



ORIGINAL ARTICLE

Simultaneous optimization of extraction and antioxidant activity from *Blumea laciniata* and the protective effect on Hela cells against oxidative damage



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Abstract *Blumea* was the resource for medicine such as l-borneol showing a great economic value. Interestingly, *Blumea laciniata* was widely used as a folk medicine in south of China and Asia. But the chemical compounds and specific pharmacological activity were rarely reported. Therefore, in this present work, the chemical components from *B. laciniata* were determined and the antioxidant ability on scavenging radicals and the protection on Hela cells against H₂O₂ were evaluated. The results showed the antioxidant ability was associated with the presence of polyphenols via response surface method. Additionally, chlorogenic acid, isoorientin, rutin, luteolin-4'-O-glucoside and cinnamic acid were firstly identified in *B. laciniata*. Moreover, the extract from *B. laciniata* (EBL) showed a strong antioxidant on clearing DPPH and ABTS free radicals with a lower EC₅₀. Besides, EBL showed no toxicity on Hela cells and even could protect cells from H₂O₂ induced damage by sharply reducing the excessive reactive oxygen species, improving the mitochondrial membrane potential and then decreasing the generation of cell apoptosis. These outcomes could provide a promising understanding on the potential antioxidant.

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1. Introduction

Blumea distributed from southeast to southwest China, Africa and Southeast Asia, and in those places many kinds possessing various medicinal effects were widely used as a herbal medicine for pharmacological activity, such as antioxidant activity, enhancing percutaneous penetration activity, hepatoprotective effects, antitumor activity and antifungal effects (Chen et al., 2009). Those effects might be linked with the content of

flavonoids, terpenoids, polyphenols and volatile oil compounds (Pang et al., 2017; Shen et al., 2019; Tan et al., 2020). Likewise, *Blumea balsamifera* could well scavenge free radicals and inhibit aldose reductase to show anti-diabetic effect (Sakee et al., 2011; Lee et al., 2012). In addition, *Blumea* was the resource for l-borneol (a widely used medicine), and the essential oil exhibited a unique aromatic odor and was widely incorporated into perfume and cosmetics industries (Pang et al., 2014a, 2014b). Therefore, the plants of *Blumea* had a great economic value. However, rarely studies were reported on *Blumea laciniata* (Roxb.) DC. about chemical and pharmacological activity.

B. laciniata was a commonly used herbal medicine in the south of China and Himalayan region. However, the relative researches about *B. laciniata* were rarely reported. Scholars from India found the essential oil from *B. laciniata* could well inhibit common pathogenic bacteria, *Escherichia Coli*, *Pseudomonas Aeruginosa*, *Salmonella Typhi*, *Protus Vulgaris*, *Salmonella Typhi*, *Staphylococcus Aureus* (Kar and Jain, 1971). And protocatechuic acid, chrysoeriol, apigenin, 4-hydroxy-3,5-dimethoxybenzoic acid and scopolet also were identified in ethyl acetate fraction of ethanol extract from *Blumea laciniata* (Yuan et al., 2009). Li et al. screened 21 herbs originating in southern China for antiviral activity and found that the extract of *B. laciniata* exhibited a good inhibitory effect on respiratory syncytial virus. Previous studies proved the extract of *B. laciniata* also showed a strong antiviral ability against human herpes simplex virus type 1 (Chen et al., 2018). Meanwhile, the chemical composition of *B. laciniata* was analyzed, and it was found that the effective components of the antiviral activity might be related to its polyphenols (Li et al., 2004). But, the reports about the antioxidant ability of the extract from *B. laciniata* was still unknown.

When body was stimulated by endogenous or exogenous oxidative stimuli, the number of reactive oxygen radicals in the body would significantly increase, which caused damage to mitochondrial DNA, mitochondrial dysfunction, and then leading to tissue damage, disease occurrence and aging of the body (Finkel and Holbrook, 2000). Hopefully, many antioxidant active substances in plant and botanical herbs had been found to play a crucial role by scavenging free radical levels and improving antioxidant enzyme activity in the organism (Zhuang et al., 2014; Chen et al., 2018). The first and important step to obtain bioactive compounds from plants was extraction, easily affected by multiple factors such as extraction time, solvent to sample ratio and so on (Santos et al., 2016). Therefore, response surface method (RSM) was established to observe the influence on the extraction rate and optimize the process. In our previous pre-experiment, *B. laciniata* contained different chemical components and exerted a great antioxidant ability. Therefore, in this present study, the first attempt to optimize the ultrasound-assisted extraction (UAE) condition for chemical compounds and the antioxidant ability by applying Box-Behnken design (BBD). Further the phenolic compounds were detected by High-Performance Liquid Chromatography (HPLC). And the protection of *B. laciniata* in cellular level was determined to comprehensively evaluate the antioxidant capacity. Those finds may be helpful to gain a better understanding on *B. laciniata* for potential pharmacological applications.

2. Materials and methods

2.1. Materials and chemicals

Blumea laciniata (Roxb.) DC. was picked up from Zigong, Sichuan, China, at 29°18'41" N, 104°47'31" E. The plant was identified as *B. laciniata* by professor Chao Hu, Department of Botanical Science, College of Life Science, Sichuan Agricultural University and the herbarium (No.3564) was collected and stored in the specimen room of College of Life Science, Sichuan Agricultural University. The whole plant was washed by up-water three times then dried at 50°C before grounded into powder.

2,2-Diphenyl-1-picryl-hydrazyl (DPPH) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethanol, gallic acid, acetic acid, vanillin, ferric chloride, sodium carbonate, perchloric acid, sodium nitrite and others were purchased from the Chengdu Kelong Chemical Factory (Chengdu, China). Folin-phenol reagent was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). MTT kits and Reactive Oxygen Species Assay Kit (ROS) was obtained from Beyotime Biotechnology (Shanghai, China). Fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), phosphate buffer solution (PBS) and digestive enzyme and Penicillin-Streptomycin solution (10X) were purchased from Hyclone (USA). All chemicals were analytical grade.

2.2. Ultrasonic-assisted extraction (UAE) of chemical compositions

The chemical compositions in *B. laciniata* were extracted with the assistance of ultrasonic cleaner (SB-4200DTD, Ningbo Scientz Biotechnology Co., LTD, Zhejiang, China). Dry powder (1 g) of *B. laciniata* was mixed with different concentration of ethanol (50%, 70% and 90%). The solvent to sample ratio was kept at 20, 40 and 60 mL/g. The solution was placed at 50°C under the UAE with power of 200, 280 and 360 W for 30, 60 and 90 min. All experiments were performed in triplicate.

