Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(11):653-660

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Study on the extraction process and seasonal variation of Toosendanin in Chinaberry (*Melia azedarach* L.)

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ABSTRACT

Toosendanin as one kind of plant pesticide exists in the fruits, barks, and leaves of Melia azedarach L. (chinaberry tree). To obtain toosendanin, the sample was defatted with petroleum ether, extracted with ethanol; then the solution was concentrated, and extracted with ethyl acetate. Uniform design and regression analysis were employed to establish a model of extraction and optimize the process. The results showed that the model was accurate to predict the toosendanin yield. Optimum extraction parameters were elicited as 1:24 g:ml for feed to solvent ratio and 20 min of extraction with petroleum ether, 1:34 g:ml for feed to solvent ratio and 390 min of extraction with ethanol, followed by extraction with ethanol:ethyl acetate (1:8, v:v) for 100 min. Under these optimal conditions, the yield was 0.74% of toosendanin on a dry weight basis. In addition, the toosendanin contents of the different part of chinaberry collected in different months for three years were analyzed. It was suggested that the toosendanin extraction from the leaves, fruits, and barks of chinaberry occurred in May and June, November and December, and June, November, and December, respectively. Thus, this study provides the direction for obtainment of toosendanin from chinaberry.

Keywords: *Melia azedarach* L.; uniform design; regression analysis; toosendanin; collecting time

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INTRODUCTION

Toosendanin (a common name in China is “chuan lian su” or “ku lian su”) is a natural triterpenoid compound (structure was shown in Fig.1) belonging to a class of limonoids [1, 2]. It is usually found in the plants of *Melia* genus. *Melia azedarach* Linn. which has a common name as chinaberry tree is recorded as the only species in *Melia* genus in the United States [3]. *Melia toosendan* Sieb. et Zucc., *Melia candollei* A. Juss., and *Melia dubia* Cav. are commonly regarded as synonyms of *M azedarach* L.. In China, *Melia toosendan* Sieb et Zucc named chuan lian is well known as a famous traditional Chinese medicine contained toosendanin and another species in Melia genus in China [4]. In this paper, all of the samples were from chinaberry.

The extract of chinaberry is famous for its biological properties such as feeding deterrent for many insects and growth disruptant for most insects and many other arthropods [5-8]. Toosendanin is a main active compound in the extract of the chinaberry tree, also has been used in traditional Chinese medicine as an anthelmintic and neuromuscular blocking agent [9]. And it shows remarkable pesticide activity and functions as an arthropod antifeedant and growth disruptor [10-12]. As a valuable natural pesticide, it was reported a very low toxicity for vertebrates [13, 14]. Up to now, several publications have dealt with different techniques for the extraction and separation of toosendanin from the bark of chinaberry. Generally, organic solvent extraction is a traditional method for toosendanin extraction. Ethanol was used for the extraction from the material, and ethyl acetate for the extraction from the solution usually [15, 16]. Unfortunately, the articles used barks as the material and did not research the condition of defatting and the interactions among the process factors. For the fruits was lack of systematical work. In addition, the supercritical CO_2 fluid extraction and microwave or supersonic assistant extractions were also

employed to obtain toosendanin from plant material [15-17]. However, they have been not yet developed on an industrial scale with economical way due to the technology limitation. Therefore, the organic solvent extraction is still used as the main method for the industrial production of toosendanin. In order to effectively improve the efficiency, the solvent extraction was optimized by uniform design and regression analysis in this paper.

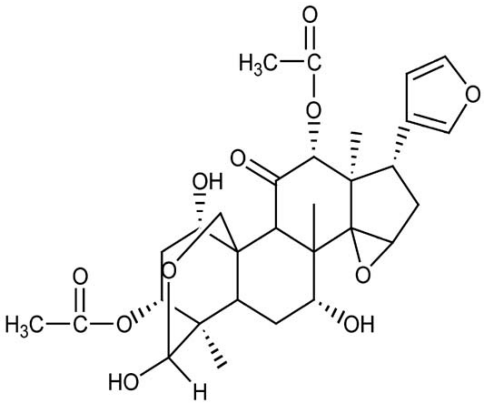

Figure 1. Chemical structure of toosendanin

Single factor experiment and orthogonal design have been employed to extract toosendanin in previous studies [16]. However, up to now, the interactions among different parameters have not been systematically evaluated during the extraction of toosendanin with organic solvent. Therefore, it is necessary to investigate various operating determinants during the extraction process. Uniform design is available and powerful statistical experiment design method especially when the experimental region has many factors and multiple levels [18]. It could describe the nonlinearity between dependent and independent variables and provide the prediction of response for given factors with the combination of regression analysis [19]. Taking those into account, uniform design method and regression analysis were applied to optimize the extraction conditions of toosendanin from chinaberry with organic solvent in this study.

