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Optimization of the Extraction Process and Comprehensive Evaluation of the Antimicrobial and Antioxidant Properties of Different Polar Parts of the Ethanol Extracts of *Cannabis sativa* L.

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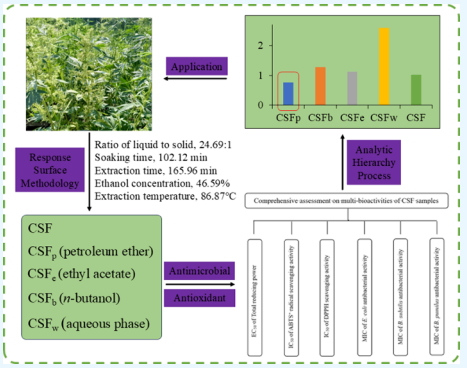
ABSTRACT: The total flavonoids of *Cannabis sativa* L. were selected as the research object, and the extraction process of *C. sativa* L. was optimized on the basis of a single factor experiment utilizing a five-factor, three-level response surface method. Subsequently, the vitro antimicrobial and antioxidant activities of the flavonoids were evaluated. The optimized extraction conditions were as follows: ratio of liquid to solid, 24.69:1 mL/g; soaking time, 102.12 min; extraction time, 165.96 min; ethanol concentration, 46.59%; extraction temperature, 86.87 °C. The extraction rate of *C. sativa* L. flavonoids (CSF) was found to be 5.51 ± 0.04 mg/g. The extraction of crude flavonoid (i.e., flavonoids extracted under the optimal extraction process) was conducted using four solvents, resulting in five *C. sativa* L. flavonoid extracts (petroleum ether, CSF_p; *n*-butanol, CSF_b; ethyl acetate, CSF_e; aqueous phase, CSF_w; and crude flavonoid, CSF). CSF contains 10 flavonoid components. In vitro, all five CSF samples demonstrated good total reducing power, effective scavenging capacity against DPPH and $ABTS^+$ radicals, and pronounced inhibitory effects against *Escherichia coli*, *Bacillus subtilis*, and *Bacillus pumilus*. Analytic Hierarchy Process (AHP) was employed to evaluate the five CSF samples in terms of antibacterial and antioxidant activity. The results indicated that petroleum-ether-extracted *C. sativa* L. flavonoids (CSF_p) exhibited the most pronounced antibacterial and antioxidant effects.



1. INTRODUCTION

Cannabis sativa L. is an annual herbaceous plant belonging to the mulberry family. It has been demonstrated that the plant contains a variety of physiologically active substances, including antibacterial, antithrombotic, antiallergic, and analgesic properties.[1−4] China is the world's primary producer of *C. sativa* L. The plant has relatively simple growth requirements and can be cultivated throughout the country, with straightforward planting technology.[5] The primary applications of this plant are in the fiber industry, seed processing industry, and cannabinoid alcohol production.[6] The use of *C. sativa* L. in China is attested for a long time. Its characteristics are sweet natured, and its flowers, roots, stems, and leaves can be employed in the preparation of medicinal remedies. It has been demonstrated that *C. sativa* L. seeds exert a moistening and sliding effect on the intestines, thereby promoting blood circulation. It has been demonstrated that *C. sativa* L. seeds exert a moistening and sliding effect on the intestines, freeing strangury and promoting blood circulation. Additionally, they possess a high food value, with the seed kernel containing a substantial quantity of high-quality protein, and the crude protein content of residue after oil preparation still reached 57.1%.[7] The application of *C. sativa* L. flowers has been demonstrated to be an effective method for the treatment of memory decline. The stem or bark of the *C. sativa* L. is a principal treatment for bleeding and bruises.[8] The secondary metabolites of *C. sativa* L., such as cannabinoid and cannabidiol, have beneficial pharmacological effects, including anti-inflammatory, anticancer, antiepileptic, cardioprotective, neuroprotective, pain management, and even the treatment of a variety of psychiatric syndromes such as depression, anxiety, and sleep disorders.[9] So far, 554 distinct compounds have been identified in *C. sativa* L., including flavonoids and their glycosides, alkaloids, coumarins, terpenoids, sterols, fatty acids, and phenanthrenes.[10,11] The four principal components of the ethanol extracts derived from the diverse parts of *C. sativa* L. are cannabinoids, terpenoids, flavonoids, and sterols. The principal components extracted from *C. sativa* L. flowers are cannabinoids (15.77−20.37%), terpenoids (1.28−2.14%), and flavonoids (0.07−0.14%). The principal components of *C. sativa* L. leaves are cannabinoids (1.10−2.10%), terpenoids (0.13−0.28%), and flavonoids (0.34−0.44%). The principal constituents of *C. sativa* L. stem bark are sterols (0.07−0.08%)

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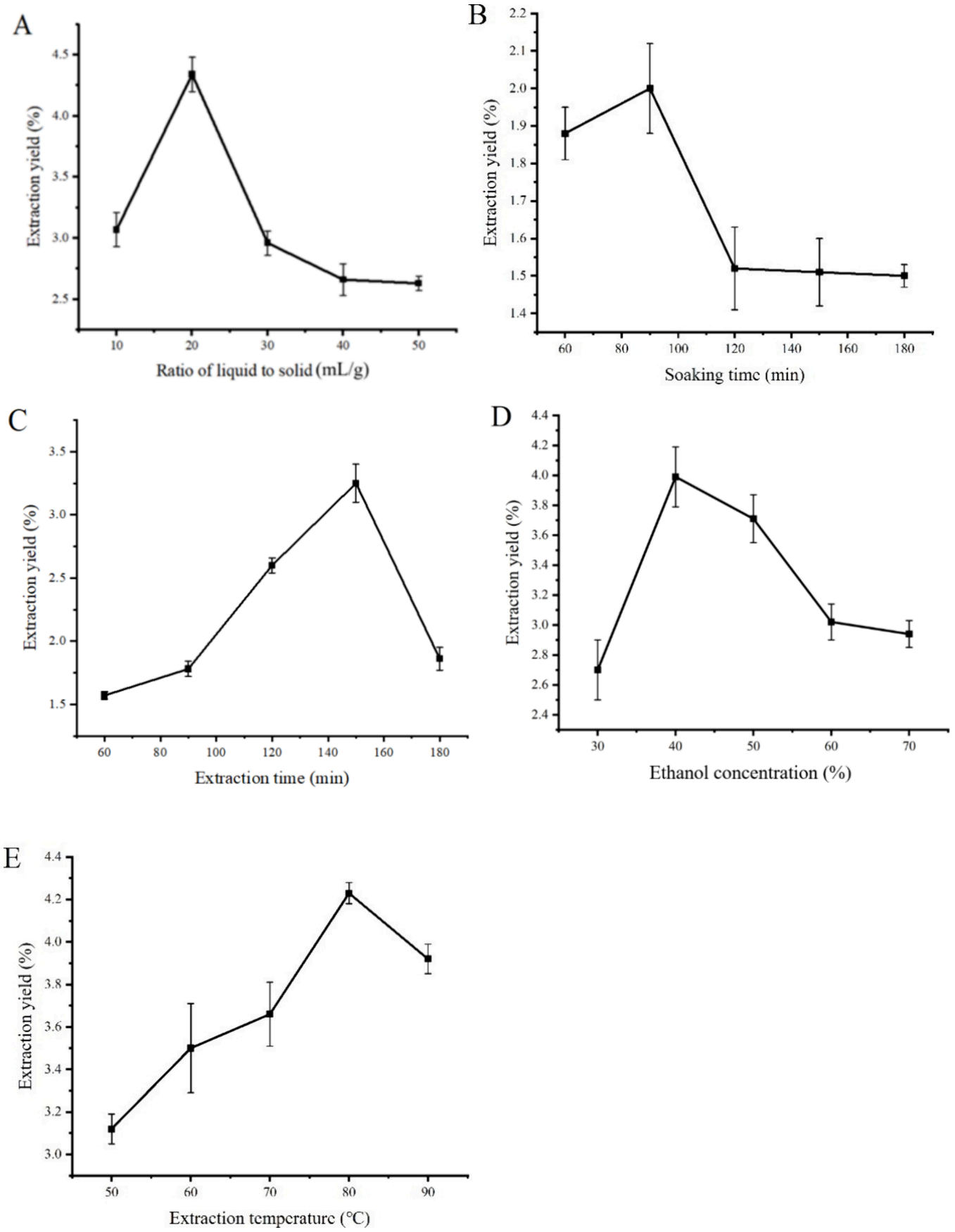

Figure 1. Effect of single factors on the extraction of *C. sativa* L. (CSF). (A) Ratio of liquid to solid (mL/g); (B) soaking time (min); (C) extraction time (min); (D) ethanol concentration (%); and (E) extraction temperature (°C). Data are shown as mean ± SD (n = 3).