2.3. Response surface methodology (RSM) design

The optimization experiments for UAE of chemical compounds from *B. laciniata* were performed by applying a three-level-four-factor Box-Behnken design (BBD) with RSM. Four chemical compounds and three antioxidant abilities were taken as the responses. The coded and actual levels of the four factors were presented in Table 1. The mean values of dependent parameters obtained from the triplicate experiments were fitted to a second order polynomial model as follows:

$$Y = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i=1}^4 \beta_{ii} X_i^2 + \sum_{1 < j < 2}^4 \beta_{ij} X_i X_j$$

where Y was the predicted extraction yield; β_0 meant a constant; β_i , β_{ii} , and β_{ij} denoted coefficients of the linear, quadratic, and interaction terms, respectively; X_i and X_j represented independent variables. Analysis of variance (ANOVA) was performed to evaluate the individual linear, quadratic and interaction regression coefficients (β) using Design Expert 11 trial version (Stat-Ease, Inc.). The significance of the depen-

Table 1 Box–Behnken designs and its results.

Run	Solvent to sample ratio mL/g (X ₁)	Power of UAE W (X ₂)	Extraction time min (X ₃)	Concentration of ethanol % (X ₄)	TP mg GAE/g	TF mg LE/g	TPA mg PAE/g	TT mg UAE/g	FRAP mM FE / g	ABTS mM TE / g	DPPH %
1	30	280	90	50	40.55	91.79	17.63	8.95	61.76	18.29	82.09
2	40	280	60	50	52.75	83.38	15.89	8.26	47.28	12.02	82.39
3	40	280	60	90	58.06	89.21	14.41	19.10	42.41	11.18	88.09
4	20	360	60	70	28.61	85.37	14.06	18.50	70.52	21.96	83.51
5	30	280	30	90	45.40	80.30	13.30	13.67	45.23	13.54	88.05
6	30	200	60	50	39.67	67.78	11.85	11.22	46.26	12.90	79.60
7	40	280	30	70	53.85	94.34	20.98	16.55	43.98	10.53	84.43
8	30	360	60	50	38.83	86.64	18.12	7.90	55.27	14.50	80.18
9	30	280	60	70	41.39	93.06	17.94	16.30	55.15	16.28	82.13
10	40	360	60	70	53.62	80.94	17.12	11.91	38.04	9.82	80.99
11	20	280	60	90	31.99	57.77	10.96	11.91	51.56	17.37	83.42
12	30	280	60	70	41.74	84.51	17.48	16.93	52.80	15.73	83.91
13	30	200	60	90	45.03	53.05	11.77	14.40	31.43	8.89	85.78
14	20	280	30	70	29.36	70.12	13.84	17.40	64.44	21.94	80.39
15	30	280	30	50	41.66	83.66	19.58	16.74	50.54	14.59	82.28
16	30	280	60	70	44.20	93.45	16.54	19.46	56.19	18.06	81.89
17	30	200	90	70	43.83	75.39	17.57	17.08	45.83	14.83	83.28
18	30	360	90	70	43.89	95.67	21.39	21.78	64.39	17.74	83.51
19	40	280	90	70	55.78	93.36	22.92	21.25	48.79	11.84	85.79
20	20	280	90	70	29.92	84.91	16.78	16.07	57.76	23.16	83.30
21	30	200	30	70	42.00	66.97	14.26	19.48	43.96	11.91	80.30
22	30	280	90	90	45.52	81.75	17.92	25.27	46.10	12.29	86.92
23	30	280	60	70	42.19	84.67	13.95	18.01	50.40	15.29	81.84
24	40	200	60	70	54.05	83.56	15.35	19.90	36.91	10.18	83.62
25	30	360	30	70	41.74	90.06	17.48	18.71	54.68	15.81	85.02
26	20	200	60	70	26.53	33.65	11.49	6.65	34.89	13.21	73.03
27	30	360	60	90	45.82	78.18	11.55	21.86	49.22	12.54	87.33
28	30	280	60	70	41.65	90.63	17.36	18.56	52.02	16.81	82.61
29	20	280	60	50	27.17	81.32	13.44	11.67	70.01	24.11	79.91

dent variables was statistically analyzed by calculating the *p* value.

2.4. Determination of chemical components

Quantifications of total polyphenols (TP), total flavonoids (TF), total phenolic acid (TPA) and total triterpenes (TT) were measured by a microplate reader and all experiments were performed in triplicate.

TP was assessed according to Folin-phenol reagent method (Zeng et al., 2020). Briefly, 0.2 mL sample solution was mixed with 0.2 mL Folin-phenol reagent (25%, v/v). After 5 min, 0.2 mL sodium carbonate (7.5%, w/v) was added in. Finally, the mixture was diluted to 2 mL with up-water. The reaction solution was placed at dark for 30 min before read at 700 nm for absorbance. The TP content was calculated according to the standard curve made with gallic acid and the TP was expressed as milligram gallic acid equivalent per gram (mg GAE / g) of dry sample.

TF was determined according to the previous method (Lim et al., 2019). Briefly, 0.2 mL sample solution was mixed with 0.3 mL sodium nitrite (5%, w/v), 0.3 mL aluminum nitrate (10%, w/v) and 0.6 mL sodium hydroxide (4%, w/v). The mixture solution was stood at dark for 15 min then diluted to 2 mL with up-water. Finally, the absorbance of reaction solution was read at 510 nm. The TF content was calculated according to the standard curve made with rutin and the TP was

expressed as milligram rutin equivalent per gram (mg RE / g) of dry sample.

TPA content was assessed by SDS-HCl method. Briefly, 0.1 mL sample solution was mixed with 0.3 mL SDS (0.3%, w/v) and 0.3 mL mixture solution made with 0.15 mL ferric chloride (0.6%, w/v) and 0.15 mL potassium ferricyanide (0.9%, w/v). Finally, HCl (0.1 mM) was added in to 2 mL. The whole reaction was operated at dark. The absorbance of the reaction solution was read at 753 nm. The TPA content was calculated with the standard curve made with protocatechuic acid. TPA was expressed as milligram protocatechuic acid equivalent per gram (mg PAE / g) of dry sample.

TT content was determined according to the method with some modifications (Luo et al., 2020). Briefly, 0.05 mL sample solution was mixed with 0.1 mL vanillin (2%, w/v, resolved in acetic acid) and 0.15 mL perchloric acid. The mixture was placed at 60°C for 15 min before diluted to 1.2 mL with acetic acid. The absorbance of reaction solution was read at 550 nm. The TT content was calculated with the standard curve made with ursolic acid. TT was expressed as milligram ursolic acid equivalent per gram (mg UAE / g) of dry sample.

