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Effect of acid hydrolysis in the microwave-assisted extraction of phenolic compounds from *Geranium sibiricum* Linne with the guidance of antibacterial activity

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The feasibility and the effect of acid or alkali hydrolysis in microwave-assisted extraction as well as the antibacterial activity of the resulting extracts were studied to select appropriate phenols extraction method. The results demonstrated that acid hydrolysis was suitable for the extraction of phenols from *Geranium sibiricum* and hydrochloric acid (HCl) hydrolysis do not only performed the highest effectiveness, but also enhanced its antibacterial capacity. After the process was optimized, the total phenols content, yields of corilagin and geraniin were increased by 28.60, 53.80 and 35.65%, respectively, as compared to only 20% in microwave-assisted extraction (MAE). Scanning electron microscopy (SEM) was employed to verify the destructive effects of the methods, the results represented that HCl hydrolysis in MAE method had dramatic destructiveness on cell wall. Thus, HCl hydrolysis in MAE provides an efficient and rapid approach for the natural products extraction and the research also provides a valuable nature resource for healthy food industry.

Key words: Phenols, Hydrolysis method, Microwave-assisted extraction, *Geranium sibiricum* Linne, Antibacterial activity.

INTRODUCTION

Geranium sibiricum Linne is widely employed for the therapy of dysentery and enteritis in traditional Chinese medicine (TCM) and it is always consumed as restorative food (Yang et al., 2010). Recent researches indicated that it has anti-inflammation (Guo et al., 2007), anti-proliferative (Shim and Lim, 2008) and antioxidant activities (Yang et al., 2010). However, the plant is always treated as weeds in farmland and turf (Wang et al., 2005; Yan et al., 2005). Therefore, lots of efforts have been devoted on the researches of full exploitation and usage. It reveals that polyphenols are the main compounds in the family (Ivancheva et al., 1992), which should be responsible for the beneficial efficacies of *G. sibiricum* (Li et al., 2008). Among the polyphenolic compounds, corilagin (CG) and geraniin (GE) (Figure 1) are consider-ed as the main pharmacological substances (Krygier et

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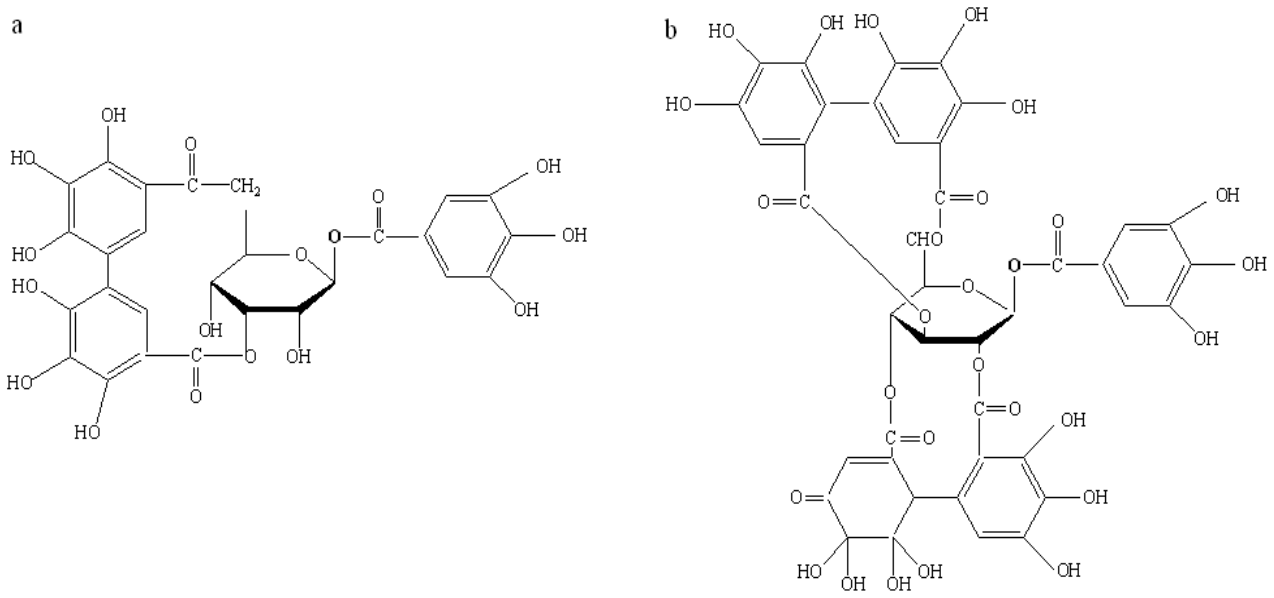

Figure 1. Chemical structures of (a) corilagin (CG) and (b) geraniin (GE).

al., 1982; Yang et al., 2010).

Currently, cases of bacterial resistance after the treatment of antibiotics and hormones have ever been reported, and received more attention (Hatano et al., 2008). It caused the escalating development of new antibacterial agents in a high number of sources, such as the secondary metabolites obtained from plants (Eloff, 1998). Polyphenols from tea, *Zanthoxylum*, *Origanum*, *Combretum*, Jerusalem artichoke and others plants have been screened for their potential uses as alternative remedies for the treatment of many bacterial (*Staphylococcus areus*, *Enterococcus faecalis, Pseudomonas aeroginosa, Bacillus cereus* and *Escherichia coli*, etc) infections (Hatano et al., 2008; Ma et al., 2011; Martini and Eloff, 1998; Oksana et al., 2012). The main mechanisms are associated with the inhibition of nucleic acid synthesis, cytoplasmic membrane function and energy metabolism (Cushnie and Lamb, 2005). Nevertheless, the antibacterial activity of *G. sbiricum* has not been evaluated yet.

To the best of our knowledge, the releasing polyphenols from the higher plant cell interfered with the presence of cell-wall (CW), which is influenced by the CW characteristics, such as structural parameters, physical traits and chemical composition and phenols chemical structure (Pinelo et al., 2006; Yang et al., 2010), anyway, cross-linking CW polysaccharides is the main barrier for the release of intracellular substances (Pinelo et al., 2008). Therefore, it thus becomes very urgent to develop the adequate hydrolysis methods to degrade CW, with the levels of environmental friendship, low-cost and high efficiency (Kim et al., 2006). Polyphenols are commonly extracted by alkaline hydrolysis (Verma et al., 2009). Phenols in coffee and apple (Kammiovirta et al., 2002), wheat (Kim et al., 2006), onion and spinach (Verma et al., 2009) have been reported to be extracted using alkaline hydrolysis. Germanò et al. (2006) found that a dramatic increase in caffeic acid, ferulic acid, *p*-coumaric acid, syringic acid, vanillic acid, protocathecuic acid and gallic acid content was obtained in *Trichilia emetica* via alkaline hydrolysis. Very limited information, however, is available regarding the effects of acidic hydrolysis. The potential reasons are that acidic hydrolysis can elevate temperature (Krygier et al., 1982) and cause the loss of some phenols acid extraction yields (Kim et al., 2004).