As we all known, the content of bioactive compound in plant materials is varied during their growing period, therefore, it is important to study the relationship between the content of bioactive compound in chinaberry and its growth season. Currently, there are three papers for providing little information about the content of toosendanin in plant material collected in different seasons [20-22]. However, the results based on the data collecting in 1 year could not be reliable for industry because of the effect of abnormal climate and other unusual factors in the year.

In this paper, uniform design and regression analysis were employed to establish a model of extraction and optimize the process. Additionally, the contents of toosendanin in fruits, barks, and leaves of chinaberry collected in different months for three years were also analyzed.

EXPERIMENTAL SECTION

Plant materials

The leaves, barks, and fruits of chinaberry were harvested on days between the 1th and 5th of a certain month in 2011, 2012, and 2013 in a garden of Northwest A&F University, Yangling, China. The sampling place (Yangling) has a loess type soil, an average elevation of 445.0 meters, annual average temperature of 13 °C, annual average sunshine time of 2,163.8 h, and annual average rainfall of 635.1-663.9 mm (data from Yangling Weather Bureau). All of the samples were collected from the east, west, south, and north branches of 20 chosen trees.

The collected samples were cleaned and air dried at 17-25 °C for 3 days, then dried further for 20 h in a thermostatic vacuum at 40 °C. After vacuum drying, the materials were grounded and sieved through a 40 mesh screen. Finally, the samples were sealed and stored at -18℃ before extraction.

Extraction process

The sample (10.0 g) was defatted with petroleum ether at 40 °C. After filtration and air drying, the material was extracted with ethanol at 95 °C. Then the solution was filtered and concentrated under vacuum condition at 45 °C to a volume of 15 ml, followed by the addition of salt-saturated solution (NaCl) with the same volume under continuous stirring. After that, ethyl acetate was used to extract toosendanin from the solution at 25 °C. Finally, the

extract obtained with ethyl acetate was filtered and ethyl acetate was removed under reduced pressure at 40 °C. The extract was stored in a refrigerator before analysis.

In order to optimize the extraction method, fruits from chinaberry were collected in October, 2012. For the seasonal variation study, all the samples were treated with the optimum method.

Experimental design and statistical analysis

Based on the results of screening out with mono-factor experiments [16], a 16^{12} uniform experiment design was used to identify the relationship between the response functions and the process variables as well as to optimize the extraction process. The six factors (X_1-X_6), in which each factor was performed at eight levels, were applied in the experiments (Table 1). All trials were carried out in triplicate and the average yield of toosendanin was taken as an evaluated response (Y). SAS software (Version 8.0) was employed to finish the statistical and stepwise regression analyses of the data.

Determination of toosendanin content

The concentration of toosendanin was measured by *p*-dimenthyl aminobenzaldehyde method [15]. Briefly, the color reagent was prepared by dissolution of 0.745 g *p*-dimenthyl aminobenzaldehyde in 100 ml of 10% (v:v) sulphuric acid. The extract solution of toosendanin (1 ml) was diluted to be 50 ml with methanol, and 1 ml of the diluted solution was mixed with 1 ml color reagent. After 20 min, the absorbance of the mixture was determined by a UV-vis detector at 512 nm to calculate the content of toosendanin.

RESULTS AND DISCUSSION

Regression analysis

Table 1 Uniform design matrix of factors and the responses of toosendanin yield.