2.5. Determination of antioxidant abilities

Antioxidant activities of sample solution were evaluated by DPPH free radical scavenging ability, ferric reducing antioxidant power (FRAP) and 2,2-azino-bis (3-ethylbenzthiazoline-

6-sulphonic acid) (ABTS) radical scavenging activity assay kit. DPPH was resolved in ethanol to 0.7 mM. 20 μ L sample solution was mixed with 180 μ L DPPH solution (0.7 mM) at dark for 10 min before the absorbance was read at 517 nm. The outcome was expressed as clearance rate (%). FRAP and ABTS were determined according to the kit instructions (Nanjing Jiancheng Bioengineering Institute, China). The FRAP results were expressed as millimoles of ferrous sulfate equivalent per gram (mM FE/g) of dry sample. ABTS results were expressed as millimoles of Trolox equivalent per gram (mM TE/g) of dry sample. All experiments were performed in triplicate.

2.6. HPLC analysis

The chromatographic analysis was performed using an Agilent 1260 HPLC (Agilent Technologies, Santa Clara, CA, USA) coupled with a UV-Vis DAD detector and a C18 reversed-phase column (5.0 μ m, 150 mm \times 4.6 mm). The phenolic compositions were analysed by a gradient elution program using (A) mili-Q water, (B) aqueous phosphoric acid (0.2%, v/v) and (C) acetonitrile were used as the mobile phase and the gradient program was set as follows: 10–16% B (0–3 min), 16–30% B (3–20 min), 30–40% B (20–25 min), 40–16% B (25–30 min), and 16–10% B (30–40 min) with a 0.5 mL/min flow rate. The column temperature was kept at 30°C. The polyphenols concentrations of chlorogenic acid, isoorientin, rutin, apigenin-8-C-glucoside, luteolin-4'-O-glucoside, quercitrin, apigenin-7-O-glucoside, luteolin, quercetin, cinnamic acid and apigenin were determined.

2.7. Determination of the antioxidant ability on cellular level

2.7.1. The cell culture

Hela cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences, Shanghai, China. Hela cells were cultured in DMEM containing 10% FBS. When the cells grew to 80% of the flask, the cells were digested from the bottom and washed with PBS for 3 times for following assays.

2.7.2. Establishment of injury model

The cell density was readjusted to 10⁵ cells/mL and the suspension was seeded into 96-well plate for 12 h. Then different concentrations of H₂O₂ was added into the plate for 5 h. After exposure, all solution was removed and fresh medium was added into the plate and the cell viability was determined with MTT method (Rajan et al., 2020).

2.7.3. The toxicity of EBL on Hela cells

The Hela cells were resuspended at a concentration of 10⁵ cells/mL. 90 μ L cell suspension was added into 96-well plate for 12 h and then different concentration of EBL was transferred into each well for 24 h treatment. After incubation, all solution was removed and new medium was added into the plate. 5 mg/mL MTT solution was transferred into each plate for 5 h. Subsequently the solution was replaced by DMSO solution to resolve formazan. The plate was incubated in a shaker for 1 h. The absorbance of the plate was read at 570 nm and the cell viability was calculated according to the following formula:

$$\text{Cell viability (\%)} = A1/A2$$

Where A1 was the absorbance of trail group and A2 was the absorbance of the blank group.

2.7.4. Determination of the protection of EBL on Hela cells against oxidative stress

Hela cells were seeded into 96-well plate for 12 h and EBL was added into each well for 24 h treatment. Then the solution was removed and new medium and H₂O₂ were added in and incubated for 5 h. 25 μ g/mL of Vc was used as the positive group. Then the cell viability was determined with MTT method.

2.7.5. Determination of the ROS level

The cell suspension was adjusted to the density of 10⁶ cells/mL and then transferred into 6-well plate for 12 h before the sample solution was added in. After 24 h treatment, the cells were exposed to H₂O₂ for 5 h. Subsequently, the solution was replaced by paraformaldehyde (4%) for 30 min at 4°C. Then PBS was used to washed the cells for 3 time to remove the fixative and ROS kit was added to stain the cell for 30 min at 37°C. Finally, the ROS level was determined by a fluorescence microscope (Olympas-BX51TRF, Japan).

2.8. Statistical analyses

The experimental results of the response surface design were analyzed statistically by one-way ANOVA using Design-Expert 11 software (trial version, State-Ease Inc., Minneapolis, MN, USA). All experimental results were expressed as mean \pm standard deviation (SD). All experiments were carried out in triplicates.

3. Results

3.1. The effect of different extraction conditions on the chemical components

3.1.1. Total polyphenols (TP)

Based on the regression coefficient (β) values, linear (X_1) revealed a major effect, which was followed by X_4 . The extraction yield value of TP could be expressed by the following second order poly-nomial equation:

$$Y = 42.23 + 12.88X_1 + 0.12X_2 + 0.46X_3 + 2.60X_4 - 0.63X_1X_2 + 0.34X_1X_3 + 0.12X_1X_4 + 0.08X_2X_3 + 0.41X_2X_4 + 0.31X_3X_4 - 0.71X_1^2 - 0.49X_2^2 + 0.75X_3^2 + 0.62X_4^2$$

The lack of fit was not significant with the p value = 0.7577 and the model was significant ($p < 0.001$) indicating the model was fit with good prediction ($R^2 = 0.9938$, adj $R^2 = 0.9875$).

The linear effects of X_1 and X_4 were positive to the yield of TP while the interactions between two factors were not significant (Table 2). The extraction time and power of UAE did not affect the yield of TP. Increasing of the solvent to sample ratio promoted the TP yield (Fig. 1A), which could help the solute dissolve in solvent. Furthermore, cell wall and particle collisions disruption produced by cavitation enhanced solvent penetration into the sample matrix (He et al., 2016).

3.1.2. Total flavonoids (TF)

As shown in Table 2, the ANOVA results showed significant linear (X_1 , and X_2), quadratic (X_2^2) and interactive (X_1X_2) effects on TP. Based on the regression coefficient (β) values,

Table 2 ANOVA for response surface quadratic model.