Microwave-assisted extraction (MAE) has been accepted as a potential and powerful alternative over conventional extraction methods in the extraction of organic compounds from solid matrices. The main advantages are shorter time, less solvent, higher extraction yield, less decomposition of the target species and better products for lower costs (Gujar et al., 2010). However, little information is available on acid or alkali hydrolysis in MAE to obtain the phenolic compounds.

Anyway, plant phenolics possess antibacterial activity, and alkali or acid hydrolysis can release phenolics from CW. However, two issues were raised since the MAE of phenolic compounds from *G. sibiricum*: (a) Whether alkali/acid hydrolysis in MAE could be feasible to break the cross-like of phenols and CW? (b) Will different hydrolysis methods affect the antibacterial activity of extracts ? Therefore, the aim of the present study was to evaluate the effect of acid and alkali hydrolysis on total phenolic content as well as CG and GE from *G. sibiricum* in MAE. Furthermore, the antibacterial activities of crude extracts obtained from different treatment methods were compared to screen the appropriate extracts preparation

methods. In addition, a critical study of the most essential parameters involved in extraction using central composite design (CCD) followed by response surface methodology (RSM) was evaluated, then mass transfer characteristics of acid hydrolysis in MAE were described and the optimal irradiation time was calculated through a pseudo first-order equation. Moreover, scanning electron microscopy (SEM) was used to detect the cell destructive degree under different treatment methods for verification of the efficiency of the extraction. We anticipate that this work will be useful in the comprehensive utilization of *G. sibiricum* and insight into the understanding of hydrolysis in MAE will be of value in the natural products extraction.

MATERIALS AND METHODS

Chemicals and reagents

CG and GE were brought from Delta Co., Ltd (Anhui province, China). Cellulase (Celluclast 1.5l, P1000 U/mg) and acetonitrile of high performance liquid chromatography (HPLC) grade were purchased from Sigma-Aldrich (Steinheim, Germany). Ethanol, NaOH, acetic acid and hydrochloric acid (HCl) were of analytical grade (Tianjin Chemical Reagents Co., Tianjin, China). All other reagents were of analytical grade. Deionized water was purified by a Milli Q Water Purification system from Millipore (Bedford, MA, USA). Appropriate amounts of reference compounds were dissolved in acetonitrile-water (12:88, v/v) to obtain the stock solutions at a concentration of 0.5 mg/ml for CG and GE, respectively. All solutions prepared for HPLC were filtered through 0.45 µm nylon membranes.

Plant

G. sbiricum was collected in July from the forest farm of Northeast Forestry University, Harbin, China, and was authenticated by Professor Shao-Quan Nie from the Key Laboratory of Forest Plant Ecology, Ministry of Education. Voucher specimens were deposited in the herbarium of the laboratory. The whole plant was air dried and then pulverized into a homogeneous size by a disintegrator (HX-200A, Yongkang Hardware and Medical Instrument Plant, China) and then it was sieved (20 to 30 mesh).

Analytical methods

HPLC analysis was performed using the Jasco LC system (Jasco Company, Japan), with Jasco PU-1580 intelligent HPLC pump, Jasco UV-1575 intelligent UV/VIS Detector and Millennium 32 system software. Chromatographic separation was performed on a HiQ Sil C18V reversed-phase column (250 × 4.6 mm i.d., 5 µm, Kya Tech, Hachioji City, Japan), with an Analytical KJ0-4282 C18 guard cartridge system (Phenomenex, Torrance, CA, USA).

The mobile phase consisted of acetonitrile (A) and water (B). Baseline separation of CG and GE was achieved with an elution program as follows: 12% A held until 35 min. The UV detector was set at the wavelength of 220 nm (Yang et al., 2010). The flow rate was 1 ml/min, injection volume was 10 µl, column temperature was maintained at 25°C, and the retention times for CG and GE were 22.25 and 27.89 min, respectively. The working calibration curves based on reference compounds of CG and GE showed good linearity over the range of 24.5 to 360 and 12.5 to 200 µg/ml, respectively. The regression lines were $Y = 22963966X - 1092572$ ($R^2 = 0.9963$, $n = 5$) and $Y = 53303406X - 1819305$ ($R^2 = 0.9907$, $n = 5$), where Y is the peak area of analyte, and X is the concentration of analyte (µg/ml).

Extraction procedure

The microwave extractor, including a time, a power and a temperature controllers, was manufactured by Shanghai Sineo microwave Products Company (Shanghai, China). One gram material in 25 ml 20% ethanol solution (the solution was alkalized or acidified with NaOH/acetic acid/HCl at 0.03 mol/L) was extracted at 20 min irradiation, 33°C, and 500 W. After extraction was finished, the vessels were allowed to cool to room temperature and the crude extracts was evaporated to about 20 ml, then it was extracted with ethyl acetate for 6 times and the supernatants were pooled, evaporated to dryness and dissolved in mobile phase to 100 ml. After filtration through 0.45 µm nylon membrane and centrifugation at room temperature for 10 min at 12000 rpm (Sigma 2-16P, Beijing Fine Best Trading Co., Ltd, China), the extraction yields of CG, GE and total polyphenol content were investigated in triplicate under the same conditions (Table 1).

According to the experimental design, 0, 10, 20, 30, and 40% (v/v) ethanol concentration; 20:1, 25:1, 30:1, 35:1 and 40:1 (v/g) ratio of solvent to material; 31, 33, 35 and 37°C extraction temperature, 300, 400, 500, 600 and 700 W irradiation power; 0.01, 0.02, 0.03, 0.04 and 0.05 mol/L concentration of HCl were investigated by single factor and then critical factors, such as irradiation temperature, HCl concentration and ethanol concentration were optimized by CCD with response surface methodology. All experiments were carried out in triplicate with 1 g material, 20 min irradiation time and three extraction cycles. The irradiation time was obtained through pseudo first-order equation.