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and terpenoids (0.05−0.15%). The principal constituents of the *C. sativa* L. root are sterols (0.06−0.09%) and terpenoids (0.13−0.24%).[12]

The CSF are a well-researched category of natural products that provide protection for the plant against ultraviolet damage and participate in the interaction between plants and other organisms.[13] The majority of flavonoids have been demonstrated to possess pharmacological effects, including antioxidant, neuroprotective, antimyocardial ischemic, antihypertensive, anti-inflammatory, antitumor, and hypoglycaemic effects.[14,15] Martínez et al. discovered that the methanol extract of *Cannabis sativa* leaves, when applied to the yellow fever mosquito *Aedes aegypti*, which is a carrier of several arboviruses, resulted in 100% mortality (LC_{50}: 4.4 ppm) within 48 h. This extract has the potential to effectively address the issue of mosquitoes becoming resistant to conventional insecticides, which are used to control vector-borne diseases.[16] Dobrucka et al. used the ethanolic extract of *C. sativa* L. seeds to prepare films with antimicrobial activity against both *S. typhimurium* and *L. monocytogenes*.[17] Vanhoenacker et al. found that the two most abundant compounds in the alcoholic extracted CSF as flavonoids were vitexin and luteolin, in which the former exhibited notable antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*. Luteolin exhibited remarkable efficacy against *S. aureus*, *B. subtilis*, *S. cerevisiae*, and *E. coli* with complete inhibitory concentrations of 300, 200, 250, and 300 mg/L, respectively, with highly effective antimicrobial activity.[18] Moreover, luteolin exhibited a more significant hydroxyl radical scavenging capacity than tea polyphenols and BHT (butylated hydroxytoluene).[19]

Cui et al. optimized the process of ethanol extraction of CSF, and the yield of flavonoids was 0.2275% under the optimum extraction process of ethanol concentration of 76%, bath ratio of 1:50, and extraction time of 139 min.[20] The ultrasonic technique was employed by Agarwal et al. for the extraction of polyphenols from *C. sativa* L., flavonoids, and cannabinoids. The impact of three independent variables (ultrasonic time, input power, and methanol concentration) on total polyphenols, flavonoids, plasma reducing power, and overall yield was assessed. The results demonstrated that under the optimal extraction conditions (ultrasonic time, 15 min; power, 130 W; methanol concentration, 80%), the total yield was 10.86%.[21] Cásedas et al. (2022) evaluated the antioxidant properties of two polar (aqueous and ethane) extracts of *C. sativa* L., performing a series of assays for in vitro antioxidant capacity (DPPH, superoxide radicals, FRAP, ORAC) as well as for the inhibition of physiological enzymes, including acetylcholinesterase (AChE) and monoamino oxidase-A (MAO-A). The results demonstrated that both extracts exhibited notable antioxidant capacity in the FRAP, ORAC assays, and positive results in DPPH and superoxide anion radical uptake assays. In enzyme inhibition assays, the aqueous extract demonstrated both AChE and MAO-A inhibitory activities.[22] Liu et al. optimized the process of total flavonoids extraction from *C. sativa* L. leaves by the reflux method using response surface methodology (RSM) and obtained the optimum extraction process conditions as 50% ethanol concentration; material to liquid ratio of 1:20 g/mL; extraction time of 2.0 h; and extraction temperature of 70 °C. The total flavonoid yield from *C. sativa* L. leaves under these conditions was 14.28 mg/g. The results of the FRAP, ABTS, and DPPH free radical scavenging assays demonstrated that the total flavonoid extract of *C. sativa* L. leaves possessed antioxidant properties.[23]

Analytic Hierarchy Process (AHP) is a hierarchical weighted decision analysis method that has been applied in numerous research fields. He et al. evaluated and optimized the optimal acidification modification scheme with the aim of improving the low permeability coalbed methane recovery rate by using the AHP-TOPSIS method and determined the optimal conditions for the Acidification technology. The results of comparing the optimal acidification modification scheme with the results of its parallel validation experiments were highly consistent, which provides evidence that the AHP method is reliable.[24] Cui et al. employed the AHP approach to conduct a comprehensive evaluation of the bioactivities associated with *Morus alba* L. leaves flavonoids extracted under varying polarities. The findings indicated that the bioactivity of *M. alba* L. leaves flavonoids extracted using ethyl acetate was the most pronounced.[25]

The contemporary extraction methodologies can be categorized into two distinct approaches: conventional and modern. The most widely employed conventional extraction technique is solvent extraction, which boasts the advantages of a relatively uncomplicated extraction apparatus, a straightforward method, and minimal expense. Modern extraction methodologies principally comprise supercritical fluid extraction, ultrasound-assisted extraction, and microwave-assisted extraction, among others. These techniques boast the advantages of reduced solvent consumption, reduced extraction times, and enhanced extraction efficiency. Marinaccio et al. employed the ultrasound-assisted extraction (UAE) technique to extract tomato byproducts, including seeds, pericarp, and rhizomes, with the objective of obtaining an extra virgin olive oil (EVOO) characterized by elevated levels of lycopene. Furthermore, Marinaccio also demonstrated that a high concentration of lycopene could be extracted from tomato waste using ultrasound-assisted extraction (UAE) in combination with a volatile natural deep eutectic solvent (VNADES) menthol: thymol 1:1.[26,27]

In consideration of the properties of *C. sativa* L., the present study employed the conventional extraction method of heat reflux and optimized the extraction process using the Response Surface Methodology (RSM) to ascertain the optimal extraction process for the alcoholic extract of *C. sativa* L. The vitro antibacterial activity, as well as antioxidant activity, of CSF extracted with different polarities was investigated. Hierarchical analysis was employed to comprehensively evaluate the biological activities of the components, and the results demonstrated that the CSF have significant potential for application in the field of medicine and warrant further investigation.

2. RESULTS

2.1. Evaluation of Single Factors Affecting CSF Extraction. *2.1.1. Effect of Ratio of Liquid to Solid on the Yield of CSF.* The soaking time was set at 120 min, the ethanol concentration at 70%, the extraction temperature at 80 °C, and the extraction time at 90 min. And the ratio of liquid to solid was set at five gradients. Figure 1A demonstrates that the yield of CSF increased from 3.07 ± 0.14 (10:1 mL/g) to 4.34 ± 0.14 mg/g (20:1 mL/g), after which it reached a plateau. It was thus established that the optimal ratio of the liquid to solid was 20:1 mL/g.

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Table 1. Response Surface Design and Experimental Data

run	ratio of liquid to solid (mL/g)	soaking time (min)	extraction time (min)	solvent concentration (%)	extraction temperature (°C)	extraction yield (mg/g)
1	20.00	90.00	150.00	30.00	70.00	3.11
2	20.00	90.00	150.00	40.00	80.00	5.03
3	20.00	60.00	150.00	40.00	70.00	2.11
4	20.00	60.00	120.00	40.00	80.00	2.34
5	30.00	90.00	150.00	40.00	70.00	2.46
6	10.00	90.00	180.00	40.00	80.00	2.30
7	20.00	90.00	120.00	40.00	90.00	2.65
8	10.00	90.00	120.00	40.00	80.00	1.96
9	20.00	90.00	120.00	30.00	80.00	3.71
10	20.00	120.00	150.00	50.00	80.00	4.29
11	20.00	120.00	180.00	40.00	80.00	3.48
12	20.00	90.00	180.00	30.00	80.00	3.10
13	20.00	60.00	150.00	50.00	80.00	2.67
14	20.00	120.00	150.00	40.00	70.00	2.86
15	30.00	90.00	150.00	40.00	90.00	4.28
16	30.00	90.00	120.00	40.00	80.00	2.43
17	20.00	90.00	150.00	40.00	80.00	5.03
18	20.00	90.00	150.00	40.00	80.00	5.02
19	20.00	90.00	150.00	40.00	80.00	5.04
20	20.00	90.00	180.00	50.00	80.00	4.55
21	10.00	90.00	150.00	30.00	80.00	2.00
22	20.00	90.00	150.00	50.00	90.00	4.81
23	30.00	90.00	150.00	50.00	80.00	3.62
24	20.00	60.00	150.00	30.00	80.00	2.77
25	20.00	90.00	120.00	40.00	70.00	2.70
26	10.00	90.00	150.00	50.00	80.00	3.18
27	30.00	60.00	150.00	40.00	80.00	2.31
28	10.00	90.00	150.00	40.00	70.00	2.63
29	20.00	90.00	120.00	50.00	80.00	2.70
30	10.00	60.00	150.00	40.00	80.00	2.26
31	20.00	90.00	150.00	40.00	80.00	5.03
32	10.00	90.00	150.00	40.00	90.00	1.88
33	20.00	90.00	180.00	40.00	90.00	4.28
34	10.00	120.00	150.00	40.00	80.00	1.97
35	20.00	120.00	150.00	40.00	90.00	3.88
36	30.00	90.00	180.00	40.00	80.00	4.01
37	30.00	90.00	150.00	30.00	80.00	3.52
38	20.00	120.00	120.00	40.00	80.00	3.03
39	30.00	120.00	150.00	40.00	80.00	4.17
40	20.00	90.00	180.00	40.00	70.00	2.77
41	20.00	60.00	180.00	40.00	80.00	3.16
42	20.00	90.00	150.00	30.00	90.00	3.06
43	20.00	80.00	150.00	40.00	80.00	4.85
44	20.00	120.00	150.00	30.00	80.00	2.83
45	20.00	60.00	150.00	40.00	90.00	2.84
46	20.00	90.00	150.00	50.00	70.00	2.86