X_1	X_2	X_3	X_4	X_5	X_6	Y（%）
80	120	160	420	160	160	0.35
80	320	280	120	140	140	0.48
120	520	80	620	100	120	0.15
120	720	200	220	80	100	0.42
160	120	320	720	40	80	0.37
160	320	80	420	180	60	0.14
200	520	200	20	160	40	0.39
200	720	320	520	120	20	0.61
240	20	120	220	100	160	0.34
240	220	240	720	60	140	0.33
280	420	360	320	40	120	0.31
280	620	120	20	180	100	0.24
320	20	240	520	140	80	0.58
320	220	360	120	120	60	0.66
360	420	160	620	80	40	0.37
360	620	280	320	60	20	0.45

X_1: volume of petroleum (ml); X_2: extraction time with petroleum ether (min); X_3: volume of ethanol (ml); X_4: extraction time with ethanol (min); X_5: volume of ethyl acetate (ml); X_6: extraction time with ethyl acetate (min); Y: toosendanin yield (%).

The design of the experiment and the results were shown in Table 1. According to a series of index coded by using the statistic software SAS 8.0, a second-order model was developed. The analysis of variance (ANOVA) results of the model displayed a good performance with $R^2 = 0.9802$. This result showed a great degree of correspondence between experimental and predicted values for the toosendanin yield.

The following mathematical model equation was obtained as equation (1) using regression analysis:

$$Y=-0.090983+0.00298X_1+0.00394X_3+0.0003955X_4+0.00884X_5-0.00000619X_1^2-0.00000582X_3^2-5.04756E-7X_4^2-0.00004427X_5^2 +0.00002398X_5X_6-0.0000155X_6^2 \quad (1)$$

The optimal conditions were obtained from the regression equation (1) using the optimization software Lingo Systems (Version 9.0, Lindo System Inc.). Optimum extraction parameters were elicited as 1:24 g:ml for feed to solvent ratio and 20 min of extraction with petroleum ether, 1:34 g:ml for feed to solvent ratio and 390 min of extraction with ethanol, followed by extraction with ethanol:ethyl acetate (1:8, v:v) for 100 min. Then it was predicted that the highest toosendanin yield of 0.72% was available. After triplicate verifying experiments, the mean yield of toosendanin was 0.74%, which was 2.77% of relative error. This result showed that the experimental value

was close to the predicted value. Therefore, from this point of review, the optimized results were reliable. Moreover, compared with the barks, it is obvious that the fruits have larger amounts and less environmental damage, which can make fruits the promising and sustainable sources of toosendanin.

Analysis of parameters
Mono-factor analysis

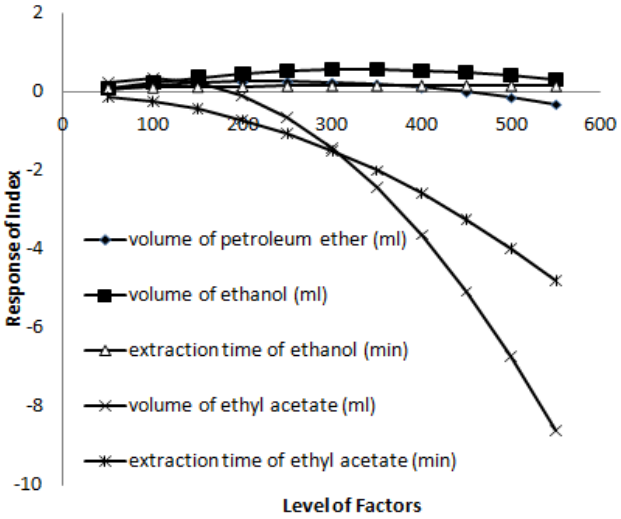

Figure 2. Effects of single factor on the index value of toosendanin yield

The regression model allowed predicating the effects of the observed parameters on the toosendanin yield. The quadratic equations of each variable were respectively created by the means of assigning other four parameters at zero level.

$Y= -0.090983+0.00298X_1-0.00000619X_1^2$ (2)
$Y= -0.090983+0.00394X_3-0.00000582X_3^2$ (3)
$Y= -0.090983+0.0003955X_4-5.04756E^{-7}X_4^2$ (4)
$Y= -0.090983+0.00884X_5-0.00004427X_5^2$ (5)
$Y= -0.090983-0.0000155X_6^2$ (6)

For all the parameters, the extraction time with petroleum ether was found to be less significant effect on the yield. In fact, the linear X_2 term did not appear in the refined model (1), although the extraction volume of petroleum ether (X_1) seemed to be significant. As can be inferred from the results, the initial step, namely the defatting with petroleum ether, played an important role in the procedure of toosendanin extraction, which appeared to be facilitating the subsequent steps.