Bacterial culture, MIC and MBC determination of the extracts

E. coli (ATCC 8739) and *S. aureus* (ATCC 6538) were obtained from the Institute of Applied Microbiology, Heilongjiang Academy of Science, China. They were maintained on an agar slant at 4°C. All strains were activated in nutrient agar at 37°C for 24 h.

The estimation of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were measured by the broth micro-dilution method (Yu et al., 2004). The samples obtained by extractions were concentrated to around 20 ml in a rotary evaporator device (RE-52AA, Shanghai Huxi Instrument Co., China) under vacuum at 35°C (Okuda et al., 1999). The crude extracts were treated as follows: ethyl acetate was added to the mixture, and was extracted for 6 times, the supernatant was pooled, evaporated to dryness, the dryness was individually dissolved in sterilized physiological saline solution (0.9% w/v) supplemented with dimethyl sulfoxide (DMSO) (Tianjin Chemical Reagents Co., Tianjin, China) at a final concentration of 0.5% (v/v). Serial doubling dilutions of the dryness were prepared in a 96-well micro liter plate in the range of 0.19, 0.38, 0.76, 1.53, 3, 10, 20, 40, 80, and 100 mg/ml.

The final concentration of each strain was adjusted to 10^5 to 10^6 colony form unit/ml. After bacterial activation, the MICs and MBCs were determined, with the positive controls of penicillin, chloramphenicol, and erythromycin (Tianjin Chemical Reagents Co., Tianjin, China). Each experiment was repeated in triplicate.

CCD assay

On the basis of the single-factor experimental results, the critical influencing factors were confirmed, and then a CCD with RSM was preformed (Myers and Montgomery., 1995). In this research, a 2^3

Table 1. Central composite design setting in the original and coded form of the independent variables and experimental results of yields of CG, GE and TPC.

Ethanol concentration (%)	Concentration of HCl (mol/L)	Temperature (℃)	Yield of CG (mg/g)			Yield of GE (mg/g)			Yield of TPC (mg GAE/g)		
			Exp[a]	Pred[b]	Resid[c]	Exp[a]	Pred[b]	Resid[c]	Exp[a]	Pred[b]	Resid[c]
1(25)	1(0.04)	1(35)	12.06	12.04	0.02	36.18	36.29	-0.11	164.46	162.58	1.88
1(25)	1(0.04)	-1(31)	13.26	13.25	0.01	40.05	39.81	0.25	192.57	189.96	2.61
1(25)	-1(0.02)	1(35)	12.47	12.77	-0.29	37.79	38.65	-0.86	179.12	187.57	-8.45
-1(15)	1(0.04)	1(35)	13.11	13.12	-0.01	39.46	39.33	0.14	181.02	178.13	2.89
-1(15)	-1(0.02)	1(35)	10.45	10.20	0.24	31.34	30.88	0.46	152.87	153.89	-1.02
-1(15)	1(0.04)	-1(31)	13.58	13.03	0.55	40.59	39.03	1.56	199.96	189.92	10.04
1(25)	-1(0.02)	-1(31)	13.91	13.65	0.26	41.45	40.89	0.56	178.68	179.98	-1.30
-1(15)	-1(0.02)	-1(31)	10.01	9.78	0.23	30.13	29.31	0.81	130.42	130.72	-0.29
1.68(28.5)	0(0.03)	0(33)	10.97	11.46	-0.48	33.14	34.57	-1.43	157.81	163.96	-6.15
-1.68(11.5)	0(0.03)	0(33)	13.92	13.80	0.12	42.18	41.74	0.43	196.18	192.28	3.90
0(20)	1.68(0.047)	0(33)	10.31	10.46	-0.14	31.15	31.39	-0.24	149.04	141.69	7.36
0(20)	-1.68(0.013)	0(33)	12.36	12.58	-0.22	36.83	37.58	-0.75	160.81	170.42	-9.61
0(20)	0(0.03)	1.68(37)	12.82	13.33	-0.51	38.33	39.89	-1.56	181.67	187.49	-5.82
0(20)	0(0.03)	-1.68(30)	12.81	12.67	0.14	38.82	38.25	0.56	187.52	183.96	3.56
0(20)	0(0.03)	0(33)	14.97	14.83	0.14	45.22	44.58	0.64	202.96	200.43	2.53
0(20)	0(0.03)	0(33)	14.73	14.83	-0.10	44.41	44.58	-0.17	197.39	200.43	-3.04
0(20)	0(0.03)	0(33)	14.86	14.83	0.03	44.28	44.58	-0.30	201.34	200.43	0.91

[a]Exp: experimental value; [b]Pred: quadratic model predicted value; [c]Resid: residue (the subtraction of predicted value from experimental value).

full factorial CCD was employed to fit a second order polynomial model which indicated 17 experiments to be required for this procedure.

In all cases, calculations were carried out with the help of the statistica package Statgraphics Plus for Windows V6.0 (StatSoft Software, Inc., Oklahoma, USA).

Effect of time on hydrolysis process

After each parameter was evaluated by single-factor and CCD design, acidic hydrolysis in MAE extraction process (irradiation time 1 to 20 min) were conducted. Extraction reaction rate constants (k) and equilibrium concentrations (C_∞) were calculated using nonlinear regression in Sigma Plot Version 10 (Systat Software, Inc., Chicago, IL) according to the first-order equation:

$$C = C_\infty[1 - \exp(-kt)]$$

Student's t-test (two-tailed) was used to compare the k and C_∞ values, results with $p \leq 0.05$ were considered significantly different. All analysis was carried out in triplicate. Statistical Analysis Software (SAS) 9.1.3 was used for the analyses (SAS Institute, Cary, NC).

Determination of total polyphenol content (TPC)

TPC were determined by using Folin-Ciocalteu method according to Singleton and Rossi (1965). The results were reported in gallic acids equivalents (GAE) per gram of the sample. All the measurements were taken in triplicate.

SEM

For the purpose of examining the effect of various extraction methods on the morphological alteration of the plant cells, *G. sbiricum* dried samples before and after hydrolysis treatment were scanned by SEM (FEI Company, USA). The samples were fixed on adhesive tape and then sputtered with gold, and examined under high vacuum condition at a voltage of 12.5 kV (20 µm, 1000 × magnification).