2.1.2. Effect of Soaking Time on the Yield of CSF. The soaking time was set at five gradients, ranging from 60 to 180 min in 30 min increments. The yield exhibited a notable increase from 1.88 ± 0.07 to 2 ± 0.12 mg/g, occurring from 60 to 90 min, followed by a gradual decline in yield. Figure 1B illustrates that the optimal soaking time is 90 min.

2.1.3. Effect of Extraction Time on the Yield of CSF. In order to ascertain the optimal extraction time, a series of experiments were conducted using the following parameters: an ethanol concentration of 70%, a ratio of liquid to solid of 30:1 mL/g, a soaking time of 120 min, and an extraction temperature of 80 °C. The extraction time was varied from 60 to 180 min in 30 min increments. As illustrated in Figure 1C, the yield exhibited a notable increase from 1.57 ± 0.03 to 3.25 ± 0.15 mg/g, from 60 to 150 min, respectively. Subsequently, a gradual decline was observed, and the optimal extraction time for *C. sativa* L. was determined to be 150 min.

2.1.4. Effect of Ethanol Concentration on the Yield of CSF. Given the high solubility of flavonoids in ethanol, this solvent was selected for use in the extraction process. To further examine the impact of solvent concentration on the extraction rate, experiments were conducted at varying concentrations within the range of 30 to 70%. Figure 1D illustrates that the yield increased from 30 to 40%, reaching a peak value of 3.99 ± 0.2 mg/g at 40% concentration, but subsequently decreased

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Table 2. Variance Analysis of the Extracted Equation of Flavonoids from *C. sativa* L

source	sum of squares	df	mean square	F	p-value prob > F	
model	43.46	20	2.17	110.56	<0.0001	significant
A-ratio of liquid to solid	4.64	1	4.64	236.30	<0.0001	
B-soaking time	2.29	1	2.29	116.40	<0.0001	
C-extraction time	3.18	1	3.18	161.67	<0.0001	
D-solvent concentration	1.95	1	1.95	99.02	<0.0001	
E-extraction temperature	2.39	1	2.39	121.46	<0.0001	
AB	1.16	1	1.16	58.80	<0.0001	
AC	0.38	1	0.38	19.56	0.0002	
AD	0.29	1	0.29	14.84	0.0007	
AE	1.65	1	1.65	84.02	<0.0001	
BC	0.034	1	0.034	1.74	0.1989	
BD	0.61	1	0.61	30.96	<0.0001	
BE	0.021	1	0.021	1.07	0.3109	
CD	0.53	1	0.53	27.12	<0.0001	
CE	0.61	1	0.61	30.96	<0.0001	
DE	1.00	1	1.00	50.88	<0.0001	
A^2	13.92	1	13.92	708.26	<0.0001	
B^2	10.50	1	10.50	534.47	<0.0001	
C^2	8.68	1	8.68	441.48	<0.0001	
D^2	4.23	1	4.23	215.27	<0.0001	
E^2	7.27	1	7.27	370.09	<0.0001	
residual	0.49	25	0.020			
lack of fit	0.46	20	0.023	4.27	0.0572	not significant
pure error	0.027	5	5.440×10^{-003}			
cor total	43.95	45				
degree of fit			$R^2 = 0.9888$			

significantly at concentration of 40 to 70%. Accordingly, the optimal solvent concentration was determined to be 40%.

2.1.5. Effect of Extraction Temperature on the Yield of CSF. The impact of varying temperatures (50−90 °C) on flavonoids was examined (Figure 1E). The yield demonstrated a gradual increase from 3.12 ± 0.07 to 4.23 ± 0.05 mg/g, followed by a decline to 3.92 ± 0.07 mg/g at temperatures between 50 and 80 °C. The optimal temperature for extraction was determined to be 80 °C.

In consideration of the aforementioned experimental outcomes, the optimal conditions for the extraction process from the single factors test were identified as follows: an extraction time of 150 min, a soaking time of 90 min, a ratio of liquid to solid of 20:1 mL/g, an ethanol concentration of 40%, and an extraction temperature of 80 °C. These conditions were determined through the application of response surface methodology (RSM).

2.2. RSM Analysis. The extraction of flavonoids was optimized using RSM. When the two commonly used experimental design methods, Box-Behnken Design (BBD) and Central Composite Design (CCD), are compared, it is important to note that the CCD method requires consideration of more interactions, leading to a larger number of experimental runs and potentially extreme conditions. Given the heat-sensitive nature of flavonoids, excessively high or low temperatures can negatively impact their yield and bioactivity. Therefore, the CCD method may not be ideal for optimizing flavonoid extraction due to these potential extremes. Therefore, the BBD method was selected for this experiment, and a total of 46 tests were designed, comprising six central experiments and 40 factorial tests. Table 1 presents the data on the designed and experimental of the CSF. By employing the multivariable regression fitting method on the data, a quadratic polynomial regression model was generated for the ratio of liquid to solid (A), soaking time (B), extraction time (C), solvent concentration (D), and extraction temperature (E). The resulting equation was generated as below:

$$R_1 = 5.00 + 0.54A + 0.38B + 0.45C + 0.35D + 0.39E + 0.54AB + 0.31AC - 0.27AD + 0.64AE - 0.092BC + 0.39BD + 0.072BE + 0.37CD + 0.39CE + 0.50DE - 1.26A^2 - 1.10B^2 - 1.00C^2 - 0.70D^2 - 0.91E^2 \quad (1)$$

The results of the analysis of variance (ANOVA) for the regression equation are presented in Table 2. The p-value for this model was <0.0001, indicating that the linear and quadratic terms were highly significant. The p-value for the lack of fit was 0.0572 (>0.05), indicating that the experimental data is consistent with the model. The degree of fit of the response surface model is evaluated using the response surface correlation coefficient R^2. A value of R^2 approaching 1 indicates an optimal fit of the response surface model. As demonstrated by the data presented in the table (R^2 = 0.9888), this substantiates the assertion that the degree of fit is superior and that the reliability of the simulation is robust.

A comparison of the F-values of the factors allows the extent to which each factor affects the results of the experiment to be ascertained (in descending order of importance: ratio of liquid to solid, extraction time, extraction temperature, soaking time, solvent concentration). As shown in Figure 2, a further comparison of the two factors can be verified in the response surface contour plots and 3D plots, where the steepness of the 3D plots, the height of the vertices, and the ellipticity of the