Source	p-value													
	TP	TF	TPA	TT	FRAP	ABTS	DPPH	TP	TF	TPA	TT	FRAP	ABTS	DPPH
X ₁	2122.86	99.87	34.47	15.37	64.57	161.47	33.78	<0.0001 [#]	<0.0001 [#]	<0.0001 [#]	0.0015**	<0.0001 [#]	<0.0001 [#]	<0.0001 [#]
X ₂	0.17	149.19	15.39	10.05	66.09	21.40	15.91	0.6829	<0.0001 [#]	0.0015**	0.0068**	<0.0001 [#]	0.0004 [#]	0.0013**
X ₃	2.66	11.23	11.05	4.33	3.65	4.94	1.39	0.1249	0.0048**	0.0050**	0.0562	0.0769	0.0432*	0.2577
X ₄	86.38	23.65	13.95	121.19	32.56	21.72	78.33	<0.0001 [#]	0.0003 [#]	0.0022**	<0.0001 [#]	0.0001 [#]	0.0004 [#]	<0.0001 [#]
X ₁ X ₂	1.68	71.00	0.10	83.22	27.38	12.76	36.80	0.2153	<0.0001 [#]	0.7617	<0.0001 [#]	0.0001 [#]	0.0003**	<0.0001 [#]
X ₁ X ₃	0.50	5.99	0.15	7.72	3.04	0.001	0.51	0.4916	0.0282*	0.7026	0.0148*	0.1031	0.9705	0.4877
X ₁ X ₄	0.06	20.75	0.15	23.71	4.24	5.36	1.02	0.8059	0.0004 [#]	0.7035	0.0002 [#]	0.0585	0.0362*	0.3298
X ₂ X ₃	0.03	0.19	0.05	6.33	1.41	0.15	4.29	0.8687	0.6695	0.8183	0.0247*	0.2545	0.7075	0.0574
X ₂ X ₄	0.71	0.95	6.42	24.54	1.78	0.65	0.20	0.4140	0.3471	0.0239*	0.0002 [#]	0.2038	0.4351	0.6588
X ₃ X ₄	0.41	1.07	6.55	79.52	2.47	3.78	0.19	0.5341	0.3181	0.0227*	<0.0001 [#]	0.1386	0.0722	0.6694
X ₁ ²	3.64	23.26	2.14	26.68	0.47	0.64	2.56	0.0770	0.0003 [#]	0.1653	0.0001 [#]	0.5059	0.4380	0.1316
X ₂ ²	1.62	78.09	7.68	4.73	16.65	24.49	5.76	0.2232	<0.0001 [#]	0.0150*	0.0473*	0.0011**	0.0002 [#]	0.0309*
X ₃ ²	3.92	4.52	25.81	22.30	1.77	0.35	10.61	0.0679	0.0517	0.0002 [#]	0.0003 [#]	0.2041	0.5656	0.0057**
X ₄ ²	2.68	26.31	17.26	57.36	2.57	8.84	11.97	0.1240	0.0002 [#]	0.0010 [#]	<0.0001 [#]	0.1311	0.0101*	0.0038**
Lack of Fit	0.61	0.35	0.51	0.64	2.35	1.57	1.82	0.7577	0.9182	0.8257	0.7437	0.2130	0.3513	0.2958
Model	159.29	36.11	10.85	35.54	16.42	19.16	14.96	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Note: * means p < 0.05; ** means p < 0.01 and [#] means < 0.001.

interactive (X₁X₂) revealed a major effect, which was followed by X₂, X₃², and X₁. The extraction yield value of TP could be expressed by the following second order poly-nomial equation:

$$Y = 89.26 + 9.30X_1 + 11.37X_2 + 3.12X_3 - 4.53X_4 - 13.59X_1X_2 - 3.95X_1X_3 + 7.35X_1X_4 - 0.7029X_2X_3 + 1.57X_2X_4 - 1.67X_3X_4 - 6.11X_1^2 - 11.19X_2^2 + 2.69X_3^2 - 6.49X_4^2$$

The lack of fit was not significant with the p value = 0.9182 and the model was significant (p < 0.001) indicating the model was fit with good prediction (R₂ = 0.9731, adj R₂ = 0.9461).

The linear effects of X₁ and X₂ showed a significant (p < 0.05) positive effect on the yield of TF. However, the interaction between X₁ and X₂ was negative to the yield. Extraction ratio was increasing with a lower solvent to sample ratio and a weaker power of UAE. Increasing the power of ultrasound, the yield of TF was enhanced (Fig. 1B). X₂² also showed a negative effect on the yield of TF. This may be multilevel effects of cavitation created by ultrasonic radiation pressure while increasing the power would contribute to the degradation of some bioactive compounds (Sang et al., 2017; Rombaut et al., 2014)

3.1.3. Total phenolic acid (TPA)

As shown in Table 2, the ANOVA results showed significant linear (X₁, X₂ and X₃), quadratic (X₂², X₃² and X₄²) and interactive (X₁X₂) effects on TPA. Based on the regression coefficient (β) values, quadratic (X₄²) revealed a major effect. The extraction yield value of TP could be expressed by the following second order poly-nomial equation:

$$Y = 16.65 + 2.17X_1 + 1.45X_2 + 1.23X_3 - 1.38X_4 - 0.1984X_1X_2 - 0.25X_1X_3 + 0.2492X_1X_4 + 0.1502X_2X_3 - 1.62X_2X_4 + 1.64X_3X_4 - 0.7375X_1^2 - 1.40X_2^2 + 2.56X_3^2 - 2.09X_4^2$$

The lack of fit was not significant with the p value = 0.8257 and the model was significant (p < 0.001) indicating the model was fit with good prediction (R² = 0.9156, adj R² = 0.8312).

The linear effects of X₁ and X₂ showed a significant (p < 0.05) positive effect on the yield of TPA while X₄ showed a significant (p < 0.05) negative effect. In addition, the interaction between X₁ and X₂ was also negative to the yield. X₂² and X₄² also showed a negative effect on the yield of TF. While as the extraction time prolonged, the extraction rate of TPA was improved (Fig. 1C). The influence of four factors on TPA was similar to that on TF. Both of them were mainly affected by the solvent to sample ratio and power of UAE.

3.1.4. Total triterpenes (TT)

The ANOVA results showed significant linear (X₄), quadratic (X₁², X₃² and X₄²) and interactive (X₁X₂, X₁X₄, X₂X₄ and X₃X₄) effects on TT. Based on the regression coefficient (β) values, interactive (X₃X₄) revealed a major effect. The extraction yield value of TP could be expressed by the following second order poly-nomial equation:

$$Y = 17.85 + 1.23X_1 + 0.9948X_2 + 0.6535X_3 + 3.46X_4 - 4.96X_1X_2 + 1.51X_1X_3 + 2.65X_1X_4 + 1.37X_2X_3 + 2.69X_2X_4 + 4.85X_3X_4 - 2.21X_1^2 - 0.9285X_2^2 + 2.02X_3^2 - 3.23X_4^2$$

The lack of fit was not significant with the p value = 0.7437 and the model was significant (p < 0.001) indicating the model was fit with good prediction (R² = 0.9726, adj R² = 0.9453).

