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EXTRACTS OF *AMBROSIA ARTEMISIIFOLIA* L. AND ITS TOXICITY EFFECTS ON MICE

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Abstract

A new and effective method of ultrasonic/microwave assisted extraction (UMAE) of the active extracts from *Ambrosia artemisiifolia* L. (AAE) and to investigate its acute toxicity had been proposed. The response surface methodology (RSM) was employed to optimize the UMAE conditions. The optimum conditions were as follows: sonication time, 28.50 min, microwave treatment, 2.40 min, microwave temperature, 70°C and liquor to material ratio, 8.00 (ml/g). Then four obtained extraction methods were compared. The results indicated that the extracts produced by UMAE had the highest yield and the highest anti-pathogenic activity. The acute toxicity of AAE was studied in mice. The LD_{50} was 4966.33 mg/kg, which belonged to a low level of mammalian toxicity. Furthermore, the AAE could activate the activity of SOD in mice livers, while inhibiting the CAT and POD activities. The results suggested that *A. artemisiifolia* was a novel antimicrobial agent. It also instigated thoughts of security.

Introduction

Application of pesticides has become an important method to improve the yield and output of agricultural products over a long period around the world and that will be continued for a long time to come. Unquestionably, chemical pesticides are quite effective. However, the harmful effects to human health and ecology have been reported frequently (Guillette and Iguchi 2012, Ríos-Díez *et al.* 2011, Pavela 2008). Hence, biological pesticides that have the advantage of non-target specific safety and easy degradation have been extensively studied (Xu *et al* 2015, Miresmailli and Isman 2014). However, some reports also showed that some of them still have high toxicity for mammals, such as the nicotine (Krishna *et al* 2010) and vauquline (Sowjanya and Kushal 2011).

Ambrosia artemisiifolia L. (Fam.: Asteraceae) is a malignant weed all around the world, which can spread aggressively with competitive seeds and promoting soil microbial carbon source utilization (Ciappetta 2016, Shi *et al*. 2011, Qin *et al* 2014). In addition, the pollen of this species is a strong an aphylactogen that can cause an allergic reaction in humans (Smitha *et al* 2013, Daniel and Tiffany 2014). Nevertheless, the extracts of ragweed proved to have various bioactivities, such as antibacterial (Solujic *et al* 2008), molluscidal (EL-Sany *et al.* 1981), anti-thrombin (Chistokhodova *et al* 2002), hepatoprotective and hypolipemic (Parkhomenko *et al.* 2006) activities. Moreover, our previous studies demonstrated that the ethanol extract of the plant exhibited insecticidal and anti-fungal activities (Zhang *et al.* 2010). Furthermore, a series of compounds have been isolated from ragweed, including terpenoids, flavonoids, phenolic acids, coumarins, and polyacetylenes (Huang *et al.* 2014), which are structurally similar to the known active ingredients of botanical pesticides. Thus, it has been suggested that *A. artemisiifolia* is a potential candidate of a botanical pesticide that can be further developed.

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Recently a few investigations have been conducted on the method of extraction of *A. artemisiifolia*, but the safety of the extracts in mammals is still unclear. Therefore, the effective ultrasonic/microwave assisted method was first applied in the extraction of the active ingredients from *A. artemisiifolia*. Then the acute toxicity of the extracts was evaluated. The results of those investigations are reported here.

Materials and Methods

Ambrosia artemisiifolia was collected from Mudanjiang city, Heilongjiang province in August. The ultrasonic cell disruptor (JY92-IIN, Ningbo Xinzhi Biotechnology Co. Ltd., China) and microwave extraction apparatus (MDS-6, Shanghai Xinyi Microwave Chemical Technology Co. Ltd., China) were used to facilitate the extraction of materials from *A. artemisiifolia* (AAE). The *Cytospora chrysosperma* was selected as the pathogen to evaluate the bacteriostatic activity of the AAE using different extraction methods. Kunming mice (18 - 25 g) were used to test the acute toxicity. All the reagents were analytical grade.