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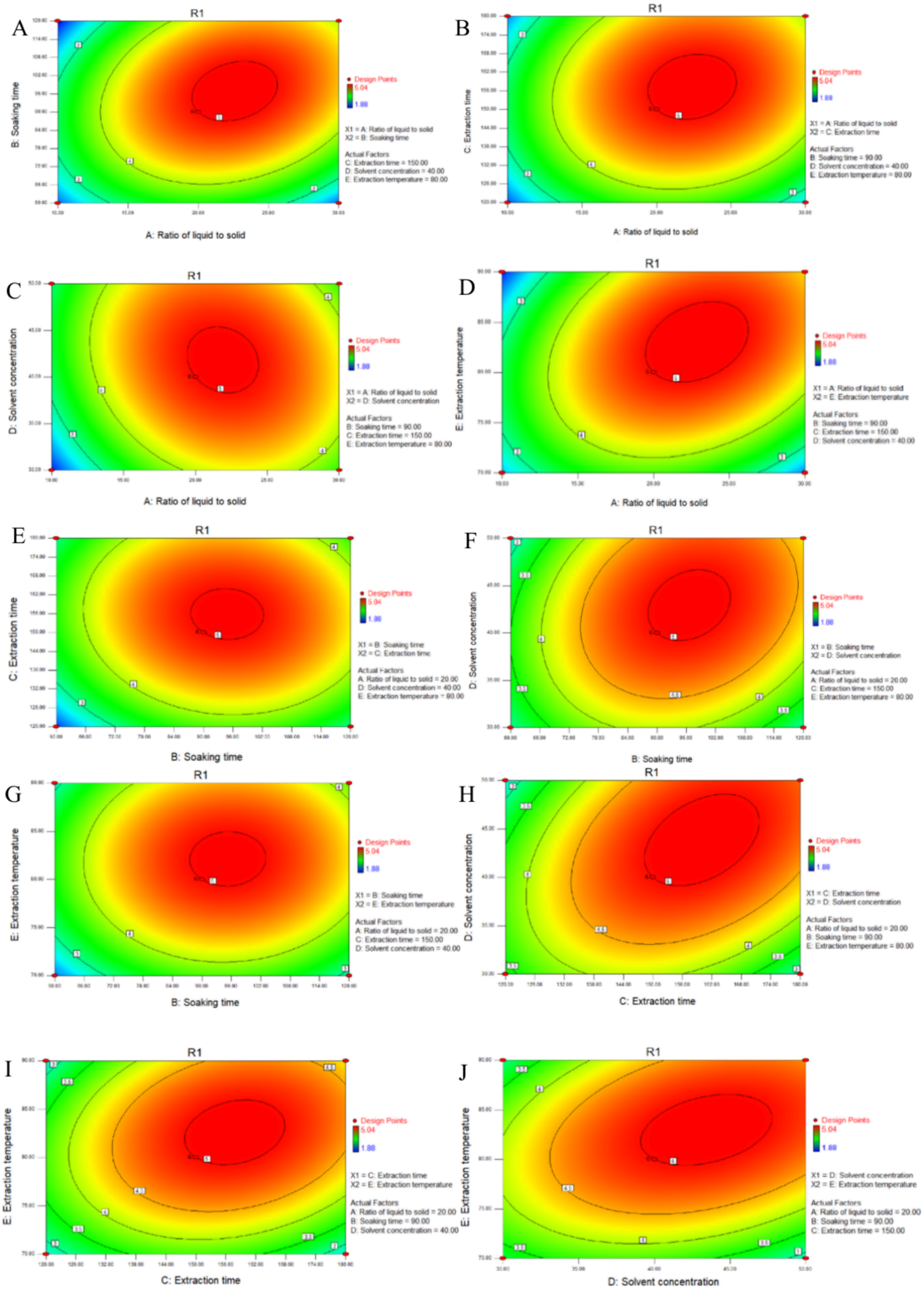

Figure 2. Contour plots showing the effects of the ratio of two single factors. (A) Ratio of liquid to solid versus soaking time; (B) ratio of liquid to solid versus extraction time; (C) ratio of liquid to solid versus solvent concentration; (D) ratio of liquid to solid versus extraction temperature; (E) soaking time versus extraction time; (F) soaking time versus solvent concentration; (G) soaking time versus extraction temperature; (H) extraction time versus solvent concentration; (I) extraction time versus extraction temperature; (J) solvent concentration versus extraction temperature.

contour lines allow for a more intuitive observation of the effect of the factors on the extraction rate of CSF.[28]

The optimal conditions for the extraction of CSF were as follows: a ratio of liquid to solid of 24.69:1 mL/g, a soaking time of 102.12 min, an extraction time of 165.96 min, a solvent concentration of 46.59%, and an extraction temperature of 86.87 °C. The predicted yield was 5.5691 mg/g. In accordance with the theoretical optimum conditions, three further sets of parallel experiments were conducted, resulting in flavonoid extraction rates of 5.51 ± 0.04 mg/g (Table 3). The actual

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Table 3. Flavonoid Extraction Rate under Optimal Process Conditions

run	ratio of liquid to solid (mL/g)	soaking time (min)	extraction time (min)	solvent concentration (%)	extraction temperature (°C)	extraction yield (mg/g)
1	24.69:1	102.12	165.96	46.59	87	5.50
2	24.69:1	102.12	165.96	46.59	87	5.48
3	24.69:1	102.12	165.96	46.59	87	5.56

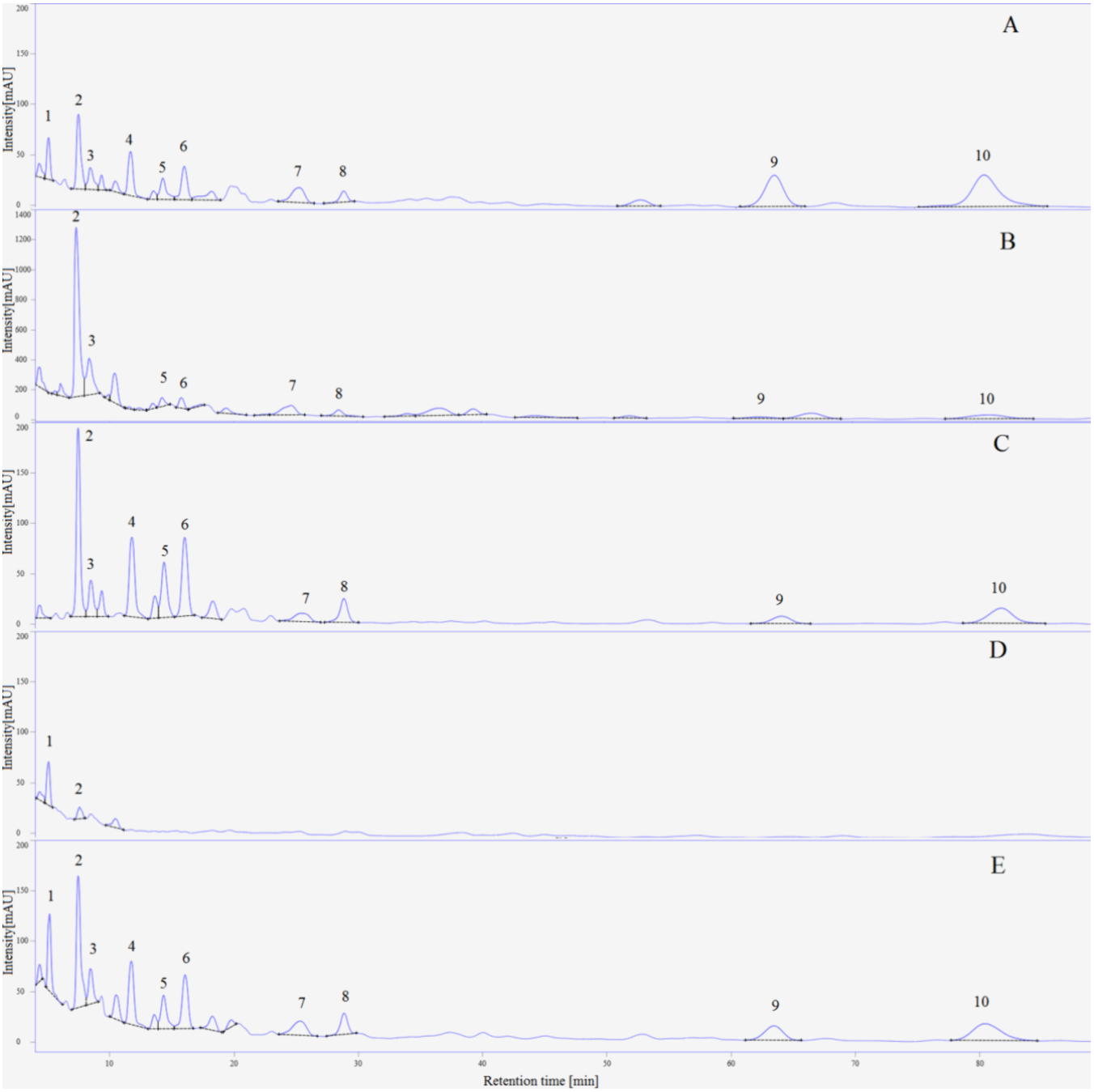

Figure 3. Liquid chromatograms of five CSF samples by HPLC (λ: 256 nm). (A) CSF_p; (B) CSF_b; (C) CSF_e; (D) CSF_w; and (E) CSF.

flavonoid extraction rate was found to be in closer alignment with the predicted value, thereby substantiating the efficacy of this modeling approach.

