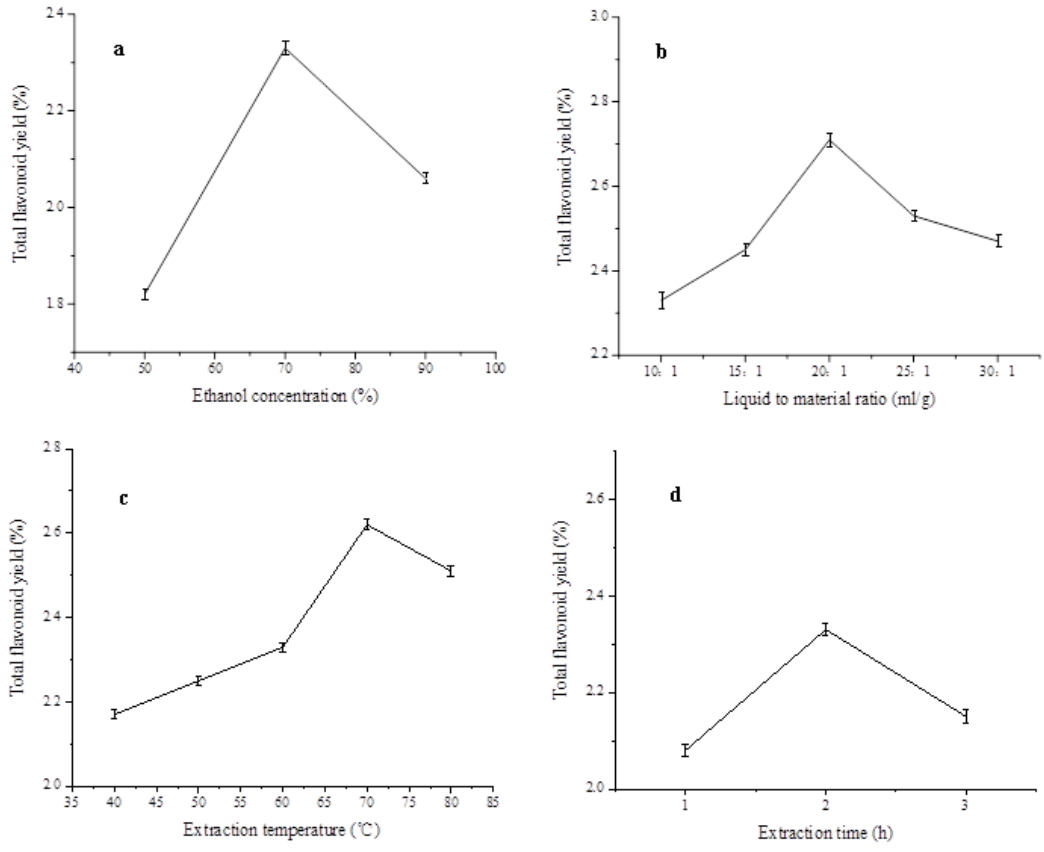

Figure 1. Effects of four single factors on yield of total flavonoids: (a) ethanol concentration, (b) liquid to material ratio, (c) extraction temperature, (d) extraction time. Note: there was a significant ($P < 0.05$) difference among the different yields of total flavonoid