RESULT AND DISCUSSION

Hydrolysis method

Many studies have revealed that the majority of polyphenol are bound by ester linkages with polysaccharides in the CW and can be hydrolyzed using an acid or an alkali method. The mechanisms of acid and alkaline hydrolysis are to obtain a tetrahedral intermediate and then hydrolyze

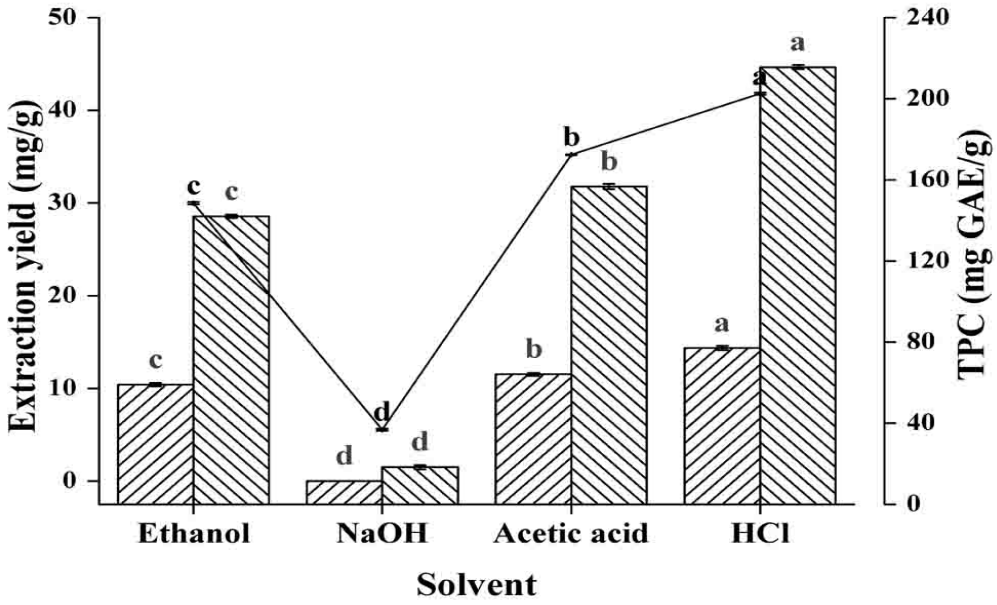

Figure 2. Extraction yields of CG (▨), GE (▨) and TPC (—•—) from *G. sibiricum* employing various hydrolysis methods (Ethanol was control). Each experiment was repeated in triplicate. The letters (a to d) indicate significant differences at a significance level of $p < 0.05$.

Table 2. Effect of antibacterial capacity of different extracts from *G. sibiricum* on *S. aureus* and *E. coli*.

Extraction method	*S. aureus* (mg/mL)		*E. coli* (mg/mL)	
	MIC[a]	MBC[b]	MIC[a]	MBC[b]
20% Ethanol	25	25	15	15
NaOH + 20% Ethanol	>100	>100	>100	>100
Acetic Acid + 20% Ethanol	2.25	2.25	3.5	3.5
HCl + 20% Ethanol	0.75	0.75	0.75	0.75
Penicillin	0.006	0.5	0.0015	0.1563
Chloramphenicol	0.003	0.25	0.0078	>1
Erythromycin	0.0015	0.25	0.125	1

Penicillin, chloromycetin and erythrocin were positive controls. [a]MIC: minimum inhibitory concentration (mg/ml).[b]MBC: minimal bactericidal concentration (mg/ml). The values are the average concentration from duplicates which obtained for the same dilution tested (coincident results).

ether linkage (Hori et al., 1999; Hori et al., 2007). However, the chemical structures of phenols are various, which introduces the position and the degree of ether bonds with CW and finally influences the hydrolysis yields and profiles of extracted phenols (Liyana-Pathirana and Shahidi, 2006; Saadi et al., 1998). For example, alkali hydrolysis was more efficient in releasing vanillic, cisferulic, etc. Comparatively, acid hydrolysis was more suitable for caffeic acid (Kim et al., 2006; Krygier et al., 1982; Verma et al., 2009). Here, acidic and alkaline hydrolysis in MAE were compared to determine CG, GE and TPC content, the results are shown in Figure 2. The yields of alkali hydrolysis were rather low, while acid hydrolysis was more efficient in releasing target constituents from the CW with acedic acid or HCl. It reveals that acid hydrolysis was suitable for CG, GE and TPC from *G. sibiricum*.

Antibacterial activity

Recent studies have reported that plant polyphenols exhibit the antibacterial activity (Alberto et al., 2001; Ma et al., 2011; Oksana et al., 2012), with the concentration-dependent relationship (Ghasemi et al., 2011). Although, the main compounds of the extract of *G. sibiricum* have been proved as the phenolic compounds (Yang et al., 2010), there is still lack of research on their antibacterial activities (Guo et al., 1987). Therefore, the antibacterial capacity of the crude extracts were examined and compared with each other to screen the appropriate extracts preparation methods.

The MICs and MBCs results of different extraction methods were presented in Table 2. HCl hydrolysis showed the best antibacterial activity against acetic acid and alkali hydrolysis, with 0.75 mg/ml MIC and MBC