2.3. Component Analysis. The components of five CSF samples were analyzed by HPLC, and 2−10 peaks were obtained at 256 nm, which showed the most peaks, respectively (Figure 3). Analysis of the MS/MS results (Figure S1) screened for ten compounds with high content in the CSF, which were scopoletin (1), vomifoliol (2), vitexin (3), kaempferol (4), dehydrovomifoliol (5), apigenin (6), cannabidiolic acid (7), cannabidiol (8), tetrahydrocannabinol (9), and docosanoic methyl ester (10). The content of these ten compounds in CSF was used as a standard to calculate the relative content in the other four samples, and the results are

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Table 4. MIC of Antibacterial Activity Results of CSF

	CSF_p (mg/mL)	CSF_b (mg/mL)	CSF_e (mg/mL)	CSF_w (mg/mL)	CSF (mg/mL)
Candida albicans				5	2.5
Staphylococcus aureus	5				
Bacillus subtilis	5	20	5	2.5	2.5
Bacillus pumilus	2.5	20	5	5	5
Escherichia coli	2.5	2.5	10	2.5	2.5
Saccharomyces cerevisiae					

shown in Table S1. CSF_p contained more tetrahydrocannabinol and docosanoic methyl ester compared to the other four samples, and CSF_b contained more vomifoliol, vitexin, and cannabidiol.

2.4. Antimicrobial Activity. The data presented in Table 4 indicates that, among the five flavonoids samples, CSF_w and CSF demonstrated antibacterial activity against *C. albicans* and CSF_p exhibited antibacterial activity against *S. aureus*. Notably, none of the five samples displayed antibacterial activity against *S. cerevisiae*. In comparison to the aforementioned bacteria, the five flavonoids demonstrated remarkable inhibitory activity against *B. subtilis* and *B. pumilus*. The minimum inhibitory concentration (MIC) of CSF_p, CSF_w, and CSF ranged from 2.5 to 5 mg/mL, indicating that these three flavonoid samples could achieve the inhibitory effect at low concentrations. The inhibitory effect of CSF_b was particularly pronounced against *E. coli*. The MIC value was 2.5 mg/mL, which was relatively inferior for *B. subtilis* and *B. pumilus*, with MIC values of 20 mg/mL for both. CSF_e demonstrated a notable bacteriostatic effect against *B. subtilis* and *B. pumilus*, with an MIC values of 5 mg/mL, and for *E. coli*, it had an MIC value of 10 mg/mL.

2.5. In Vitro Antioxidant Activity. The total reducing ability and scavenging against the 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) ($ABTS^+$) radical and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical of CSF was investigated using vitamin C (VC) as a positive control. The antioxidant activity of the CSF was characterized by the effective concentration of attainment of a 50% scavenging rate (IC_{50}) or the attainment of an absorbance of 0.5 absorbance units (EC_{50}). As illustrated in Table 5, the extraction of organic solvents with varying

Table 5. Results of In Vitro Antioxidant Activities of CSF Samples and Vitamin C (VC)

	DPPH (IC_{50})	$ABTS^+$ (IC_{50})	total reducing power (EC_{50})
CSF_p (mg/mL)	0.2951 ± 0.0054	0.5076 ± 0.0039	0.9023 ± 0.0125
CSF_b (mg/mL)	0.2429 ± 0.0095	0.3012 ± 0.0060	0.8424 ± 0.0073
CSF_e (mg/mL)	0.2763 ± 0.0074	0.3280 ± 0.0038	0.7091 ± 0.0079
CSF_w (mg/mL)	0.3905 ± 0.0106	0.7049 ± 0.0130	3.7279 ± 0.1113
CSF (mg/mL)	0.3801 ± 0.0092	0.3358 ± 0.0015	1.1957 ± 0.0070
VC (mg/mL)	0.1973 ± 0.0025	0.2588 ± 0.0035	0.0798 ± 0.003

polarities markedly influenced the antioxidant activities observed in vitro. The IC_{50} and EC_{50} values were observed to demonstrate enhanced antioxidant activity with diminutive values. Among the simples, CSF_b was observed to scavenge DPPH and $ABTS^+$ radicals with the smallest IC_{50} value, while CSF_e demonstrated the highest total reducing power with the smallest EC_{50} value.

A one-way analysis of variance (ANOVA) was conducted on samples of five polarities (CSF_p, CSF_b, CSF_e, CSF_w, and CSF) within three groups (IC_{50} of $ABTS^+$ radical-scavenging activity; IC_{50} of DPPH scavenging activity; and EC_{50} of total reducing power). The results demonstrated that within the IC_{50} of $ABTS^+$ radical-scavenging activity group, the data exhibited a significant difference in terms of statistical analysis ($F_{(4,10)}$ = 1247.0, $P < 0.0001$), with the five polar samples displaying notable differences. Similarly, within the IC_{50} of DPPH scavenging activity group, the five polar samples displayed a significant difference ($F_{(4,10)}$ = 115.8, $P < 0.0001$). Moreover, the data for the EC_{50} of total reducing power group also exhibited a significant difference ($F_{(4,10)}$ = 1907, $P < 0.0001$). The data for all five CSF were subjected to a normal distribution test, logistic completeness test, and a one-way ANOVA. The resulting *p*-values were found to be less than the pre-established significance level of 0.05, indicating that the data were statistically significant.

The analysis of Figure 4 demonstrated that CSF_b and CSF_e exhibited the highest antioxidant activity among the five CSF. However, the magnitude of the antioxidant activity of these two samples could not be confirmed when they were compared with each other in the three antioxidant experiments. To illustrate, the IC_{50} values for the $ABTS^+$ radical scavenging activity and DPPH radical scavenging activity of CSF_e were greater than those of CSF_b, whereas the EC_{50} values for total reducing power were smaller than those of CSF_b. Accordingly, the data from this experiment were subjected to an Analytic Hierarchy Process in order to evaluate the antioxidant activity and inhibitory activities of the different polarities in a comprehensive manner.

2.6. AHP Model for Weight Calculation. A multicriterion model was constructed for the purpose of evaluating the aforementioned process parameters. Figure 5 shows the structure of the AHP model, which was constituted of two levels. The top level represented the goal of the model, namely, a comprehensive assessment of the multiple-bioactivities of CSF samples. The second level comprised the criteria, which were as follows: f_1, EC_{50} of total reducing power; f_2, IC_{50} of $ABTS^+$ radical-scavenging activity; f_3, IC_{50} of DPPH scavenging activity; f_4, MIC of *E. coli* antibacterial activity; f_5, MIC of *B. subtilis* antibacterial activity; and f_6, MIC of *B. pumilus* antibacterial activity.

Subsequently, the results were then transformed into positive pairwise comparison matrices *N* as follows:

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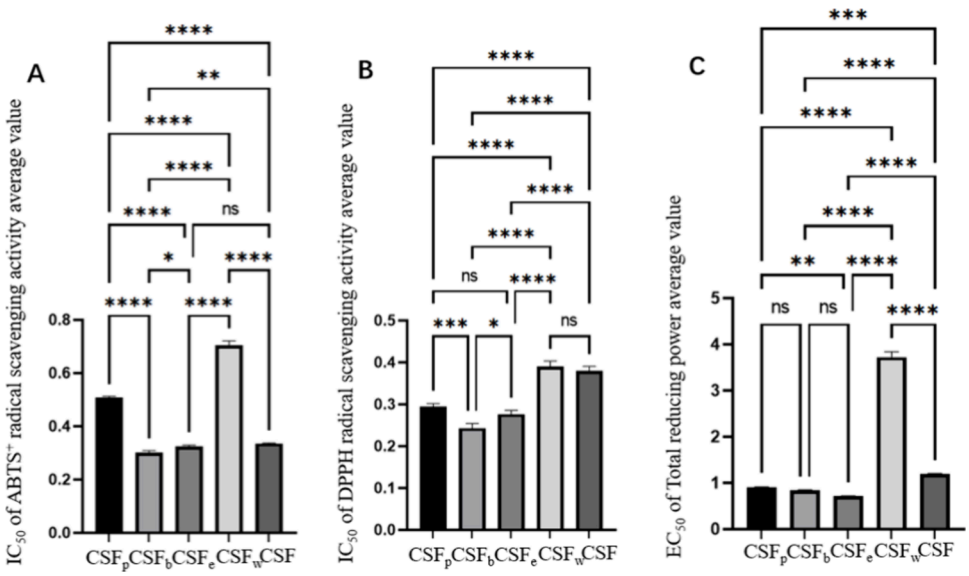

Figure 4. Significant two-by-two differences between the five polar samples within each group. (A) Two-by-two differences within groups of five polar samples in the IC_{50} of $ABTS^+$ radical-scavenging activity group; (B) two-by-two differences within groups of five polar samples in the IC_{50} of DPPH scavenging activity; (C) two-by-two differences within groups of five polar samples in the EC_{50} of total reducing power group. *, **, ***, and **** represent significant differences at the $P < 0.05$, $P < 0.01$, $P < 0.001$, and $P < 0.0001$ levels, respectively. ns represents insignificant differences.