The dried powder (10 g) of ragweed was weighed accurately and mixed with a certain amount of 80% ethanol. The extraction process was first performed in the ultrasonic cell disruptor chamber with a different time and ultrasonic power. Then the flask was transferred into the microwave extraction apparatus. The program of different microwave powers and temperatures was set. After the extraction procedure, the treated mixture was cooled to room temperature and filtrated by vacuum suction. The filtered solution was concentrated by a rotary evaporator at 45°C. Finally, it was lyophilized to obtain the dry AAE. All experiments were performed in triplicate. The lyophilized extract was weighed and the extraction yield was calculated as followed: Extraction yield (%) = (extracts weight / material weight × 100).

Single factor tests were carried out to screen the appropriate variables in order to determine their experimental domain for further extraction conditions optimization. In this study, independent variables were preliminary tested. These included ultrasonic power, sonication time, microwave temperature and time, as well as liquor to material ratio. The basic extraction conditions were the following: A sonication time of 25 min; an ultrasonic power of 350 W; a microwave time of 3 min; a temperature of 70°C and a ratio of liquor to material of 7.5: 1 (ml/g). Single factor experiment factors and levels have been shown in Tab. 1. From the preliminary experiments, sonication time, microwave time, temperature and liquor to material ratio were the significant selected variables. Therefore, the combined effects of these variables on AAE extraction yield were studied using the Box-Behnken design (Table 2).

Table 1. Single factor test protocols.

Levels	Factor				
	Sonication (time/min)	Ultrasonic (power/W)	Microwave (time /ºC)	Microwave (temp./ºC)	Liquor to material ratio (ml/g)
1	5	300	0.5	40	2.5:1
2	15	350	1	50	5:1
3	25	400	2	60	7.5:1
4	35	450	3	70	10:1
5	45	500	4	80	15:1

The ambient temperature extraction (ATE), traditional heating extraction (THE), ultrasonic bath assisted extraction (UAE) and ultrasonic/microwave assisted extraction (UMAE) were selected to compare the extractive effects. The ATE was carried out at the following conditions: an

extraction temperature of 25ºC, an extraction time of 48 hrs, a water/solid ratio of 10 : 1, and a speed of 150 rpm was performed in the condition of 80ºC for 1 hr under the water/solid ratio of 10 : 1. The UAE was carried out under the condition as follows : ultrasonic power of 500 W, sonication time of 45min and liquor to material ratio of 10 : 1. The UMAE was performed at the optimum extraction condition based on the results above. Then the AAE was prepared by the method of 2.2.1. Each method was repeated three times.

Table 2. Scheme of Box-Behnken design.

Levels	Factor			
	Sonication (time/min)	Microwave (time/min)	Microwave (Temp./ ºC)	Liquor to material ratio (ml/g)
–1	15	1	60	5 : 1
0	25	2	70	7.5 : 1
1	35	3	80	10 : 1

The mycelia radial growth technique was used to compare the anti-pathogenic activity of the AAE using four methods against *C. chrysosperma* (El Ghaouth *et al.* 1994). The AAE aqueous solutions were prepared in 5%Tween 80and added to the melted PDA medium at concentrations of 300, 600, 1200, 2400 and 4800 mg/l. The 5% Tween 80 only was used as the control. After inoculation, the plates were incubated in the dark at 25ºC. The colony diameter was measured when the mycelial growth of control was fully-grown. The per cent inhibition rate of the radial growth of *C. chrysosperma* was calculated relative to the control. The effective concentration that inhibited 50% of the mycelial growth (EC_{50}) was estimated according to the probit analysis.

The preliminary studies were firstly conducted to find the dose interval, which can cause a 0 and 100% death rate of mice before the formal test. The ratio of female to male mice was 1:1. The mice were fasted for 3 hrs prior to the experiment and were weighed. Then the mice were administered with a single dose of the AAE solution above (doses ranges from 1982.998 - 9914.989 mg/kg at various levels). Then they were observed for mortality for up to 48 hrs (short-term toxicity). Based on the short-term toxicity, the dose of the next animal was determined as per OECD guideline 425. The LD_{50} of the extracts was calculated using a graphical method.