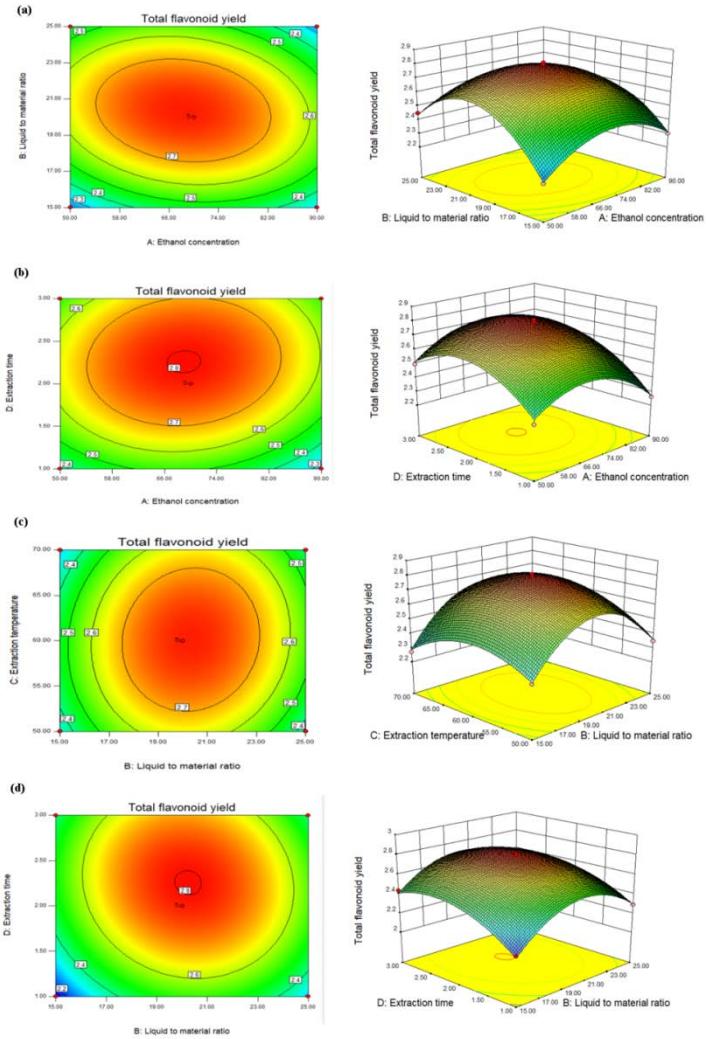

Figure 2. Effect of the interaction of various factors on yield of total flavonoids, including (a) ethanol concentration and liquid-solid ratio, (b) ethanol concentration and extraction time, (c) liquid-solid ratio and extraction temperature, (d) liquid-solid ratio and extraction time

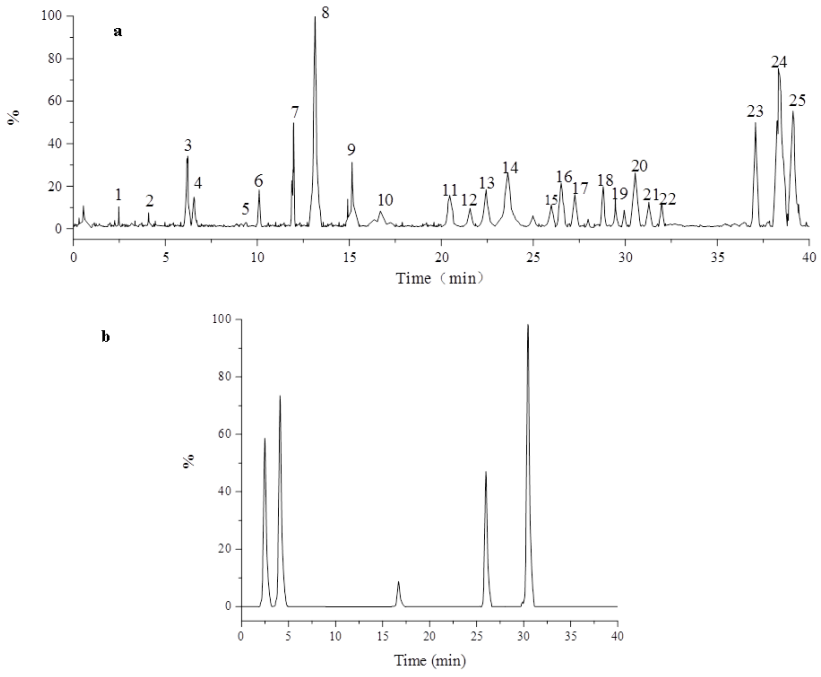

Figure 3. HPLC-ESI–MS/MS analysis of extract of Corylus mandshurica leaves. (a) the ESI-MS total ion current diagram (TIC) of the extract in negative ion mode, (b) the ESI-MS total ion current diagram (TIC) of the standards substance in negative ion mode

Using Design Expert 8.0 software, the optimum process conditions were obtained and recommended as the actual optimum process conditions: an ethanol concentration of 68.74%, a liquid-solid ratio of 20.28 ml/g, and an extraction temperature of 60.32°C. Under the conditions of an extraction time of 2.25 hours, the maximum yield of total flavonoids was 2.80411%. Considering the actual operation in the experiment, we adjusted the test value: ethanol concentration was 70%; the liquid-solid ratio was 20ml/g; the extraction temperature was 60°C; the extraction time was 2h.