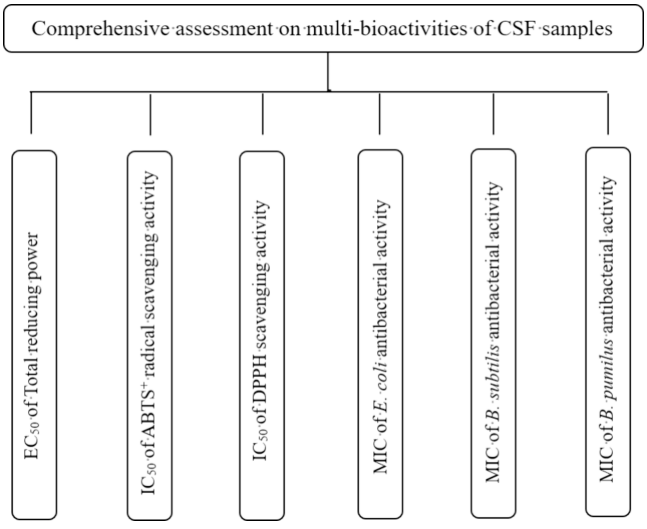

Figure 5. Proposed analytical hierarchy process model.

$$N = \begin{bmatrix} 1 & 2 & 3 & 4 & 5 & 5 \\ 1/2 & 1 & 2 & 3 & 4 & 4 \\ 1/3 & 1/2 & 1 & 2 & 3 & 3 \\ 1/4 & 1/3 & 1/2 & 1 & 2 & 2 \\ 1/5 & 1/4 & 1/3 & 1/2 & 1 & 1 \\ 1/5 & 1/4 & 1/3 & 1/2 & 1 & 1 \end{bmatrix}$$

The calculated initial weights w_1', w_2', w_3', w_4', w_5', and w_6' were 8.4343, 3.6342, 1.4423, 0.2027, 0.2027, and 0.5503, respectively. In a subsequent normalization process, the priority weights w_1, w_2, w_3, w_4, w_5, and w_6 were found to be 0.5830, 0.2512, 0.0997, 0.0140, 0.0140, and 0.0380, respectively. The maximum eigenvalue (λ_{max}) was 6.0808, the CI was 0.0162, and the consistency ratio (CR) was 0.0131 < 0.1, indicating that consistency check was passed. The value of w, representing the priority weight of the criterion relative to the goal, was provided as follows:

$$N = [0.5830 \quad 0.2512 \quad 0.0997 \quad 0.0140 \quad 0.0140 \quad 0.0380]$$

The comprehensive assessment score S for CSF_p, CSF_b, CSF_e, CSF_w, and CSF was 0.7703, 1.2809, 1.1203, 2.5893, and 1.0193, respectively. The lowest S-value was observed for CSF_p, indicating that it exhibited the greatest combined evaluation of antimicrobial activity and antioxidant activity in vitro.

3. DISCUSSION

C. sativa, a traditional medicinal and economic crop, is widely distributed throughout China. The entire plant is a rich source of flavonoid. This paper presents an investigation into the extraction process of ethanol extract as well as a comprehensive analysis of its antimicrobial and antioxidant activities. The ratio of liquid to solid has a significant impact on the yield of CSF. An increase in the ratio of liquid to solid from 10:1 to 20:1 resulted in a gradual enhancement in the yield of CSF. Following a maximum at 20:1, the yield of CSF demonstrated a decline with an increase in the ratio of liquid to solid. This phenomenon could be attributed to the fact that as the amount of the solvent increases, the contact area between the *C. sativa* L. and the solvent also increases. This results in an increase in the active diffusion force, which in turn leads to an increase in the CSF extraction rate. However, when the ratio of liquid to solid exceeded 1:20, a majority of the CSF present in the reaction had been extracted. The high concentration of solvent may have resulted in the subsequent dissolution of other soluble components, which affected the extraction of CSF to a certain extent, thereby causing a decline in the yield of flavonoids.[23] The soaking and extraction times significantly influence the extraction of flavonoids due to their heat sensitivity. Both elevated and reduced temperatures can compromise the stability and biological activity of these compounds. Therefore, it is essential to extend the soaking and

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extraction times to protect heat-sensitive substances from degradation and decomposition, thereby enhancing both the bioactivity and the yield of flavonoids. When the extraction time is between 60 and 90 min, the extraction rate of CSF is relatively slow. The emergence of this phenomenon may be attributed to certain components of flavonoids being susceptible to heat-induced decomposition. Prolonged heating results in the breakdown of these compounds, which in turn impedes the extraction process. This interaction between the extraction and decomposition steps ultimately results in a slowing down of the overall yield of CSF. In comparison to the initial 30 min, the yield of CSF increased markedly between 90 and 150 min. This can be attributed to the thermally unstable flavonoids having undergone decomposition, resulting in a rapid increase in the extraction rate, which reaches its peak at 150 min. The prolonging of the extraction time resulted in an increased leaching rate of impurities in the reaction. Subsequently, the yield of CSF exhibited a gradual decline after 150 min.[29] The concentration of the solvent has a considerable impact on the rate of extraction of CSF. The yield of CSF is observed to increase continuously when the solvent concentration is between 30 and 40%, and it reaches its peak at 40%. However, beyond this concentration, the extraction rate declines rapidly. This phenomenon may be attributed to the fact that when the ethanol concentration is excessively high, a low water content results in a rapid decrease in ethanol polarity, which in turn reduces the affinity between ethanol and the hydroxyl and other polar groups present in flavonoids. This ultimately impedes the extraction of flavonoids into the ethanol phase, resulting in a notable decline in the extraction rate.[30] It can be observed that the extraction temperature also exerts a certain influence on the extraction of flavonoids. An elevated temperature facilitates the diffusion rate of the solvent, thereby enhancing the solubility of soluble components in flavonoids and consequently augmenting the extraction rate of CSF. The extraction rate of CSF was observed to increase significantly when the extraction temperature was between 50 and 80 °C. However, beyond 80 °C, the extraction rate continues to decline. This phenomenon may be attributed to the high temperature causing the destruction of flavonoid glycosides in the *C. sativa* L. extract.[31] Consequently, when the temperature exceeds 80 °C, the total CSF extraction rate decreases.

The extraction methods can be categorized into five distinct types based on their mechanisms: simple molecular extraction, neutral complex extraction, metal chelating extraction, ion-conjugated extraction, and synergistic extraction. In this study, ethanol was selected for extracting CSF samples using a simple molecular extraction method. The extracted components existed as neutral molecules in the two phases without undergoing any chemical reactions with the solvents; they were simply physically partitioned between the phases as simple molecules. As illustrated in Formula 2, extraction efficiency (E') is influenced by three key metrics: Extraction yield (E): The proportion of the target compound transferred from the feedstock to the solvent during the extraction process. Distribution coefficient (K): Describes how the compound distributes between two different solvents. Extraction equilibrium constant (K_{ex}): Defined as the ratio of the compound's concentration in the two phases at equilibrium during chemical extraction. Extraction efficiency is positively correlated to these three factors, all of which are influenced by the choice of solvent, extraction temperature, pH, and extraction time. By careful control of these parameters, the extraction conditions can be optimized to enhance both the extraction efficiency and the purity of the compounds. The formula is as follows:

$$E'(\%) = E \times K \times K_{ex} \times 100\% \quad (2)$$

The composition and content of ingredients in a plant extract directly determine its biological activity directly. A total of 26 flavonoids from seven major classes, including vitexin, isovitexin, apigenin, luteolin, kaempferol, orientin, and quercetin, have been identified and detected in the *C. sativa* L.[32] The four major components are vitexin (1.0−1.5 mg/g), orientin (0.6−0.8 mg/g), luteolin-7-*O*-*β*-D-glucuronide (3.0−4.5 mg/g), and apigenin-7-*O*-*β*-D-glucuronide (1.0−1.5 mg/g).[18] The extraction of CSF in petroleum ether results in the removal of certain oils and pigments, and a total of 14 compounds were extracted using petroleum ether, the majority of which were sterols and terpenoids, with relatively few flavonoids extracted.[33] In this experiment, higher levels of tetrahydrocannabinol were found in CSF_p compared to the other four samples as well as cannabidiolic acid and cannabidiol, among others. These compounds are widely used cannabinoids with good anti-inflammatory, antibacterial, and antioxidant effects. Among these compounds, cannabidivarinic acid (CBDVA) and cannabidivarin (CBDV) have been observed to exert a certain degree of damage to the cell membrane, cell wall, and other structures of *E. coli* to a certain extent. This results in the inhibition of bacterial growth, with the MIC being 0.5 and 0.01 mg/mL, respectively.[34] The extraction of *C. sativa* L. in *n*-butanol included the highest content of vitexin.