The tested mice were gavaged with an AAE solution by a dose of the LD_{50}value, while the physiological saline was used as the control group. The survived mice were chosen after feeding 1, 6, 12 and 24 hrs, respectively. The livers of mice were obtained after taking off the neck, which resulted in death. Then they were rubbed into a homogenate with PBS (pH = 7.0, 0.05 mol/l) to obtain the crude enzyme. The SOD activity was measured according to the method of Beauchamp and Fridovich (1973). The CAT activity assay was based on the method of Cohen (Cohen *et al.* 1970). The POD activity was measured with guaiacol as a substrate (Xu *et al.* 1991). All of the assays were done in triplicate.

Design-expert 8.0 was used to design the RSM experiment and to analyze the data. The variance (ANOVA, $p < 0.05$ and $p < 0.01$) and probit analyses were performed by the SPSS 17.0

Results and Discussion

The single factor experiments were designed to evaluate the effects of each factor on the extraction yield of the AAE (data not shown). The results of ANOVA showed that the variables of the sonication time, microwave time, microwave temperature and liquor to material ratio were the significant ones. However, the effect of the ultrasonic power was not obvious. This may be due to

the destruction of cell walls of ragweed under the joint action of the ultra sound and microwave, which was similar to the previous report (Wu *et al.* 2015). Thus, the ultrasonic power of 400 W was screened out as the appropriate parameter.

Taking extraction yield as an index, the RSM design of four factors and three levels was carried out based on the single factor test results. Twenty-nine experiments were performed and the results are listed in Table 3.

Table 3. Scheme and results of Box-Behnken design.

No.	Factors				Yield (%)
	X_1	X_2	X_3	X_4	
1	(15)-1	(2)0	(70)0	(5:1)-1	20.47
2	(25)0	0	0	(7.5:1)0	26.08
3	0	(3)1	(80)1	0	24.02
4	0	0	(60)-1	(10:1)1	23.52
5	(35)1	0	–1	0	25.02
6	0	0	0	0	26.08
7	0	1	0	–1	24.43
8	0	1	–1	0	25.42
9	–1	(1)–1	0	0	19.12
10	0	0	1	1	25.01
11	0	–1	–1	0	19.77
12	0	0	0	0	25.98
13	–1	0	–1	0	21.97
14	–1	0	1	0	22.87
15	1	–1	0	0	24.12
16	0	–1	1	0	21.97
17	1	0	0	–1	24.22
18	0	0	–1	–1	22.02
19	1	1	0	0	25.17
20	0	1	0	1	25.13
21	1	0	1	0	24.97
22	0	0	0	0	26.03
23	0	0	1	–1	21.52
24	1	0	0	1	25.52
25	0	–1	0	1	22.85
26	–1	1	0	0	25.62
27	0	0	0	0	26.13
28	0	–1	0	–1	18.52
29	–1	0	0	1	24.22

Through polynomial regression analysis of the experimental data, the quadratic regression equation model fitting all variables was obtained as follows:

$$Y = -107.91 + 1.18X_1 + 21.30X_2 + 2.17X_3 + 4.19\ X_4 - 0.13\ X_1\ X_2 - 0.003\ X_1\ X_3 - 0.02\ X_1\ X_4 - 0.09\ X_2\ X_3 - 0.35\ X_2\ X_4 + 0.02\ X_3\ X_4 - 0.009\ X_1^2 - 1.79\ X_2^2 - 0.01\ X_3^2 - 0.25\ X_4^2$$

Where, Y represents the yield of AAE; X_1, X_2, X_3 and X_4 are the coded variables for the sonication time, microwave time, microwave temperature and liquor to material ratio, respectively.

The analysis of variance of the regression and the partial regression coefficient have been shown in Tab. 4. The data indicated that the proposed regression model was highly significant (P<0.001). The P-value of the lack of fit was 0.0712, which implied that the lack of fit was insignificant and the test error was small. The model determination coefficient (R^2) was 0.9987 and the adjusted determination coefficient (Adj R^2) was 0.9975, which indicated that the model showed a favorable goodness of fit between the experimental results and the theoretical values predicted by the polynomial model. The linear terms (X_1, X_2, X_3, X_4), quadratic terms (X_1^2, X_2^2, X_3^2, X_4^2) and three inter-action ($X_1 X_2$, $X_1 X_3$, $X_1 X_4$, $X_2 X_3$, $X_2 X_4$, $X_3 X_4$) were all significant ($p < 0.001$).