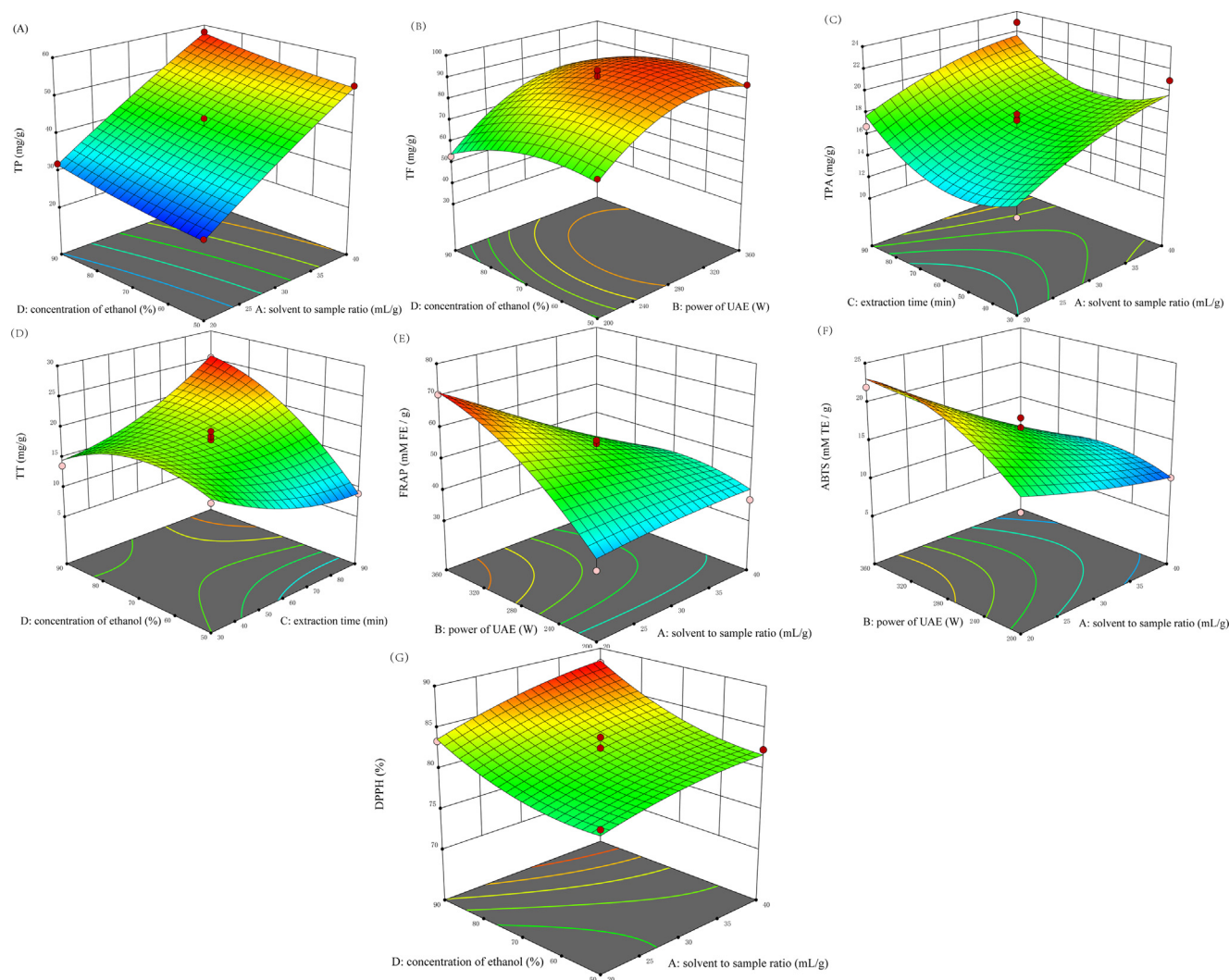


Fig. 1 Response surface plots (3D) showing the interactive effect of extractions variables on (A) the total polyphenols, (B) Total flavonoid, (C) Total phenolic acid, (D) Total triterpenes, (E) ferric reducing antioxidant power, and the scavenging ability on (F) ABTS and (G) DPPH free radicals.

The linear effects of X_4 showed a significant ($p < 0.05$) positive effect on the yield of TT while quadratic X_4^2 showed a significant ($p < 0.05$) negative effect. In addition, the interactions of X_1X_4 , X_2X_4 and X_3X_4 were also positive to the yield. Under a high temperature, extending the extraction time could help extract more TT from *B. laciniata* (Fig. 1D). Nevertheless, the interaction between solvent to sample ratio and power of UAE was negative to the yield of TT. Increasing the concentration of ethanol helped the solute (like TT) dissolve in solvent (Belwal et al., 2016).

3.2. The effect of different extraction conditions on the antioxidant abilities

FRAP was mainly affected by X_1 followed by X_2 , X_4 and X_1X_2 . Enhance the single factor of power of UAE showed a positive effect on FRAP. However, the interaction of X_1X_2 revealed that a lower solvent to sample ratio and a weaker power of UAE could enhance FRAP. In addition, X_1 and X_4 also demonstrated a negative effect on the yield.

ABTS was mainly affected by X_1 followed by X_2 , $X_2X_1X_2$ and X_4 . The linear effects of X_1 and X_4 were negative to ABTS while X_2 showed a positive. Hence interactive effect of X_1X_2 also suggested a negative on the activity of ABTS, which was similar to the outcome of FRAP.

DPPH radical scavenging ability was dominated by X_4 followed by X_1 , X_3 , X_1X_2 , X_1^2 and X_3^2 . Linear effects of X_1 and X_4 were positive to the ability. While increasing extraction time would lower the DPPH scavenging ability.

The second order poly-nomial equation of three antioxidant abilities were present as followed:

$$\text{YFRAP} = 53.31 - 7.65X_1 + 7.74X_2 + 1.82X_3 - 5.43X_4 - 8.63X_1X_2 + 2.87X_1X_3 + 3.40X_1X_4 + 1.96X_2X_3 + 2.20X_2X_4 - 2.59X_3X_4 - 0.8838X_1^2 - 5.28X_2^2 + 1.72X_3^2 - 2.08X_4^2$$

$$\text{YABTS} = 16.43 - 4.68X_1 + 1.70X_2 + 0.8187X_3 - 1.72X_4 - 2.28X_1X_2 + 0.024X_1X_3 + 1.48X_1X_4 - 0.2443X_2X_3 + 0.5127X_2X_4 - 1.24X_3X_4 - 0.40X_1^2 - 2.48X_2^2 + 0.2948X_3^2 - 1.49X_4^2$$

$$\text{YDPPH} = 84.47 + 1.81X_1 + 1.24X_2 + 0.3682X_3 + 2.76X_4 - 3.28X_1X_2 - 0.3852X_1X_3 + 0.5457X_1X_4 - 1.12X_2X_3 +$$

$$0.2439X_2X_4 - 0.2357X_3X_4 - 0.6796X_1^2 - 1.02X_2^2 + 1.38X_3^2 + 1.47X_4^2$$

The non-significant values of the lack of fit ($p = 0.2130$, 0.3513 , and 0.2958 , respectively) showed that the models were fit with good prediction ($R^2 = 0.9426$, 0.9504 and 0.9373 , respectively; adj $R^2 = 0.8852$, 0.9008 and 0.8747 , respectively)