Vitexin, which has apigenin as its fundamental carbon framework, has been demonstrated to possess a plethora of pharmacological effects, including antimyocardial infarction, anti-inflammatory and analgesic, antioxidant, and antitumor properties. The total flavonoids present in vitexin have been demonstrated to possess robust antioxidant properties, effectively scavenging both oxygen-derived free radicals and DPPH free radicals.[35] Vitexin was observed to exert considerable inhibitory effects against *E. coli*, *S. aureus*, and *B. subtilis*, with the MICs of 100, 25, and 100 μg/mL, respectively.[36] The administration of orientin and vitexin was observed to enhance the antioxidant capacity of superoxide dismutase, catalase, and glutathione peroxidase in the blood, brain, liver, and kidney of aged mice. Orientin and vitexin have been shown to enhance the activity of antioxidant enzymes and to scavenge free radicals.[37] Orientin has been demonstrated to exert an efficacious antibacterial effect on a range of bacterial species, including *E. coli*, *S. aureus*, *S. mutans*, and *C. albicans*. The three hydroxyl groups at positions 4′, 5, and 7 and the double bond at positions C_2 and C_3 in the apigenin molecule possess the capacity to bind with free radicals. Moreover, the hydroxyl groups at positions 5 and 7 possess the ability to chelate metal ions and inhibit the production of free radicals, thereby determining the antioxidant activity of apigenin.[38,39] The MIC values for apigenin against Gram-positive and Gram-negative bacteria ranged from 32.5 to 62.5 μg/mL.[40] Luteolin demonstrated antioxidant activity in scavenging of DPPH radicals (IC_{50} = 2.099 μg/mL), while luteolin and apigenin also exhibited an excellent scavenging ability against $ABTS^+$ radicals (IC_{50} = 0.59, 0.8243 μg/mL).[41]

In conclusion, the flavonoids obtained from ethyl acetate and n-butanol phase extraction exhibited a higher flavonoid content than those obtained from petroleum ether extraction. Therefore, CSF_b and CSF_e exhibited augmented antioxidant

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Table 6. Extraction Conditions of the Single Element Test

	ratio of liquid to solid (mL/g)	soaking time (min)	extraction time (min)	ethanol concentration (%)	extraction temperature (°C)
ratio of liquid to solid	10:1,20:1,30:1,40:1,50:1	120	90	70%	80
soaking time	30:1	60, 90, 120, 150, 180	90	70%	80
extraction time	30:1	120	60, 90, 120, 150, 180	70%	80
ethanol concentration	30:1	120	90	30%, 40%, 50%, 60%, 70%,	80
extraction temperature	30:1	120	90	70%	50, 60, 70, 80, 90

activity in comparison to CSF_p.[42] The main components in CSF_p are cannabinoid analogues, which are much more effective at inhibiting bacteria. The ethanol-extracted flavonoids demonstrated a broad-spectrum antimicrobial effect, with all five CSF samples exhibiting significant antibacterial activity against *E. coli*, *B. subtilis*, and *B. pumilus*. To ensure the integrity of the experimental data, an analysis of variance (ANOVA) was performed to determine whether the flavonoid samples of varying polarities exhibited significant differences within their respective groups. The experimental data were validated through the application of the appropriate statistical techniques. Given the flavonoids present in *C. sativa* L. are a class of compounds with multiple biological activities, the AHP method employed in this study is a decision analysis method that combines qualitative and quantitative methods for solving complex problems with multiple objectives. The individual activities of the flavonoids were aggregated to calculate their composite score, designated as the *S*-value. A lower *S*-value indicates a more favorable *C. sativa* L. flavonoid activity for this polarity. Among the compounds under consideration, CSF_p exhibited the most favorable composite score, reaching 0.7703.

4. CONCLUSIONS

The conditions for the extraction of CSF were optimized using the Box-Behnken design (BBD), and the following parameters were identified as optimal: ratio of liquid to solid, 24.69:1 mL/g; soaking time, 102.12 min; extraction time, 165.96 min; ethanol concentration, 46.59%; extraction temperature, 86.87 °C. And the yield of CSF was 5.51 ± 0.04 mg/g. The antibacterial activity of flavonoids following extraction with organic solvents is superior to that of crude flavonoids. Of the flavonoids tested, CSF_p exhibited the most pronounced antibacterial effect. In the antioxidant experiments, the total reducing power, DPPH, and $ABTS^+$ scavenging rate of the flavonoids after extraction with different solvents were determined. CSF contains 10 flavonoid components. It was observed that all of the flavonoids exhibited a certain degree of scavenging activity and that their scavenging capacity was positively correlated with the concentration of flavonoids within a certain range. The AHP analysis of the five CSF samples revealed that CSF_p exhibited the greatest efficacy among them for antibacterial activity and antioxidant activity. The results demonstrate that flavonoids present in *C. sativa* L. possess significant potential for utilization in both medical and industrial applications. Further investigation into these possibilities is recommended.

5. MATERIALS AND METHODS

5.1. Materials and Microbials. *C. sativa* L. was collected from the Longfeng Lake Protected Area in Changling County, Jilin Province, in September. The specimen was identified by Prof. Xianpu Ni of Shenyang Pharmaceutical University and preserved in the herbarium of the Jilin Institute of Chemical Technology, with the designation No. CS-01. Subsequently, samples of *C. sativa* L. were dried at room temperature. The bacterial strains used in this study were *Staphylococcus aureus* ATCC 29213, *Bacillus pumilus* ATCC 6633, *Bacillus subtilis* ATCC 700814, *Escherichia coli* ATCC 25922, *Saccharomyces cerevisiae* ATCC 9763, and *Candida albicans* ATCC 14053. All other reagents were of analytical grade.

Table 7. Response Surface Analysis Factors and Levels Design

	coded levels		
independent variables	−1	0	1
ratio of liquid to solid (mL/g)	10	20	30
soaking time (min)	60	90	120
extraction time (min)	120	150	180
solvent concentration (%)	30	40	50
extraction temperature (°C)	70	80	90

Table 8. Value Meaning of the Nine-Scale Method

description of *fi*/*fj*	scale
fi is equally important as *fj*	1
fi is moderately more important than *fj*	3
fi is strongly more important than *fj*	5
fi is very strongly more important than *fj*	7
fi is extremely more important than *fj*	9
the median of adjacent scales, when a compromise is needed	2, 4, 6, 8

5.2. Examination of Single Factors in the Extraction Process. A specific quantity of *C. sativa* L. was taken, and the total flavonoids were extracted by ethanol solution. The influence of the ratio of liquid to solid (mL/g, *A*), soaking time (min, *B*), extraction time (min, *C*), ethanol concentration (%, *D*), and extraction temperature (°C, *E*) on the flavonoid extraction rate was examined in accordance with the extraction conditions outlined in Table 6. The resulting extract was then concentrated in a rotary evaporator and then in a vacuum desiccator, thereby obtaining the ethanol extract of *C. sativa* L. (CSF).[20]

5.3. Determination of Flavonoid Content. The flavonoid content was determined by using the $NaNO_2$−$Al(NO_3)_3$−NaOH method. A standard solution of rutin was prepared using a concentration gradient with a 60% ethanol solution as the solvent. The absorbance was measured at 510 nm, and a standard curve for rutin was constructed. The concentration of the *C. sativa* L. total flavonoid was obtained by the curve. The formula for calculating the yield of flavonoid extraction is provided by the following equation:[43]

$$Y(\mathrm{mg/g}) = \frac{C \times V \times N}{m} \quad (3)$$

where *Y* is the yield of flavonoid, mg/g; *C* is the concentration of total flavonoids, mg/mL; *V* is the volume of the sample

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Table 9. Average Consistencies of Random Matrices

matrix order	1	2	3	4	5	6	7	8	9
random consistency index	0.00	0.00	0.58	0.90	1.12	1.24	1.32	1.41	1.45

solution, mL; N is the dilution of the solution to be measured; and m is the mass of *C. sativa* L., g.

5.4. Optimization of the Flavonoid Extraction Process by Response Surface Methodology. The Box-Behnken design principles were employed using the Design-Expert 8.0.6 software based on the results obtained from the single-factor experiments. The design of a five-factor, three-level response surface analysis experimental scheme was undertaken (Table 7).