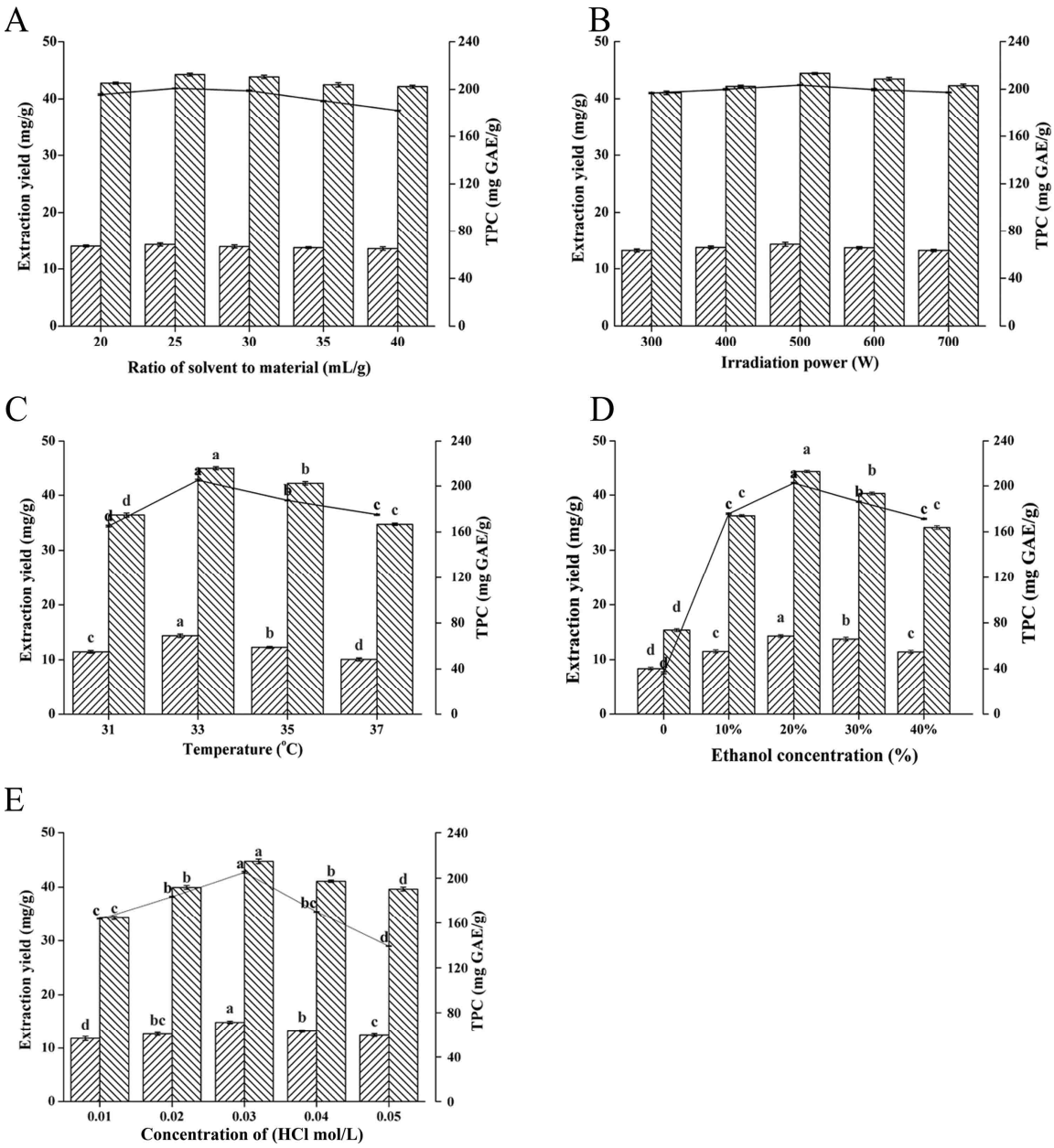

Figure 3. Extraction yields of CG (▨), GE (▨) and TPC (—•—) from *G. sibiricum* during the evaluation of the following parameters: (A) ratio of solvent to material (ml/g), (B) irradiation power (W), (C) temperature (℃), (D) ethanol concentration (%) and (E) concentration of HCl (mol/L). Each experiment was repeated in triplicate. The letters (a to d) indicate significant differences at a significance level of $p < 0.05$.

values for *S. aureus* and *E. coli*, respectively. The MBC value of HCl hydrolysis against *E. coli* was higher than those of chloromycetin and erythrocin. As shown in Table 2 and Figure 2, the inhibitory capacity was also increased, with the increasing yields of CG, GE and TPC. It indicated that the extract of HCl hydrolysis in MAE was the most effective ones, in contrast to other methods. Therefore, HCl hydrolysis extraction in MAE method was chosen and the effect of influence factors during the process would be investigated in the following sections.

Effect of influence factors on HCl hydrolysis in MAE process

As earlier discussed, HCl hydrolysis with ethanol in MAE presented its superiority. Thus, several factors during the process were evaluated, such as irradiation power, ratio of solvent to material, irradiation temperature, concentration of HCl and ethanol concentration. All experiments were carried out in triplicate with 1 g material, 20 min irradiation time and three extraction cycles. Figure 3A to

Table 3. The least squares fit and parameter estimates (significance of regression coefficients).

Model term	CG			GE			TPCs		
	Coefficient estimate	Standard error	p-value	Coefficient estimate	Standard error	p-value	Coefficient estimate	Standard error	p-value
Intercept	14.83	0.24	-	44.58	0.71	-	200.43	4.65	-
X_1	1.39	0.11	0.000437	2.14	0.33	0.000358	8.43	2.19	0.006242
X_1^2	-1.56	0.12	0.000397	-2.28	0.37	0.000440	-7.90	2.41	0.013453
X_2	1.26	0.11	0.000798	1.84	0.33	0.000873	8.55	2.19	0.005805
X_2^2	-2.35	0.12	<0.0001	-3.58	0.37	<0.0001	-15.72	2.41	0.000325
X_3	-0.39	0.11	0.122844	-0.49	0.33	0.186746	-1.05	2.19	0.645443
X_3^2	-1.30	0.12	0.001170	-1.95	0.37	0.001090	-5.21	2.41	0.067217
X_1X_2	-1.82	0.15	0.000435	-2.70	0.43	0.000437	-12.31	2.85	0.003516
X_1X_3	-0.65	0.15	0.061114	-0.95	0.43	0.064492	-3.90	2.85	0.214497
X_2X_3	-	0.15	0.586283	-0.32	0.43	0.485762	-8.74	2.85	0.018250
Lack of fit	-	-	0.0575	-	-	0.1318	-	-	0.0877

E showed the effects of these factors on the extraction yields of CG, GE and TPC. Ethanol concentration, irradiation temperature and concentration of HCl preformed stronger effects than irradiation power and ratio of the solvent to material. Hence, the three factors were chosen for further optimization in CCD. Note that the conditions of irradiation power and ratio of solvent to material were 500 W and 25 ml/g, respectively. Then, ethanol concentration, temperature and concentration of HCl were selected as variables in the RSM. The extraction yields of CG and GE and TPC (mg GAE/g) were employed as response values, and a 2^3 full factorial central composite design was designed. All experimental data were obtained from 17-run-experiment, and predicted data were from response surface analysis model, the results are as shown in Table 3.

The significance and adequacy of the model via RSM were performed by the analysis of variance (ANOVA). The significance of each coefficient is determined by p-values. It was found that quadratic main effects of ethanol concentration and concentration of HCl were significant ($p < 0.01$). It means that a small change can cause large variation in response and then affect the yields of CG, GE and TPC, while temperature and interactions with temperature and other factor were not significantly obtained by statistical analysis. In addition, lack of fits of the model were not significant ($p > 0.05$), it reveals that they were precise and applicable model. Thus, the mathematical models were built (Table 3). To investigate the interactive effects of operational parameters on the extraction yields, the three-dimensional profiles of multiple non-linear regression models are depicted in Figure 4.