3.3. Structural Identification

3.3.1. HPLC/MS Analysis of Compounds in *CML*

The methanol extract of *CML* was analyzed by HPLC-ESI-MS/MS. ESI negative ion mode was selected for mass spectrometry analysis because it was easy to provide a wide range of information through collision-induced dissociation (CID) fragments. Under the optimized HPLC-ESI-MS/MS conditions, by comparing the retention time (tR) of each standard with the retention time (tR) of the mass spectrometry data and the reference standard, 25 compounds in the extract of *CML* could be identified [37]. The total ion flow diagram was manifested in Figure 3. As demonstrated in Figure 3 that under the same analytical conditions, the retention time and molecular ion m/z of compounds 1, 2, 10, 15, and 20 (molecular ion m/z were 289, 169, 285, 609, and 447, respectively) in the methanol extract of *CML* were consistent with those of the five standard substances. It could be preliminarily judged that these five substances were epicatechin, gallic acid, kaempferol, rutin, and quercitrin [38]. Through HPLC-ESI-MS/MS analysis and identification, 25 compounds in the extracted *CML* were inferred, including 14 flavonoids, 6 phenolic acids, 1 tannin, and 4 other compounds, as displayed in Table 4.

Table 4. The structures of compounds in Corylus mandshurica leaves were identified by HPLC-ESI-MS/MS method

Peak no.	T_R(min)	$[M-H]^-$ (m/z)	Molecular formula	ESI-MS^2	Identification	Type
1	2.47	289.2707	$C_{15}H_{14}O_6$	137$[M-H-C_8H_8O_3]^-$, 123$[M-H-C_9H_{10}O_3]^-$	Epicatechin	Flavonoids
2	4.09	169.1203	$C_7H_6O_5$	125$[M-H-CO_2]^-$, 107$[M-H-CO_2-H_2O]^-$	Gallic acid	Phenolic acids
3	6.22	353.3220	$C_{16}H_{18}O_9$	163$[M-H-C_7H_{10}O_6]^-$	Chlorogenic acid	Phenolic acids
4	6.56	167.3594	$C_8H_8O_4$	152$[M-H-CH_3]^-$, 108$[M-H-CH_3-CO_2]^-$	Vanillic acid	Phenolic acids
5	9.38	471.7313	$C_{30}H_{48}O_4$	435$[M-H-H_2O_2]^-$, 407$[M-H-H_2O_2]^-$	Crategolic acid	Phenolic acids
6	10.10	357.5302	$C_{20}H_{22}O_6$	342$[M-H-CH_3]^-$, 327$[M-H-CH_2O]^-$	Pinoresinol	Flavonoids
7	11.97	575.8660	$C_{35}H_{60}O_6$	413$[M-H-C_6H_{10}O_5]^-$	Daucosterol	Other kinds
8	13.13	507.5539	$C_{23}H_{24}O_{13}$	345$[M-H-C_6H_{10}O_5]^-$, 327$[M-H-C_6H_{10}O_5-H_2O]^-$	Syringetin 3-O-glucoside	Flavonoids
9	15.12	633.9125	$C_{36}H_{58}O_9$	471$[M-H-C_6H_{10}O_5]^-$, 427$[M-H-C_6H_{10}O_5-CO_2]^-$	Siaresinolic acid 28-O-beta-D-glucopyranosyl ester	Other kinds
10	16.69	285.2363	$C_{15}H_{10}O_6$	151$[M-H-C_8H_6O_2]^-$	Kaempferol	Flavonoids
11	20.46	301.2482	$C_{15}H_{10}O_7$	180$[M-H-C_7H_5O_2]^-$,151$[M-H-C_8H_6O_3]^-$	Quercetin	Flavonoids
12	21.56	491.5802	$C_{26}H_{20}O_{10}$	311$[M-H-C_9H_8O_4]^-$,293$[M-H-C_9H_{10}O_5]^-$	Salvianolic acid C	Phenolic acids
13	22.44	153.2798	$C_7H_6O_4$	109$[M-H-CO_2]^-$	2,3-Dihydroxybenzoic acid	Phenolic acids
14	23.62	179.1701	$C_6H_{12}O_6$	87$[M-H-C_3H_8O_3]^-$	1,2,3,4,5,6-Cyclohexanehexol	Other kinds
15	25.98	609.6490	$C_{27}H_{30}O_{16}$	463$[M-H-C_6H_{10}O_4]^-$, 301$[M-H-C_6H_{10}O_4-C_6H_{10}O_5]^-$	Rutin	Flavonoids
16	26.52	433.5003	$C_{20}H_{18}O_{11}$	354$[M-H-C_2H_7O_3]^-$, 301$[M-H-C_5H_8O_4]^-$	Guaijaverin	Flavonoids
17	27.26	477.4457	$C_{21}H_{18}O_{13}$	301$[M-H-C_6H_8O_6]^-$, 164$[M-H-C_6H_8O_6-C_7H_5O_3]^-$	Quercetin-3-O-glucuronide	Flavonoids
18	28.80	299.4262	$C_{16}H_{12}O_6$	285$[M-H-CH_2]^-$, 256$[M-H-CH_2-CHO]^-$	Chrysoeriol	Flavonoids
19	29.49	463.5081	$C_{21}H_{20}O_{12}$	301$[M-H-C_6H_{10}O_5]^-$, 135$[M-H-C_6H_{10}O_5-C_8H_6O_4]^-$	Hyperoside	Flavonoids
20	30.54	447.4915	$C_{21}H_{20}O_{11}$	301$[M-H-C_6H_{10}O_4]^-$, 135$[M-H-C_6H_{10}O_4-C_8H_6O_4]^-$	Quercitrin	Flavonoids
21	31.28	463.7099	$C_{21}H_{20}O_{12}$	301$[M-H-C_6H_{10}O_5]^-$, 135$[M-H-C_6H_{10}O_5-C_8H_6O_4]^-$	Isoquercitrin	Flavonoids
22	31.98	331.2936	$C_{16}H_{12}O_8$	300$[M-H-CH_3O]^-$, 215$[M-H-CH_3O-H_5O_5]^-$	Laricitrin	Flavonoids
23	37.08	492.5662	$C_{23}H_{25}O_{12}$	330$[M-H-C_6H_{10}O_5]^-$, 314$[M-H-C_6H_{10}O_6]^-$	Malvidin 3-O-glucoside	Other kinds
24	38.32	935.8254	$C_{41}H_{28}O_{26}$	633$[M-H-C_{14}H_6O_8]^-$	casuarictin	Tannins
25	39.12	431.6691	$C_{21}H_{20}O_{10}$	285$[M-H-C_6H_{10}O_4]^-$, 135$[M-H-C_6H_{10}O_4-C_8H_6O_3]^-$	Ternatumoside II	Flavonoids