Table 4. Analysis of variance for the fitted quadratic polynomial model.

Source	Sum of squares	DF	Mean (square)	F-value	P-value
Model	135.82	14	9.70	798.17	< 0.0001
X_1	17.64	1	17.64	1451.51	< 0.0001
X_2	45.71	1	45.71	3760.69	< 0.0001
X_3	0.63	1	0.63	51.47	< 0.0001
X_4	18.73	1	18.73	1540.62	< 0.0001
$X_1 X_2$	6.63	1	6.63	545.54	< 0.0001
$X_1 X_3$	0.28	1	0.28	22.68	0.0003
$X_1 X_4$	1.50	1	1.50	123.47	< 0.0001
$X_2 X_3$	3.24	1	3.24	266.58	< 0.0001
$X_2 X_4$	3.15	1	3.15	259.22	< 0.0001
$X_3 X_4$	0.99	1	0.99	81.46	< 0.0001
X_1^2	4.99	1	4.99	410.16	< 0.0001
X_2^2	20.85	1	20.85	1715.55	< 0.0001
X_3^2	13.93	1	13.93	1146.06	< 0.0001
X_4^2	15.97	1	15.97	1314.08	< 0.0001
Residual	0.17	14	0.012		
Lack of fit	0.16	10	0.016	4.84	0.712
Pure error	0.013	4	3.250×10^{-3}		
Cor. total	135.99	28			

The contour plots were constructed to expound the interactions between the four factors and determine their optimal levels (Fig. 1). Each plot exhibited the function of any two factors while the other factor was fixed. All of the six response surface plots presented steep slopes and a saddle diagram. The variations of the saddle lead to a certain decrease in flexural strength. As observed in

Fig.1, there existed significant interactions between any of the two factors, which were in line with the variance analysis above.

According to the analysis of the regression model, the optimum conditions were predicted as follows: a sonication time of 28.46 min, a microwave time of 2.37 min, a microwave temperature of 69.70ºC and a liquor to material ratio of 8.05 (ml/g). Considering the actual operation convenience, the optimal parameters of 28.50, 2.40 min, 70.00ºC and 8.00 (ml/g) were selected to verify the accuracy of prediction. Under these conditions, the actual extraction yield of AAE was 26.51 mg/g, which was close to the theoretical value of 26.77%. The results indicated that the predicted model presented an adequate fitness and high effectiveness of AAE extraction using UMAE.