Phenols are more mainly linked to the CW and several factors including concentration of HCl, ethanol concentration and temperature can affect phenols releasing from CW and vacuoles. Concentration of HCl is a critical variable among them, to improve the extraction of phenols from CW via the cleavage of the ether and/or ester linkages (Nuutila et al., 2002). Here, the concentration of HCl for extraction was taken into account with two main aims: (1) evaluation of the concentration is necessary for the accelerated extraction, and (2) the excess usage of HCl is avoided for the compounds structure change and the environmental problems. As shown in Figures 3E, 4A, C and E, with an increasing concentration of HCl from 0.01 to 0.03 mol/L, the extraction yields of CG, GE and TPC increased accordingly. While increasing the concentration of HCl from 0.03 to 0.05 mol/L, the extraction yields slowed down, which may be due to the fact that high acid concentration can cause structure change. The results indicated that a 0.03 mol/L HCl was sufficient to hydrolyze CW.

Proper ethanol concentration can offer suitable polarity, vapor pressure, viscosity and surface tension of extraction solvent, and suitable water could enhance swelling plant material and increase the contact surface area between the plant matrix and the solvent (Hemwimon et al., 2007). As shown in Figure 4A, C and E, increase of ethanol concentration from 15 to 20% with increase of concentration of HCl from 0.02 to 0.03 mol/L enhanced the yields of target compounds, whilst about 20% ethanol concentration showed the maximum yields, because of the synergistic effect of HCl and ethanol, which could modify the diffusion coefficient of the phenolic compounds and increase their solubility in the solvent (Pompeu et al., 2009).

Although, temperature has less effect on the yields of CG, GE and TPC in contrast to others, it also plays an important role in the releasing of phenols from CW (Figures 3C, 4B, D and F), which is able to modify equilibrium and mass transfer conditions in the solid-liquid extraction. Increases of temperature can accelerate molecular movement of ethanol and active compounds and then, affect the extraction efficiency. Interaction effects between the temperature and ethanol concentration had positive effect before 33°C and there was a

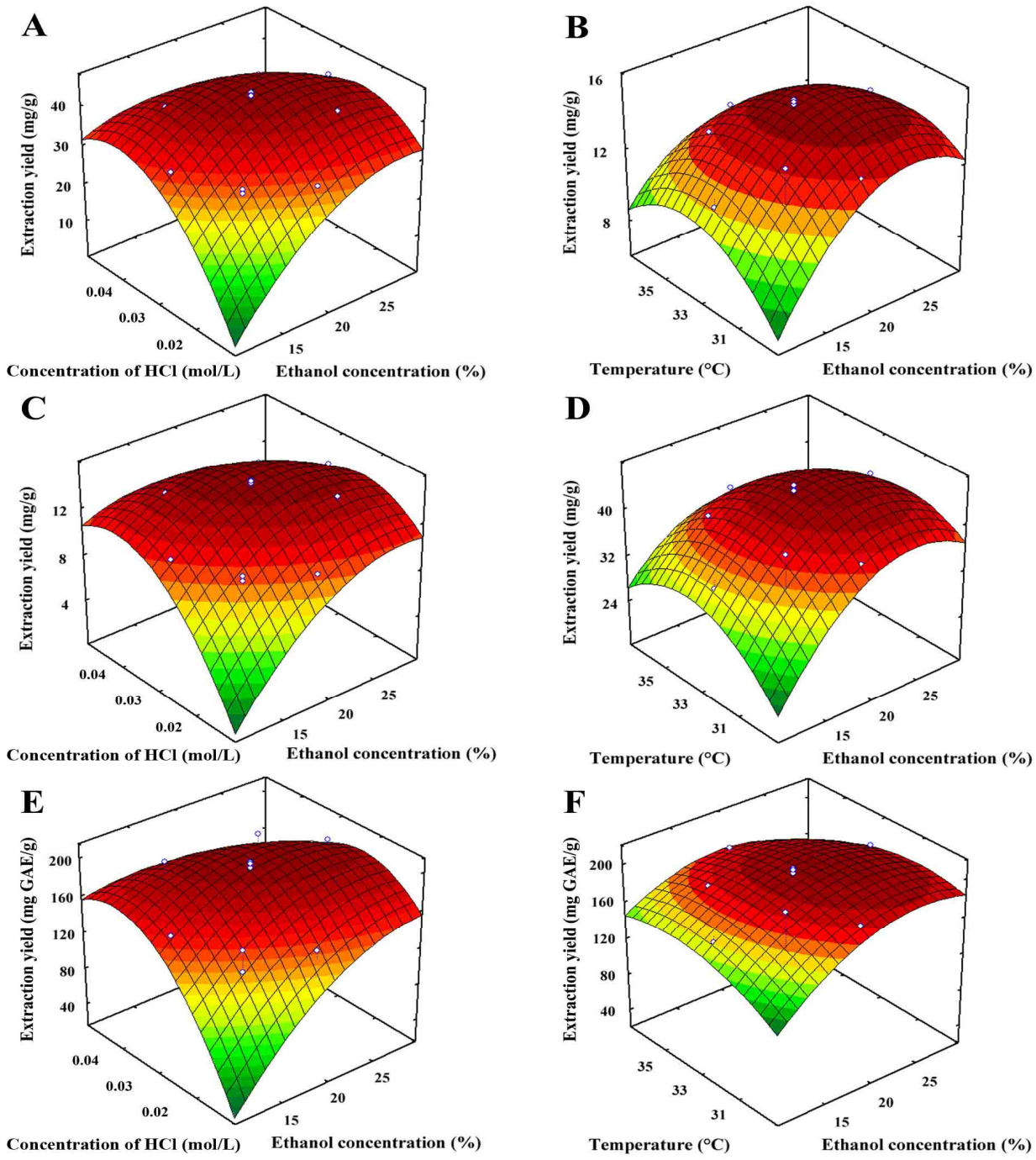

Figure 4. Three dimensional profiles of multiple non-linear regression models (Yang et al., 2010).

negative effect at the higher temperature. It indicated that the maximum mobilization of active compounds from the solid matrix may occur up to a certain temperature, avoiding the decomposition at the higher temperature (Okuda et al., 1980; Yang et al., 2010).