3.3.2. Analysis of Mass Spectrometry Fragmentation Characteristics of Compounds

Analysis and identification of flavonoids.

Flavonoids were chemical constituents found in many Chinese herbal medicines, and their mass spectrometric cleavage laws have been extensively reported in the literature by Shiqi Fang, Ana Plazonić, and Qingguo Tian et al [39,40,41]. A total of 14 flavonoids were identified in the experiment. Taking compound 8 as an example, in ESI- mode, the molecular ion mass charge ratio m/z 507$[M-H]^-$, the collision produces fragment ions m/z 345$[M-H-C_6H_{10}O_5]^-$ and m/z 151$[M-H-C_6H_{10}O_5-C_{10}H_9O_4]^-$, and the fragment ion m/z 345 was created by the molecular ion losing a molecule of glucoside. The fragment ion m/z 151 was generated by the further loss of a molecule of dimethyl phthalate by m/z 345, which is inconsistent with the results of fragmentation ions m/z 283 $[M-H-C_8H_{16}O_7]^-$ and m/z 151 $[M-H-C_8H_{16}O_7-C_8H_4O_2]^-$ reported by Luca Calani [42]. This might be due to different ion sources in mass spectrometry conditions and different fragmentation voltages. According to the broken fragment ions, it could be inferred that compound 8 was syringetin-3-O-glucoside, and the possible cleavage mode was exhibited in Figure 4.

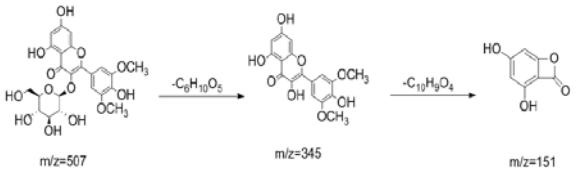

Figure 4. MS^2 fragmentation pattern of syringetin-3-O-glucoside

Analysis and identification of phenolic acid compounds.