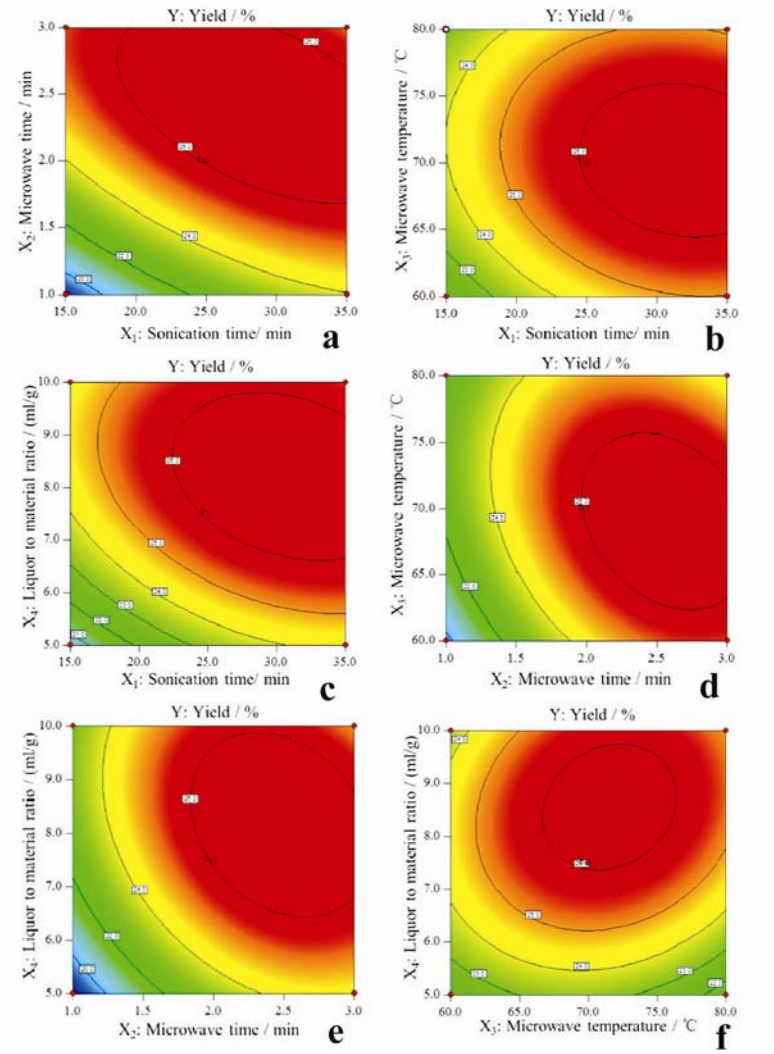

Fig.1. Contour plots showing the interactive effects of four extraction parameters on the yield of AAE.

As was shown in Table 5, the UMAE could effectively, shorten the extraction time and reduce the organic solvent usage, which had higher extraction yield and higher anti-pathogenic activity.

The extraction yield of the UMAE was 26.51%, which significantly increased by 36.4, 23.42 and 19.63% compared to the ATE, THE and UAE, respectively ($p < 0.01$). As to the bacteriostatic activity, the AAE prepared by UMAE exerted the most potent inhibitory effects to *C. chrysosperma* with the lowest EC_{50} value of 1285.73 mg/l. This was significantly higher than others ($p < 0.05$). Interestingly, the ATE could obtain relatively higher activity that the EC_{50} of the AAE and there was not significant difference between the method of UMAE at the level of $p < 0.01$. However, the yield was unsatisfactory. This may be caused by the excessive ultrasonic mechanical effects or thermal treatment, which lead to part of the active ingredients failure.

Table 5. Effects of different extraction methods.

Methods	Extraction (time/hr)	Material-to-solvent ratio (ml/g)	Extraction yield (%)	Bacteriostatic activity EC_{50} (mg/l)
UMAE	0.5	8:1	26.51^{Aa}	1285.73^{Aa}
ATE	48	10:1	19.43^{Cd}	1378.41^{Ab}
THE	1	10:1	21.48^{Bc}	1853.82^{Cd}
UAE	0.75	10:1	22.16^{Bb}	1530.46^{Bc}

*Different small or capital letters in the same column mean significant difference at $p < 0.05$ or $p < 0.01$ level, respectively.

The result of the acute toxicity test has been shown in Table 6. The tested mice showed some characteristics after being injected with the AAE solution, including convulsions, tachypnea, balance disorder of the body and loss of vigor. The mice with an average weight of 22.35g were used for calculating the mass concentration. According to the experimental data, the median lethal dose (LD_{50}) of AAE in mice was 4966.33mg/kg, while the R^2 of the regression analysis of the log dose and probit was 0.9629.

Table 6. The results of acute toxicity test in mice.

Dose (mg/kg)	Log dose	No. of mice	No. of death	Corrected mortality (%)	Probit
9914.989	3.996	30	27	88.89	6.22
7931.991	3.899	30	24	77.78	5.77
5948.993	3.774	30	20	62.96	5.33
3965.996	3.598	30	15	44.44	4.86
1982.998	3.297	30	8	18.52	4.10
Control		30	3		

The result of the protective enzymatic (SOD, CAT and POD) activity assays on mice livers are shown in Fig. 2. The activity of SOD was restrained at first. Then it was activated after treating for 1h. The activity was all lower than the control group within 1-6 hrs. Then it became higher than the control group until 24 hrs. The highest inhibition rate was 15.88% in the treatment group when compared with the control group. The highest activity of SOD in the treatment group increased by 1.13 times compared to that of normal group (Fig. 2a), which was activated after 12 hrs ($p < 0.05$).