As regarding the built mathematical models, the experimental conditions for CG should be: concentration of 0.031 mol/L HCl, 22.2% ethanol concentration, and 32.5℃ temperature; conditions for GE were: 0.031 mol/L HCl, 22.3% ethanol concentration, 32.5℃ temperature; conditions for TPC were: 0.032 mol/L HCl, 22.4% ethanol concentration, and 32.1℃ temperature. When these parameters were applied, the predictive value of extraction yields of CG, GE and TPC were 15.05 and 45.22 mg/g, and 203.60 mg GAE/g, respectively. Since it was difficult in operating them in the actual extraction process, they were carried out with slight modifications: concentration of HCl at 0.03 mol/L, ethanol concentration

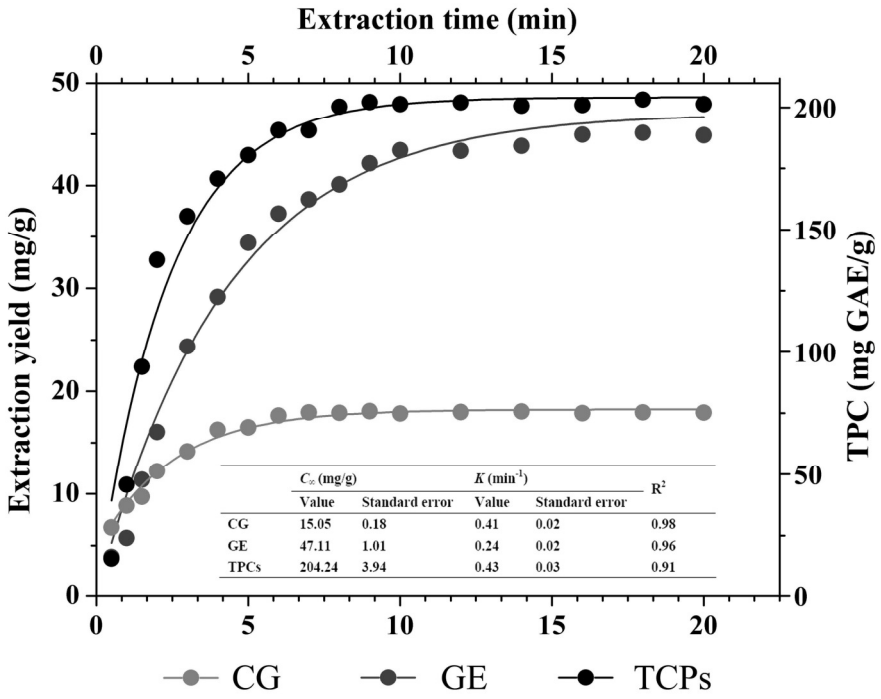

Figure 5. Extraction process (Yang et al., 2010).

at 22% and temperature at 33°C, respectively.

Effect of irradiation time on HCl hydrolysis in MAE process

In our works, the irradiation time of HCl hydrolysis was selected from the preliminary experiment. As in the literatures, secondary metabolites released from the sample to the solvent generally took place at three different stages during extraction process (Figure 5). (I) Washing-type (Pinelo et al., 2008), the compounds of non CW and CW are dissolved quickly into the solution and form broken CW, as a combined result of microwave energy enhancing extraction efficiency and speed due to the localized heating, pressure builds up within the cells of the sample and finally leading to a fast transfer of the compounds from the cells into the extracting solvent, and acid hydrolysis cross-linking of CW polysaccharides, took place at 0 to 4 min. When phenols are extracted from matrixes, the effect of this initial 'washing' type is commonly reduced. (II) A rapid type, constant speed of diffusion occurring following the washing type (Pinelo et al., 2008). From 4 to 8 min, the extraction rate fell because a reverse flow of solvent occurred, impeding the solvent transfer from the intercellular to the outside, however, CW was destroyed severely as former result and the phenols cross-link with polysaccharides exposed more accessible surface area to acid, consequently, it improved acid biocatalysis and increased the yields of desired compounds gradually. Therefore, this effect of mutual offset keeps certain speed of CG, CE and TPC release to the solvent. (III) At a slower type, following an exponential decay curve from 8 to 20 min, a slower migration was likely ascribable to the exhaustion of phenols contained in the matrix (Figure 5). Hence, we can conclude that 8 min was enough to obtain the maximum extraction yields of CG, GE and TPC simultaneously.

The overall extraction process was stimulated by pseudo first-order kinetics, as shown in Figure 5. The R^2 value was above 0.91 in all cases, indicating a good data matching. As comparison of K value of CG and GE, the former was larger than the latter. It means that the transfer rate of CG was faster than that of GE under the same extraction process. This may due to the factor of chemical structure, seven additional –OH of GE could introduce more hydrogen-bondings with the cross-linking ether bonds of polysaccharides in the CW.

Verification of predictive model

For the actual operation and further verifying the reliability of the method, the verification experiments were carried out (Table 4). Compared with traditional MAE, HCl hydrolysis in MAE preformed more efficiently. The former extraction yields of CG, GE and TPC were 11.47, 29.37 mg/g and 148.55 mg GAE/g, respectively, while those of the latter were 14.75, 45.17 mg/g and 201.51 mg GAE/g, respectively, increased by 28.60, 53.80 and 35.65%, respectively. It reveals that the experimental values were

Table 4. Comparison of the extraction conditions of MAE and HCl hydrolysis in MAE and the results of experimental verification on the extraction yields of CG, GE and TCPs.

Extraction method	Extraction yield	Ratio of solvent to material (ml/mg)	Irradiation power (W)	Temperature (℃)	Ethanol concentration (%)	Concentration of HCl (mol/L)	Predict	Experiment
MAE	CG (mg/g)	25	500	33	22	-	-	11.47
	GE (mg/g)							29.37
	TPCs (mg GAE/g)							148.55
HCl hydrolysis in MAE	CG (mg/g)	25	500	33	22	0.03	14.99	14.75
	GE (mg/g)						45.06	45.17
	TPCs (mg GAE/g)						202.53	201.51

consistent with the predictive values. Therefore, the extraction conditions obtained by response surface methodology were not only accurate and reliable, but also the practical value reflecting the expected optimization.

Comparison of effects of various extraction methods on structure change

MAE can disrupt the recalcitrant structures of CW through accelerating ionic conduction and rapid dipole rotation, while acidic hydrolysis can destroy the structures of the CW by degrading ester linkages of CW. Through structure of micrographs, the CW structure change and the extraction effects of different treatment methods can be examined as a validation (zhang et al., 2010). Thus, SEM was used to obtain structure change of samples over various methods.