Phenolic acid compounds were widely distributed in the roots and leaves of many Chinese herbs, are their mass spectrometric cleavage patterns were reported in the literature by Meng Ding, Tian-Min Wang, Taiping Li, et al [43,44,45]. A total of six phenolic acid compounds were identified in the experiment. In the case of compound No. 2, for example, the molecular ion mass-to-charge ratio in ESI-mode was m/z 169$[M-H]^-$, and after the collision produced fragment ions m/z 125 $[M-H-CO_2]^-$ and m/z 107 $[M-H-CO_2-H_2O]^-$, fragment ion m/z 125 was produced by the loss of one molecule of CO_2 by the molecular ion. Fragment ion m/z 107 was produced by further losing one water molecule by m/z 125, which agrees with the study's results by Ram M. Uckoo et al [46], proposed for both the cleavage pattern and polarity. Therefore, it was inferred that compound 2 was gallic acid, and the possible fragmentation mode was manifested in Figure 5.

Analysis and identification of tannin compounds.

Tannins were phenolic compounds with complex structures widely distributed in plants, especially bark. However, there were few reports on tannins in *CML* [47]. One tannin compound was identified in the experiment. Taking compound 24 as an example, the molecular ion mass-to-charge ratio in ESI-mode was m/z 935$[M-H]^-$, and the fragment ion m/z 633$[M-H-C_{14}H_6O_8]^-$ was produced by the collision. Fragment ions m/z 633 were produced by the loss of one molecule of ellagic acid by the molecular ion, consistent with the cleavage law proposed by Fang Chen et al [48]. Therefore, compound 24 was casuarictin. The possible cleavage mode is displayed in Figure 6.

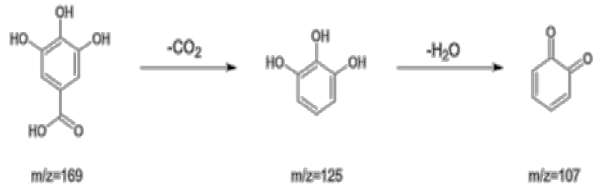

Figure 5. MS^2 fragmentation pattern of gallic acid

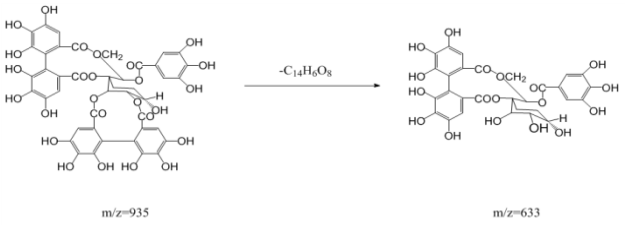

Figure 6. MS^2 fragmentation pattern of casuaricti

3.4. Cell Anti-inflammatory Ability

IL-6, IL-1β, and TNF-α were the three major pro-inflammatory cytokines responsible for activating the immune response. Their overexpression was associated with a variety of chronic diseases, including cancer, type II diabetes, and rheumatoid arthritis [49]. The pleiotropic nature of IL-6 was not only involved in cancer-related inflammation but also plays a vital role in DNA damage repair, the antioxidant defense system, proliferation, invasion, metastasis, angiogenesis, liver acute phase protein synthesis, lymphocyte differentiation, and metabolic remodeling [50]. IL-1β was essential in the inflammatory response and was involved in various cellular activities, including cell proliferation and differentiation. It was also associated with autoimmune metabolism, type 2 diabetes, Alzheimer's disease, obesity, cancer, and atherosclerosis. TNF-α was mainly secreted by macrophages and lymphocytes in response to cell damage caused by infection or malignant transformation [51]. Therefore, reducing IL-6, IL-1β, and TNF-α levels was significant.

3.4.1. The Effect of total flavonoids from *CML* on the Expression of TNF-α in RAW264.7 Cells Induced by LPS

In this experiment, the expression level of TNF-α in RAW264.7 cells induced by LPS was detected by ELISA. The results revealed that, compared with the model group (the LPS group), the total flavonoids of *CML* could significantly inhibit the expression of TNF-α in RAW264.7 cells, and the presentation of TNF-α decreased with the increase in total flavonoids concentration. [52]And when the concentration of total flavonoid of *CML*(CMLTF) was 4 μg/ml and 40 μg/ml, the expression of TNF-α could be significantly reduced ($P < 0.01$) (Figure 7a).