Solvent to sample ratio (X_1) was the main influencing factor to FRAP, ABTS and DPPH scavenging ability. Meanwhile X_1 also affected the yield of TP, TT and TPA. The interaction effect of X_1 and X_2 showed the same effect on FRAP and ABTS scavenging ability (Fig. 1E and F). Nevertheless, when the extraction time and temperature were fixed, a high concentration of ethanol could help to extract a stronger matrix on clearing DPPH (Fig. 1G). The similar effect of interaction between X_1 and X_2 indicating that the antioxidant ability was associated with the presence of phenolic compounds rather than total triterpenes (Li et al., 2004). Therefore, the antioxidant of *B. laciniata* was closely linked with the content of polyphenols.

3.3. Verification of the model

The yields of TP, TF, TPA and TT were determined on the optimum condition for each compound. And the antioxidant ability was also conducted under the optimal extraction condition. The experimental and predicted values were present in Table 3. The outcomes were close to the predicted values, suggesting the response surface method was successfully applied to optimize the extraction process for different constituents and antioxidant abilities of *B. laciniata*.

3.4. HPLC analysis of phenolic compositions

To further investigate the phenolic compounds in EBL, HPLC was applied to determine the presence of compounds. As shown in Fig. 2, chlorogenic acid, isoorientin, rutin, luteolin-4'-O-glucoside and cinnamic acid five kinds phenolic compounds were firstly identified in EBL among the 11 kinds standards. Moreover, among the five compounds, chlorogenic acid was highly contained while other four compounds were in trace amounts (Fig. 2).

3.5. The scavenging ability on free radicals

This was the first time to determine the antioxidant ability of *B. laciniata*. DPPH free radical scavenging ability was applied to evaluate the antioxidant of extract from *B. laciniata* (ELB). As shown in Fig. 3A, with the increasing concentrations, the clearance rate on DPPH was enhanced. The EC50 was 0.41 mg/mL . Moreover, EBL could also significantly scavenge ABTS radicals with an EC50 of 0.13 mg/mL (Fig. 3B). Natural

extracts from plants were demonstrated to show a strong antioxidant ability. Likewise, polyphenols from *Rosa banksiae* Ait. flowers showed a strong clearance rate on DPPH radical with an EC50 of 0.34 mg/mL (Zeng et al., 2020). And the EC50 of polyphenols from two types of *Solanum gilo* Raddi on DPPH radical ranged from 0.17 to 0.81 mg/mL (De et al., 2019). Therefore, EBL like other extracts could well scavenge free radicals.

3.6. The protection of *B. Laciniata* on Hela cells against oxidative stress

3.6.1. The toxicity of *B. Laciniata* on Hela cells

Nevertheless, single antioxidant assays in vitro were not enough to elucidate the antioxidant comprehensively (Granato et al., 2018). Hence, Hela was used to evaluate the antioxidant ability of EBL in cell level. Firstly, the toxicity of EBL should be determined and a serial of suitable concentrations may be picked up. As shown in Fig. 4, different concentrations of EBL (0 – $400 \text{ }\mu\text{g/mL}$) showed no significantly negative effect on the cell viability of Hela cells after 24 h treatment. The results obtained in this work was in accordance with the previous research that extracts from *Blumea balsamifera* was also showed a low cell toxicity on Hep G2 cells (Toshio et al., 2008). Therefore, those five concentrations were suitable for following experiments.

3.6.2. The cell viability of Hela cells under oxidative stress

Under the oxidative stress induced by H_2O_2 , the cell viability was decreased. A high concentration of H_2O_2 leded a sharply reducing on live cell numbers. Hence, it was necessary to find a suitable concentration to establish the injury model. After exposed to different concentrations of H_2O_2 , the cell viability of Hela cells was reduced, and on the condition of $4 \text{ mM H}_2\text{O}_2$, the cell viability was about 50% to the blank group (Fig. 5A). Therefore, $4 \text{ mM H}_2\text{O}_2$ was suitably picked up for the following assays. Natural polyphenols extract from plants were proved to show a protection on cells against stress environments. Likewise, phenolics from *Tamarix gallica* protect IEC-6 cells against H_2O_2 induced stress under the concentration of no-toxicity (Bettaib et al., 2017). Hence, different concentrations of EBL were used to pretreated Hela and then the cells were exposed to H_2O_2 to evaluate the protection of EBL in cellular level. As shown in Fig. 5B, 25 and $400 \text{ }\mu\text{g/mL}$ of EBL could not promote the cell viability ($p > 0.05$) while 50 – $200 \text{ }\mu\text{g/mL}$ of EBL and Vc could significantly improve the survival of Hela cells under oxidative stress. 50 – $200 \text{ }\mu\text{g/mL}$ of EBL exhibited a protection on Hela cells against cell death induced by H_2O_2 , thus the three concentrations were applied to explore the effect on the ROS level.

Table 3 The optimally predicted values and experimental values.

	TP mg GAE/g	TF mg LE/g	TPA mg PAE/g	TT mg UAE/g	FRAP mM FE / g	ABTS mM TE / g	DPPH %
Predicted	59.59	102.20	22.00	29.74	80.91	26.6	91.37
Experimental	60.29 ± 1.21	101.41 ± 1.66	21.04 ± 0.70	29.12 ± 0.77	80.97 ± 0.84	26.06 ± 0.40	91.04 ± 0.31

Note: the experimental assays were conducted three times and expressed as mean \pm SD.

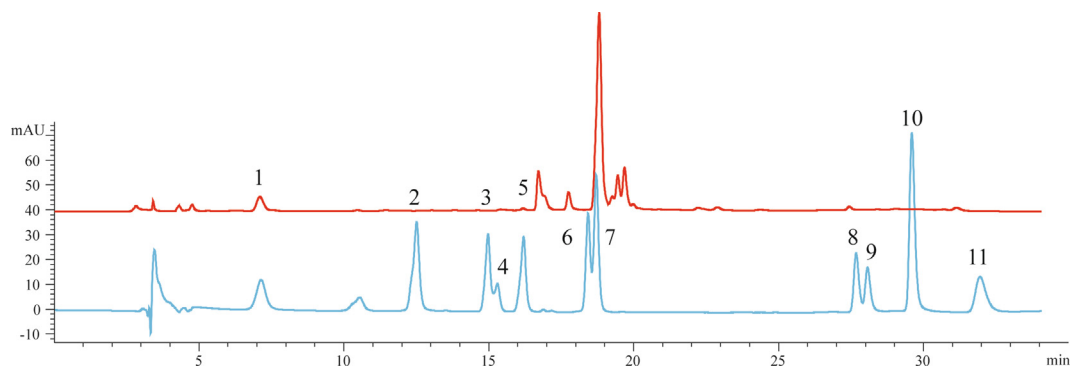


Fig. 2 The HPLC analysis on the phenolic compounds of EBL. 1 chlorogenic acid, 2 isoorientin, 3 rutin, 4 apigenin-8-C-glucoside, 5 luteolin-4'-O-glucoside, 6 quercitrin, 7 apigenin-7-O-glucoside, 8 luteolin, 9 quercetin, 10 cinnamic acid, 11 apigenin. The red line was the sample curve and blue line was the standard curve.