As shown in Figure 6, different extraction methods produced distinguishable physical changes, and an SEM micrograph of the untreated material, can be contrasted to the structures of the treated sample presented in Figure 6B (only 20% ethanol in MAE), Figure 6C (alkali hydrolysis in 20% ethanol in MAE), Figure 6D (acetic acid hydrolysis in 20% ethanol in MAE) and Figure 6E (HCl hydrolysis in 20% ethanol in MAE) with conditions at 1 g material in 25 ml solvent, 20 min irradiation, 33℃ and 500 W in MAE, respectively. Some holes and apparent pitting can be observed as shown in Figure 6B, which demonstrated that microwave energy had certain damage on the external surface of CW. However, Figure 6C, D and E showed the serious damages on CW surface after alkali or acid hydrolysis effect in MAE and a number of spiral vessal were observed, which can support that alkali or acid hydrolysis could destroy cross-link between phenols and CW and thus disrupt the recalcitrant structure of CW. Furthermore, alkali hydrolysis can break CW, however, the yield of the compounds was very low when compared with any acid one, it can be concluded that under acid condition the compounds were more stable than higher pH condition (Figure 2), which maybe mainly due to the structure change. At the same time, HCl hydrolysis had more destructiveness effect than that of acetic acid hydrolysis on rigid CW, which demonstrated that the former preformed more effectively and extracted more marker compounds than the latter.

Conclusions

Studies had demonstrated that alkali hydrolysis method was more efficient in releasing phenolic compounds from botanies and acidic hydrolysis can elevate extraction solvent temperature and then result in the loss of target compounds (German`o et al., 2006; Kim et al., 2006; Krygier et al., 1982; Verma et al., 2009), however, this study proved that acid hydrolysis can enhance the extraction concentration of CG, GE and TPC. Furthermore, the result indicated that *G. sibiricum* extracts antibacterial capacity was in connection with the concentration of phenolic compounds, consistent with Kim et al. (2004).

This work showed that HCl hydrolysis in MAE increased the yields of the compounds in contrast to other methods. Nevertheless, increasing HCl concentration cannot be unlimited (Laopaiboon et al., 2010) and 0.03 mol/L HCl was suitable. The other factors involved in HCl hydrolysis process were determined and optimized as follows: irradiation power at 500 W, ratio of solvent to

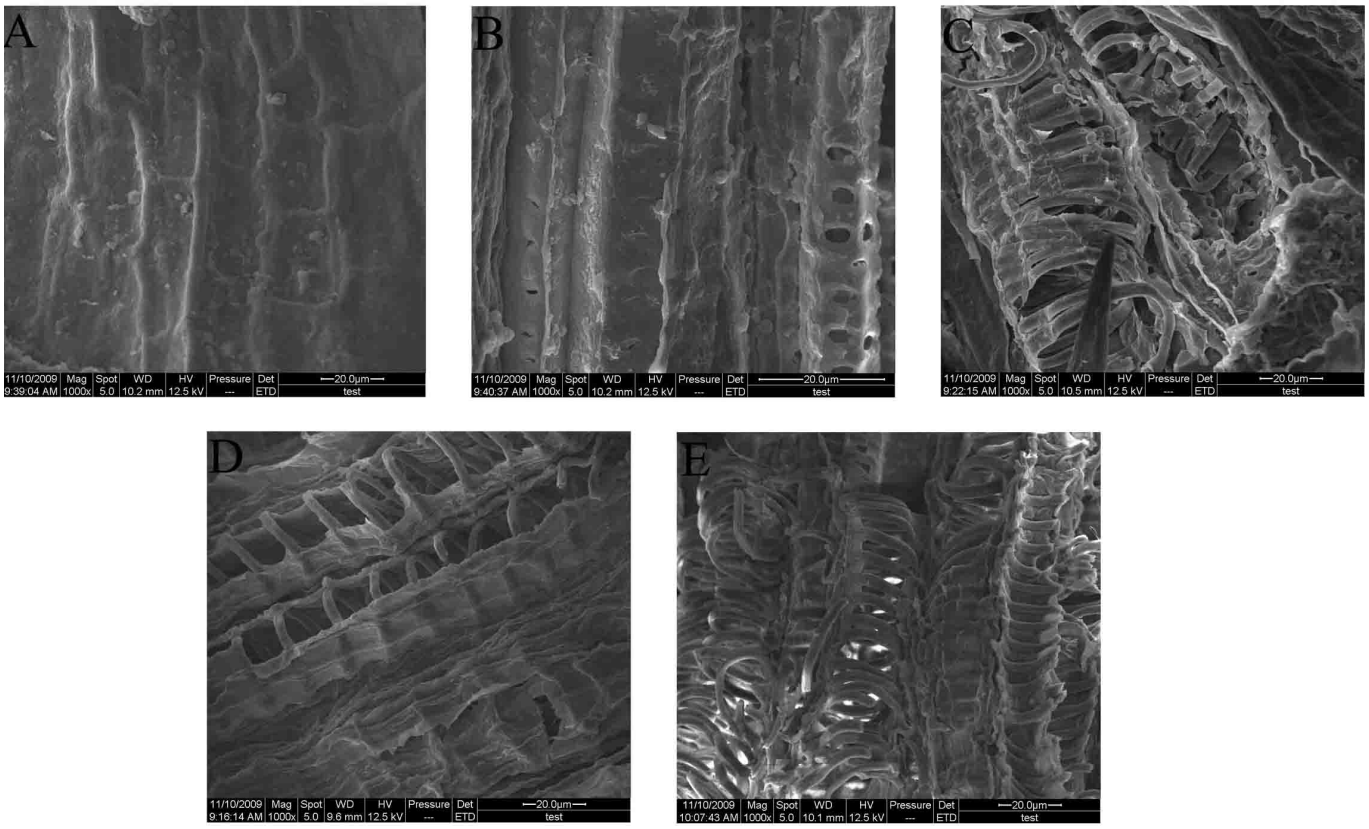

Figure 6. Damages caused by Different extraction methods (Yang et al., 2010).

material at 25 ml/g, irradiation temperature at 33℃, ethanol concentration at 22% and irradiation time at 8 min during the extraction process, the extraction yields of TPC, CG and GE were 201.51 mg GAE/g, 14.75 and 45.17 mg/g, which represented an increase of 28.60 , 53.80 and 35.65%, respectively, when compared with only 20% in MAE methods. From SEM micrograph observation, HCl hydrolysis had more destructiveness effect on plant cells than other extraction methods, which can be a confirmation for its strong extraction efficiency.

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