The enzyme activity of the CAT in the treatment group was significantly lower than the control group after treating for 1 hr with AAE ($p < 0.01$). As time went by, the changing tendency of the CAT activity was restrained at first, and then was recovered. It recovered slowly after 6h.

However, it still did not recover to the normal level until 24 hrs. The highest inhibition rate was 42.06% during the enzymatic activity change process (Fig. 2b).

The POD activity in the treatment group was invariably in the state of inhibition. The activity was significantly lower than the control level within 1 - 24 hrs ($p < 0.01$). However, it fluctuated under the state of inhibition. The activity of the POD decreased within 1 - 6 hrs. It then increased within 6 - 12 hrs. Moreover, it decreased again in 12-24 hrs. The highest inhibition rate was 79.06% in the treatment group (Fig. 2c).

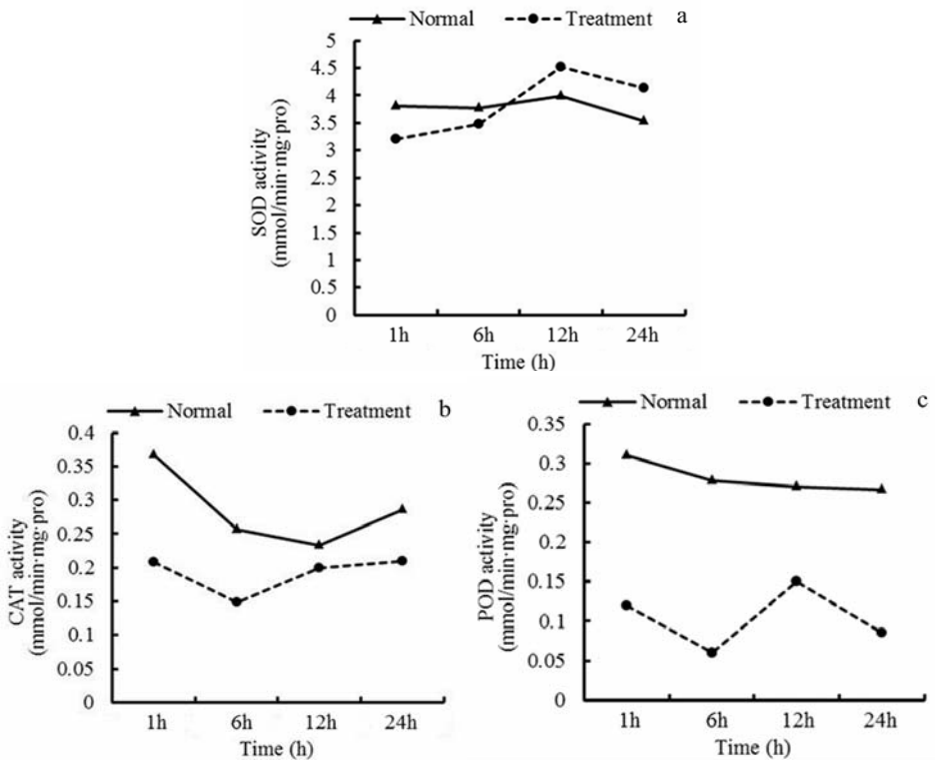

Fig. 2. Effects of AAE on SOD (a), CAT (b) and POD (c) activities of mice liver.

The response surface technique was used to establish the UMAE method in order to extract AAE based on single factor experiments. The optimum conditions for UMAE were as follows: ultra-sonication time, 28.50 min, subsequent microwave treatment for 2.40 min, microwave temperature 70ºC, and liquor to material ratio 8.00 (ml/g). Four extraction methods were compared. The results indicated that the UMAE could effectively shorten the extraction time and reduce the use of organic solvents. It also had the highest extraction yield and highest anti-pathogenic activity, which could be considered as ideal and effective method for the extraction of AAE. The acute toxicity of AAE was evaluated in mice. The LD_{50} of the AAE was estimated to be 4966.33 mg/kg, which belongs to a low level of mammalian toxicity. The results of the protective enzymatic activity test on mice livers showed that the AAE could activate the performance of the SOD. There was also a suppression effect on the CAT and POD activities. In conclusion, *A. artemisiifolia* is a novel anti-microbial agent for controlling pests which has good security.

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