3.4.2. The Effect of total flavonoids from the *CML* on the Expression of IL-6 in RAW264.7 Cells Induced by LPS

ELISA detected the expression level of IL-6 in RAW264.7 cells induced by LPS. The results indicated that compared with the model group, the total flavonoids of *CML* could significantly inhibit the expression of IL-6 in RAW264.7 cells. With the increase in total flavonoids, the expression of IL-6 decreased. When the concentration of total flavonoids in *CML* was 4 μg/ml and 40 μg/ml ($P < 0.01$), the expression of IL-6 could be significantly inhibited ($P < 0.01$)(Figure 7b).

3.4.3. The Effect of total flavonoids from the *CML* on LPS-induced IL-1β Expression in RAW264.7 Cells

ELISA detected the expression level of IL-1β in RAW264.7 cells induced by LPS. The results exhibited that, compared with the model group, the total flavonoids of *CML* could significantly inhibit the expression of IL-1β in RAW264.7 cells. With the increase in the concentration of total flavonoids in *CML*, the expression of IL-1β decreased. When the concentration of total flavonoids in *CML* was 4 μg/ml and 40 μg/ml, the level of IL-1β could be significantly reduced ($P < 0.01$) (Figure 7c).

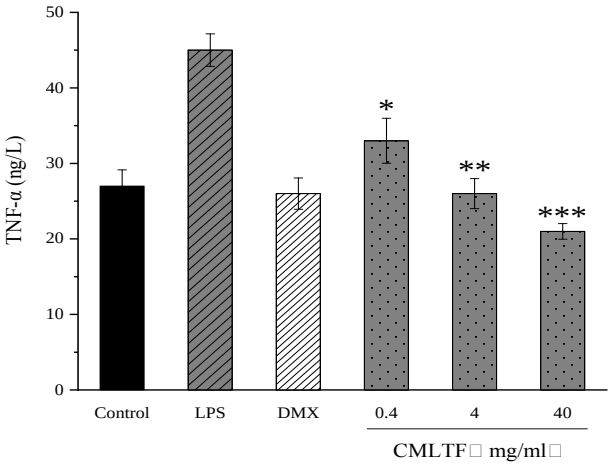

Figure 7a. The effect of CMLTF on the content of TNF-α in LPS-induced RAW264.7 cells (*P<0.05, **P<0.01 vs LPS group.)

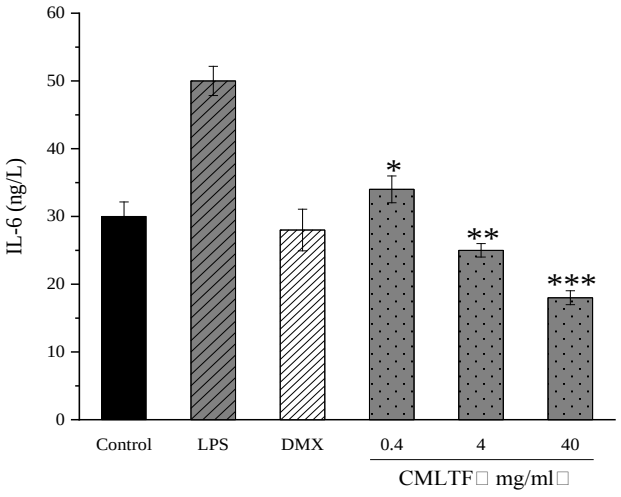

Figure 7b. The effect of CMLTF on the content of IL-6 in LPS-induced RAW264.7 cells (*P<0.05, **P<0.01 vs LPS group.)

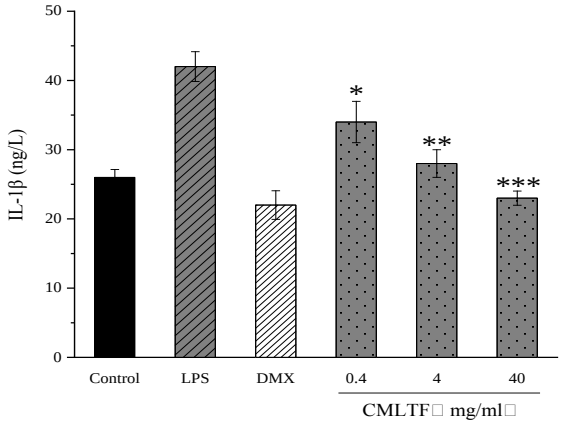

Figure 7c. The effect of CMLTF on the content of IL-1β in LPS-induced RAW264.7 cells (*P<0.05, **P<0.01 vs LPS group.)