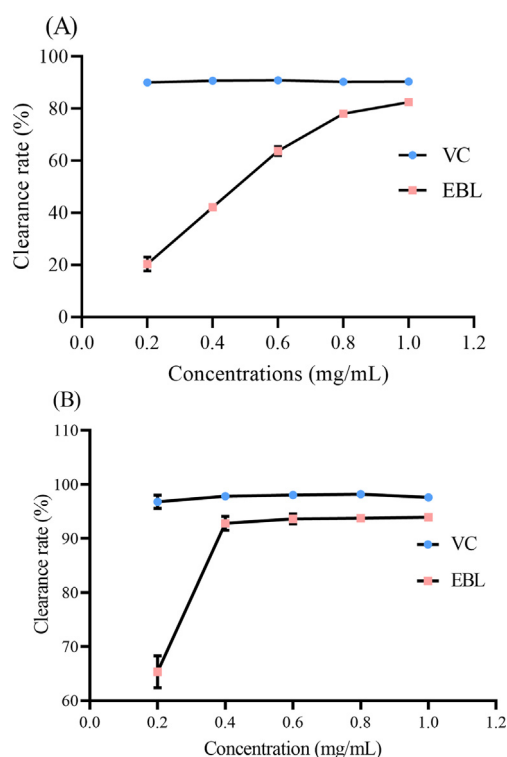


Fig. 3 The antioxidant ability of EBL on scavenging (A) DPPH and (B) ABTS free radicals.

3.6.3. The ROS level of HELA under oxidative stress

H₂O₂ was the trigger of ROS, causing more free radicals such as •OH, O₂, leading to cell damage, lipid peroxidation and even cell death (Stadtman, 2004). Hence, a high level of ROS could bring harm to the cell. As shown in Fig. 6A, compared with the blank group, the ROS in injury group was more while pretreated with EBL and Vc, the fluorescent brightness was weak. With the increasing concentration of EBL, the ROS in HeLa cells were decreasing and 400 µg/mL of EBL could lower more ROS than Vc and reduce the ROS level to that in blank group. A high level of ROS could lead to the reducing of mitochondrial membrane potential (MMP) then causing cell death (Hseu et al., 2014). As shown in Fig. 6B, the MMP was sharply

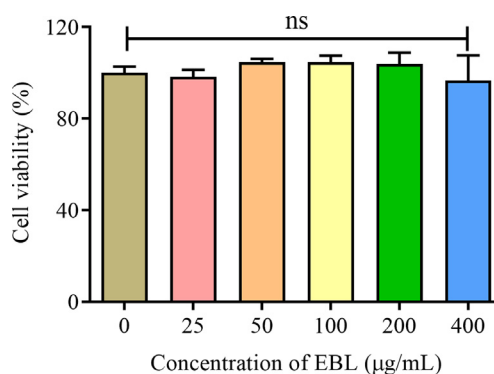


Fig. 4 The cell viability under the treatment with different concentration of EBL for 24 h. Note: ns meant not significant.

lowered when the cell was exposed to H₂O₂, while pretreated with EBL, the MMP could significantly restrain the decrease of MMP. Therefore, the cell apoptosis was then suppressed (Fig. 6C). Hence, EBL showed the protection on HeLa cells against H₂O₂ by reducing the increasing ROS and improving the MMP then reducing cell apoptosis.

4. Discussion

Rarely reports were led to focusing on the chemical and pharmacology of *B. laciniata*. Therefore, this was the first time to use response surface method to simultaneously optimize the total polyphenols, total flavonoids, total phenolic acid and total triterpenes from *B. laciniata*. EBL exhibited a strong antioxidant ability which was closely connected with the content of polyphenols, like the conclusion obtained from *B. lacera* (Alam et al., 2020). Polyphenols from natural plants were considered to be beneficial for human health through exerting various biological effects such as metal chelation, free radicals scavenging, promotion on antioxidant enzymes activity and alteration of signal transduction pathways (Stocker, 1999). Meanwhile, five phenolic compounds chlorogenic acid, isoorientin, rutin, luteolin-4'-O-glucoside and cinnamic acid were firstly identified with HPLC in *B. laciniata*. In this genus, luteolin-7-methyl ether, rutin, luteolin and quercetin were found in *B. balsamifera* (Tan et al., 2020; Chen et al., 2018).

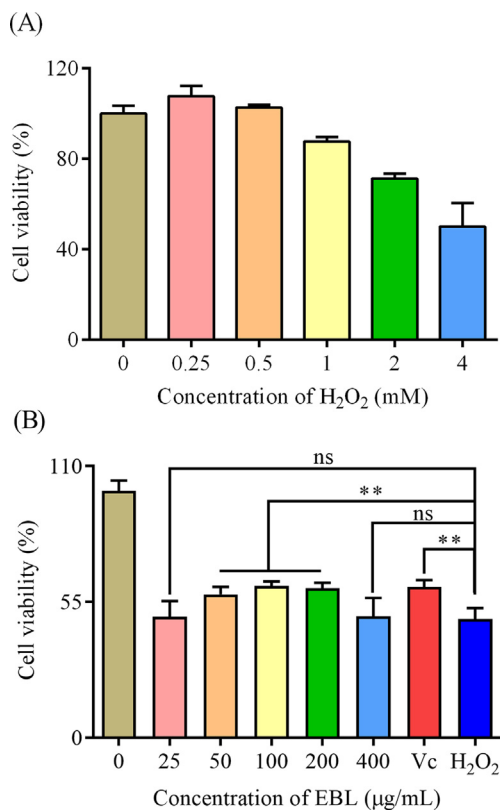


Fig. 5 The effect of EBL on HeLa cells under oxidative stress. (A) the establishment of injury model and (B) the cell viability of HeLa cells pretreated with EBL for 24 h in the presence of H₂O₂. Note: ns meant not significant and ** meant p < 0.01. Injury meant the group only treated with H₂O₂, and Blank meant the group treated with same volume of DMEM.