5.5. Extraction of CSF with Different Solvents. The crude flavonoid (CSF) was prepared as a suspension with a specified concentration. This was accomplished by combining 50 mL of the CSF suspension with 50 mL of petroleum ether in a dispensing funnel. Subsequently, the mixture was agitated and allowed to stand for a period of 30 min. The upper organic phase was isolated, and the lower extract was retained. The extraction was repeated on three occasions, and the collected organic phase was subjected to swirl steaming in order to obtain the desired flavonoids, which were extracted in petroleum ether (CSF_p). The lower extract retained from the preceding experiment was subsequently added to ethyl acetate and *n*-butanol for extraction, thereby obtaining ethyl acetate-extracted flavonoids (CSF_e) and *n*-butanol-extracted flavonoids (CSF_b), respectively. Subsequently, an aqueous extraction was conducted, and the lower aqueous phase was retained to obtain water-extracted flavonoids (CSF_w).

5.6. Analysis of CSF Components via HPLC and UPLC-MS/MS. *5.6.1. HPLC.* A Diamonsil C_{18} column (200 × 4.6 mm) was used as the HPLC (Dalian Elite 3100 series). The flow rate was 0.7 mL/min; the detector was a DAD detector; the column temperature was controlled at 28 °C; and the running time was 90 min. The mobile phases were 30% methanol, 70% ultrapure water, and 1% formic acid. The sample was dissolved with chromatographic grade methanol at a concentration of 0.1 g/mL with an injection volume of 20 μL.

5.6.2. UPLC-MS/MS. The compounds in five CSF samples were identified using ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). A Q-TOF TripleTOF 6600$^+$ system (SCIEX, USA) equipped with SCIEX OS data processing software was employed for analysis. Chromatographic separation was performed on a Phenomenex XB-C18 column (100 × 2.1 mm, 2.6 μm) under the following conditions: scan mode: negative electrospray ionization (-ESI); mass range: 50−1500 m/z; ion source temperature: 550 °C; cone gas flow: 50 L/h; column temperature: 35 °C. Mobile phase gradient: mobile phase A, ultrapure water; mobile phase B, methanol. Gradient program: 0−5 min, phase A 98 to 98%, 0.4 mL/min; 5−10 min, phase A 98 to 70%, 0.3 mL/min; 10−28 min, phase A 70 to 0%, 0.3 mL/min; 28−32 min, phase A 0 to 98%, 0.3 mL/min; 32−40 min, phase A 98 to 98%, 0.3 mL/min. The samples were dissolved in chromatographic-grade methanol to a concentration of 0.1 g/mL, and 1 μL of the solution was injected for analysis.

5.7. In Vitro Antibacterial Activity Assay. The vitro antibacterial activity experiment was conducted using the paper diffusion method. Six bacterial suspensions were prepared separately as solid media (1.0 × 10^6 cfu/mL) in plates. The *C. sativa* L. flavonoid samples were diluted into five concentration gradients, with concentrations of 1.25, 2.5, 5, 10, and 20 mg/mL, respectively. Subsequently, 15 μL of each spot sample was aspirated on a paper plate (φ = 5 mm), which was then covered with medium until the medium in the flat dish had solidified. The bacterial strains *S. aureus*, *B. subtilis*, *B. pumilus*, and *E. coli* were incubated at 37 °C for 16 h, while the fungal strains *S. cerevisiae* and *C. albicans* were incubated at 28 °C for 24 h. The inhibitory effect on bacterial growth was observed, and the minimum inhibitory concentration (MIC) was determined.

5.8. In Vitro Antioxidant Assay. *5.8.1. Total Reducing Power Test.* The Ferric ion reducing antioxidant power (FRAP) method was used to determine the total reducing power of the samples by reducing Fe^{3+} to Fe^{2+} in the solution to be tested, and the reducing power of the samples was detected by the amount of reduced Fe^{3+}.[44] The CSF samples were prepared in five concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL), with each concentration represented by 1.0 mL. VC was employed as a positive control, and five concentrations were prepared (each of which was taken as 4.0 mL). The following solution should be added to each component: 2.5 mL of PBS (0.2 mol/L) and 2.5 mL of potassium ferricyanide ($K_3Fe(CN)_6$) solution (1%). The mixture was then incubated at 50 °C for 20 min. Subsequently, 2 mL aliquots of trichloroacetic acid (10%) were added, and the solution was centrifuged after being shaken well. The upper layer of the solution (2.5 mL) was combined with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. The obtained data were used to analyze the total reducing power of the samples by taking the CSF concentration at the time of reaching 0.5 (EC_{50}) as the response value.

5.8.2. DPPH Scavenging-Activity Test. A series of five concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) of CSF samples were prepared. A DPPH solution (2.0 mL, 0.1 mmol/L) was combined with 2.0 mL of each sample solution, and the absorbance of the mixture was measured at 517 nm after 30 min of incubation in the dark (A_i). A control solution comprising DPPH without samples and anhydrous ethanol without DPPH was prepared (A_j), as well as a blank solution (A_0). The data obtained were employed as an index of 50% inhibitive concentration (IC_{50}) to examine the DPPH clearance of each polar CSF sample.[22]

The DPPH radical-scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging activity}(\%) = [1 - \frac{A_i - A_j}{A_0}] \times 100\% \quad (4)$$

5.8.3. ABTS$^+$ Radical-Scavenging Activity Test. The CSF samples were prepared in five concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL) for analysis. A volume of 2.0 mL of each concentration sample was combined with an equal volume of the diluted ABTS$^+$ radical solution and incubated for 6 min. The absorbance (A_s) was determined at 743 nm, as previously

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described.[45,46] In the blank system (A_0), the samples were replaced with distilled water.

The inhibition rate of $ABTS^+$ radical scavenging of samples was calculated by using the following formula:

$$ABTS^+ \text{ scavenging activity}(\%) = [\frac{A_0 - A_s}{A_0}] \times 100\% \quad (5)$$

5.9. Calculation of Priority Weights by the Analytic Hierarchy Process. The priority weights of the antibacterial activity of *B. pumilus*, *B. subtilis*, *E. coli*, $ABTS^+$ radical scavenging-activity, DPPH scavenging-activity, and total reducing power were determined by AHP according to a previously reported method.[47] At the outset of the examination process, the objectives and criteria were defined, and a criteria matrix was constructed. The weights were assigned a value on a nine-point scale, as illustrated in Table 8. A total of six objectives were identified for examination in this experiment, resulting in the establishment of a six-order matrix, in accordance with the criteria outlined in Table 8.

Once the matrices have been established, the consistency ratio (CR) of the pairwise matrices is calculated in order to assess the reasonableness of the results, ensuring that the findings are logical and coherent.

The formula is as follows:

$$CR = \frac{CI}{RI}$$

$$CI = \frac{\lambda_{max} - 1}{N - 1}$$

$$\lambda_{max} = \frac{1}{n}\sum_{i=1}^{n}\left(\sum_{j=1}^{n} f_{ij} \times \frac{w_j}{w_i}\right) \quad (6)$$

where RI is the random consistency index whose value for computation is presented in Table 9.

A value of CR ≤ 0.1 is indicative of successful completion of the consistency test. Subsequently, the normalized weight coefficient was employed for a comprehensive assessment of the multiple bioactivities exhibited by the CSF samples. The initial and normalized weight coefficients, w_i' and w_i, were calculated according to the following formulas:

$$w_i' = \sqrt[n]{f_1 f_2 \cdots f_n}$$

$$w_i = \frac{w_i'}{\sum_{i=1}^{n} w_i'} \quad (7)$$

The comprehensive assessment score for multiple bioactivities was determined to be S:

$$S = \sum_{i=1}^{n} f_i \times w_i \quad (8)$$

5.10. Statistical Analysis. All experiments were conducted in triplicate. A one-way analysis of variance (ANOVA) with multiple comparisons was conducted using SPSS statistical software, version 19.0, to ascertain whether significant differences existed within the sample groups for the five polarities under investigation in this experiment. The results are expressed as the mean ± the standard error. A value of $p < 0.05$ indicated a significant effect.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c10986.

Results of mass spectrometry analyses of major components in CSF samples and relative content of major components in CSF samples by HPLC (PDF)

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Conceptualization, H.C.; methodology, H.Z.; software, S.P.; validation, H.C.; formal analysis, H.J.; investigation, H.J. and Y.D.; resources, Y.D.; data curation, H.Z.; writing—original draft preparation, H.J.; writing—review and editing, H.C.; visualization, H.Z.; supervision, Y.D.; project administration, H.C.; funding acquisition, H.C.; H.J. and Y.D. contributed equally to this work.

Notes

The authors declare no competing financial interest.

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