3.5. Anti-tumor Effect of *CML* in Vitro

3.5.1. Effect of Ethanol Extract from the *CML* on the Proliferation of SGC-7901 Cells

Table 5 displayed that the ethanol extract of *CML* with different concentrations was given to the human gastric cancer cell SGC-7901. After 60 h, it had a significant inhibitory effect on the proliferation and growth of cells, and the greater the drug concentration, the stronger the inhibitory effect, which was consistent with the results of Liyan Song [53]. This might be due to the tumor cells not fully contacting and absorbing the drug before 60 hours, resulting in an insignificant inhibitory effect. After 60 h, with the tumor cells entirely in contact with the drug, the inhibitory effect was more apparent, and the larger the concentration, the stronger the inhibitory effect. Compared with the blank group, the OD values of each dose group were significantly different ($P < 0.05$), and the IC_{50} was 125.3 μg/ml.

Table 5. Inhibitory Effect of Ethanol Extract from Leaves of Corylus mandshurica on Proliferation of SGC-7901 Cells ($\bar{X}\pm S$, n=6)

group	dosage (μg/ml)	hole count	OD value	inhibitory rate (%)
blank group	-	6	0.459±0.014	-
5-Fu	20	6	0.248±0.005*	45.96
ethanol extract	50	6	0.386±0.002*	15.90
ethanol extract	100	6	0.265±0.011*	42.26
ethanol extract	150	6	0.179±0.017*	61.00
ethanol extract	200	6	0.144±0.012*	68.62
ethanol extract	250	6	0.123±0.005*	73.20
ethanol extract	300	6	0.106±0.008*	76.90
ethanol extract	350	6	0.091±0.013*	80.17

annotation: *Compared with the blank group $p < 0.05$

3.5.2. Effect of Ethanol Extract from the CML on the Proliferation of Caco-2 Cells

Table 6 demonstrated that the human colorectal adenocarcinoma cell Caco-2 was given differentconcentrations of ethanol extract from CML. After 60 h, the growth and proliferation of cells were significantly inhibited. With the increase in drug concentration, the inhibitory effect was enhanced. Compared with the blank group, the OD value of each dose group was significantly different ($P < 0.05$), and the IC50 was 121.2 μg/ml.

The MTT assay was based on the fact that the dehydrogenase in the mitochondria of living cells could metabolize and reduce MTT, which could reduce the yellow MTT to blue-purple insoluble formazan, which was soluble in dimethyl sulfoxide (DMSO). There was no such enzyme in dead cells, and MTT cannot be reduced [54]. Therefore, whether the tumor cells were affected could be determined by the color change and the depth of the change. This method is simple and has high sensitivity, good reproducibility, and a good signal-to-noise ratio. The experimental results manifested that the ethanol extract of *CML* had different inhibitory effects on the proliferation of SGC-7901 cells and Caco-2 cells, and the inhibitory effect on the addition of Caco-2 cells was more substantial than that on the expansion of SGC-7901 cells. Caco-2 cells and SGC-7901 cells were given different concentrations of extracts; after 72 h, the growth of tumor cells was significantly inhibited, and the effect was enhanced with the increase in drug concentration, which was different from the results obtained in Ali H. Ad'hiah [55]. This might be due to the different extraction solvents and extract concentrations.The results of the MTT assay showed that the ethanol extract of *CML* could inhibit the proliferation of SGC-7901 and Caco-2 tumor cells in a dose-dependent manner within a specific concentration range.

Table 6. Inhibitory Effect of Ethanol Extract from Leaves of Corylus mandshurica on Proliferation of Caco-2 Cells ($\bar{X}\pm S$, n=6)

group	dosage (μg/ml)	hole count	OD value	inhibitory rate (%)
blank group	-	6	0.384±0.016	-
5-Fu	100	6	0.204±0.005*	46.87
ethanol extract	50	6	0.322±0.013*	16.15
ethanol extract	100	6	0.217±0.015*	43.49
ethanol extract	150	6	0.146±0.005*	61.98
ethanol extract	200	6	0.119±0.012*	69.01
ethanol extract	250	6	0.097±0.011*	74.74
ethanol extract	300	6	0.076±0.008*	80.21
ethanol extract	350	6	0.063±0.011*	83.59