Rutin hydrate, gallic acid, and other six phenolic compounds except quercetin were also detected in *Blumea lacera* and the antioxidant ability of *B. lacera* was associated with the high presence of polyphenols (Alam et al., 2020). But quercetin

was not detected in *B. laciniata*. Polyphenols like luteolin-4'-O-glucoside and chlorogenic acid was proved to possess a strong antioxidant on scavenging free radicals in vitro (Süzgeç-Selçuk and Birteksöz, 2011; Naveed et al., 2018; Kono et al., 1998). And chlorogenic acid also protected against DNA breakage (Shibata et al., 1999). *Thymus vulgaris*, *Salvia officinalis* and *Origanum majorana* three extracts exerted a great antioxidant due to the combination of different phenolic compounds (Roby et al., 2013). Different phenolic compounds also existed in EBL. Hence the excellent antioxidant of EBL may be the synergistic effect of those phenolic compounds.

As was well known, single antioxidant methods using colorimetric assays are not enough to comprehensively assess the antioxidant properties of a potential plant (Granato et al., 2018). Hence, HeLa cells were applied to evaluate the antioxidant ability of EBL in cellular level to further explore the antioxidant quality of *B. laciniata*-H₂O₂ could cause oxidative stress to cell and in many reports, H₂O₂-induced cell injury model was widely used to estimate the protection effect of drugs (Fang et al., 2018; Sim et al., 2017). After exposed to H₂O₂, the cells pretreated with EBL survived more than injury group, indicating EBL showed a great protection on HeLa cells against H₂O₂ (Fig. 5B).

A low level of ROS played a vital role in physiological cell but excessive ROS could harm to cells, causing damage to DNA, lipids, proteins and other biomacromolecules (Valko et al., 2006). Under the condition of H₂O₂, the ROS in cell was quickly accumulated (Fig. 6A), then the cells were undergone the oxidative stress thus the cell viability was decreased (Fig. 5B). Extracts from plants were proved to alleviate the oxidative damage. *Hedyotis diffusa* could protect human hepatocyte cells (LO2) from H₂O₂-induced cytotoxicity by attenuating cell apoptosis via ERK pathway (Gao et al., 2015). *Rhus verniciflua* Stokes extract also reduced the ROS level in protect PC-12 cells under H₂O₂ conditions (Nam et al., 2017). In this work, EBL could significantly lower the ROS level compared with the injury group. In the cell system, antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) were the main defender to eliminate the excessive free radicals causing by high level of ROS (Michiels et al., 1994). And extracts

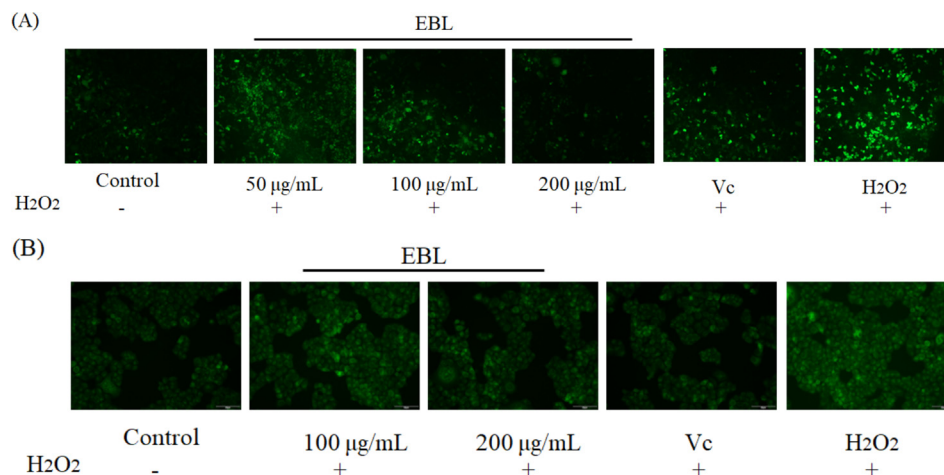


Fig. 6 The effect of EBL on (A) the ROS level, (B) the mitochondrial membrane potential and (C) the cell apoptosis stained by Hoechst 33,258 in HeLa cells. Note: * meant p < 0.05 and ** meant p < 0.01 compared with injury group. The red arrows meant the apoptotic bodies and red bar = 50 µm. Injury meant the group only treated with H₂O₂, and Blank meant the group treated with same volume of DMEM.

from plants could stimulate the antioxidant system to exert the antioxidant ability such as promoting the ability of CAT and SOD (Zal et al., 2017). Extracts from papaya seed offered protection to Hep G2 cells from oxidative stress by promoting antioxidant enzymes and then improving the cell survivals (Salla et al., 2016). *B. laciniata* showed a strong antioxidant ability in cell level, which might be linked with the antioxidant system to clear increasing ROS. Meanwhile, a high level of ROS could reduce MMP which was the mark of cell apoptosis (Smith et al., 2000; Kroemer et al., 2007). Under the oxidative stress, the content of ROS was increasing and MMP was reducing then leading to cell apoptosis (Fig. 6). However, EBL could alleviate the damage induced by ROS to MMP in the representation of preventing the decreasing of MMP and reducing the generation of apoptotic bodies. As the concentration of EBL showed no obvious negative effect on the growth of Hela cells (Fig. 4), and the effect of EBL was quite better than Vc on reducing the ROS level. Therefore, it was assumed that EBL could become a safe natural alternative in pharmaceutical industry applying in antioxidants.

But *in vitro* antioxidant assays were still not sufficient to fully evaluate the antioxidant of EBL. More further *in vivo* experiments will be carried out to explore the antioxidant capacity as well as the antioxidant mechanism.

5. Conclusions

Response surface method was successfully applied to optimized the components and the antioxidant ability of *B. laciniata*. Moreover, the antioxidant ability of *B. laciniata* was closely associated with the content of polyphenols. Besides, five phenolic compounds, chlorogenic acid, isoorientin, rutin, luteolin-4'-O-glucoside and cinnamic acid were firstly identified in EBL. Furthermore, EBL could well scavenge DPPH and ABTS free radicals. Additionally, EBL also exhibited a no-toxicity on Hela cells and a great protection on Hela cells against H₂O₂ induced damage by reducing the accumulating ROS. These outcomes could help to provide a better understanding on *B. laciniata* in pharmacological industry.

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CRedit authorship contribution statement

Lijun Zhou: Writing - review & editing. **Siyuan Luo:** Investigation. **Jiajia Li:** Investigation. **Yiling Zhou:** Investigation. **Tao Chen:** Methodology. **Shiling Feng:** Software. **Chunbang Ding:** Supervision.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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