annotation: *Compared with the blank group $p < 0.05$

3.6. Antibacterial Results

After 24 hours, the medium was taken out and observed. The medium inoculated with *Staphylococcus aureus* and *Escherichia coli* displayed noticeable bacteriostatic circles (see Figure 8). The diameter of the inhibition zone of the *Staphylococcus aureus* group was 4.42 ± 0.02mm, 7.55 ± 0.06mm, and 11.25 ± 0.05mm, respectively. The inhibition zone diameter in the *Escherichia coli* group was 6.74 ± 0.05mm, 13.31 ± 0.01mm, and 13.31 ± 0.03mm, respectively. I indicated that the total flavonoid extract of CML exhibited inhibitory activity against *Staphylococcus aureus* and *Escherichia coli* and demonstrated different degrees of inhibitory effect on *Staphylococcus aureus* and *Escherichia coli*. As displayed in Figure 8, when the extract concentration was 500μg / ml and 1000μg/ml, the inhibitory effect on *Escherichia coli* was the same, and the inhibitory effect on *Staphylococcus aureus* was different, which was similar to the results of Elahe Aleebrahim-Dehkordy [56]. This might be because the drug's inhibitory effect on Escherichia coli reached the maximum at 500μg/ml, but the inhibitory effect on *Staphylococcus aureus* did not reach the maximum.

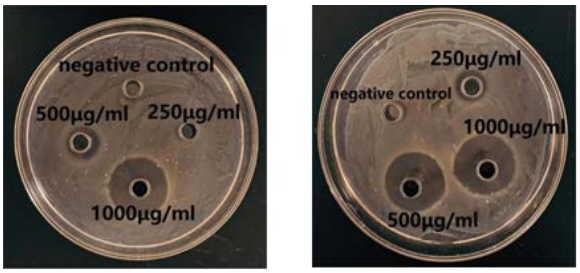

Figure 8. The results of bacteriostatic experiment of total flavonoids extract from Corylus mandshurica leaves

Table 7. Minimal Inhibitory Concentration (MIC) of total flavonoids from Corylus mandshurica Leaves

strain	Negative	Concentration (μg/ml)					Positive	MIC
		2	3	4	5	6		
		1000	500	250	125	62.5		
Staphylococcus aureus	—	—	—	+	+	+	+	250
Escherichia coli	—	—	—	—	+	+	+	125
Note: ' + ' indicates bacterial growth ; ' - ' means no bacterial growth.								

The MIC of total flavonoids extracted from *CML*: As shown in Table 7, the MICs of total flavonoids extracted from *CML* against *Staphylococcus aureus* and E*scherichia coli* were 250 μg/ml and 125 μg/ml, respectively.

4. Conclusion

In this experiment, the *CML* in CBM was used as raw materials to extract flavonoid compounds by alcohol extraction. The extraction process of total flavonoids was optimized by a single-factor test and response surface optimization method, and the yield of total flavonoids was determined by the best extraction process. The results displayed the optimum extraction conditions: ethanol concentration of 70%, liquid-solid ratio of 20 ml/g, extraction temperature of 60 °C, and extraction time of 2 h. The yield of total flavonoids was 2.8±0.004%. Through HPLC-ESI-MS/MS, the components in the *CML* were successfully analyzed and structurally identified. A total

of 25 compounds were isolated and identified, including 14 flavonoids, 6 phenolic acids, 1 tannin, and 4 other compounds. ELISA determined the contents of TNF-α, IL-6, and IL-1β in RAW264.7 cells. It was observed that the contents of TNF-α, IL-6, and IL-1β in cells increased significantly after LPS induction. The contents of TNF-α, IL-6, and IL-1β in cells were significantly decreased after administration of total flavonoids from CML in a dose-dependent manner. The inhibitory effect of ethanol extract from CML on the proliferation of Caco-2 cells was more potent than that on SGC-7901 cells. The IC50 value of Caco-2 cells was 121.2 μg/ml, and the IC50 value of SGC-7901 cells was 125.3 μg/ml. The total flavonoid extract of *CML* had different degrees of inhibitory effect on *Staphylococcus aureus* and *Escherichia coli*, and the MICs were 250 μg/ml and 125 μg/ml, respectively. This study provides a scientific basis for developing the medicinal value of local germplasm resources. However, the potential therapeutic effect and mechanism of *CML* on diseases are still unclear and need further study.

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Declaration of Funding

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List of Abbreviations

Corylus mandshurica (CM)
Corylus mandshurica leaves (CML)
Changbai Mountain (CBM)
Minimal Inhibitory Concentration (MIC)

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