As can be seen from Fig. 2, the five curves of single factor drawn according to the equation (2)- (6) showed the variability of toosendanin yield with the changes of elevating levels. It was found that the yield increased at first, then decreased with the increasing level of each factor. This result illustrated that a larger extraction volume presented a positive effect on the yield of toosendanin in comparison with extraction time, that is, extraction with a long time restrained the production of toosendanin. This phenomenon was probably caused by the fact that toosendanin tended to be dissolved in organic solution.

Dual-factor analysis
Two variables of volume and time of extraction were depicted in one three-dimensional surface plots while the other variables was fixed at zero level. Then an equation was obtained as following:

$Y=-0.090983+0.00394X_3+0.0003955X_4-0.00000582X_3^2-5.04756E^{-7}X_4^2$ (7)

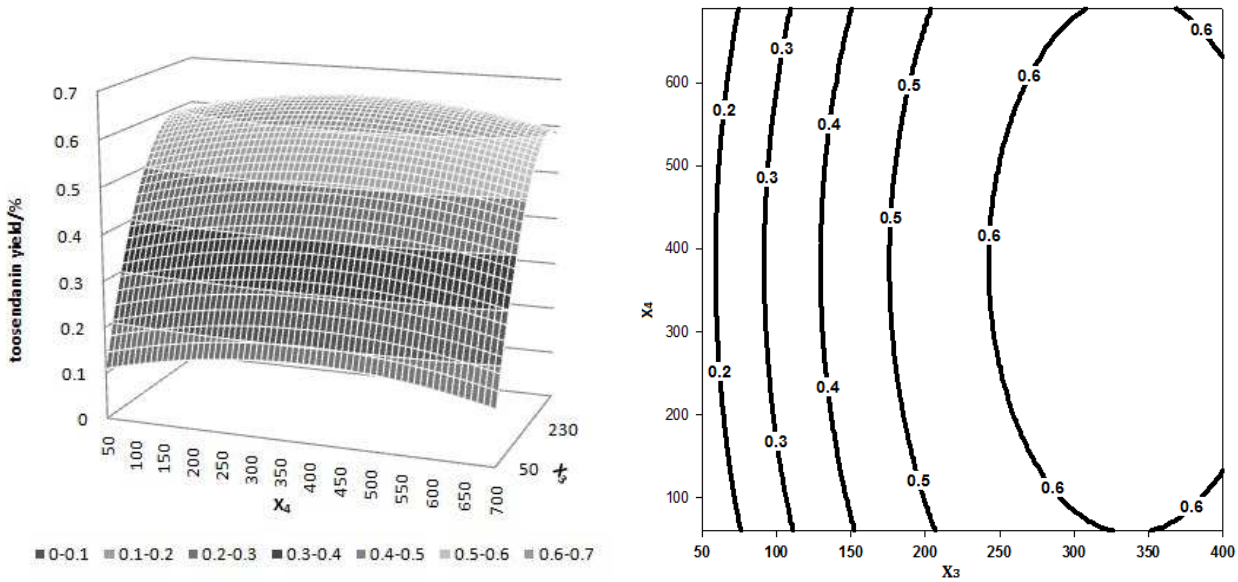

Figure 3. Three-dimensional graphic surface (left) and contour plots (right) for the effects of the volume (X_3, ml) and extraction time (X_4, min) of ethanol on the toosendanin yield

According to the equation (7), three-dimensional graphic surface and contour plots (Fig. 3) were drawn by sigmaplot software 11.0 (Systat Software International), which exhibited the interaction effects between extraction volume and extraction time (X_3 and X_4). The circular contour plot indicated that the interactions between extraction volume and extraction time had influences on the toosendanin yield. However, it did not show a significant effect. In addition, the extraction volume (X_3) had stronger impact on the extraction yield than that of the extraction time (X_4), which correspond to the result in Table 1. Interestingly, the extraction with more volume and the less time could bring a higher yield of toosendanin.

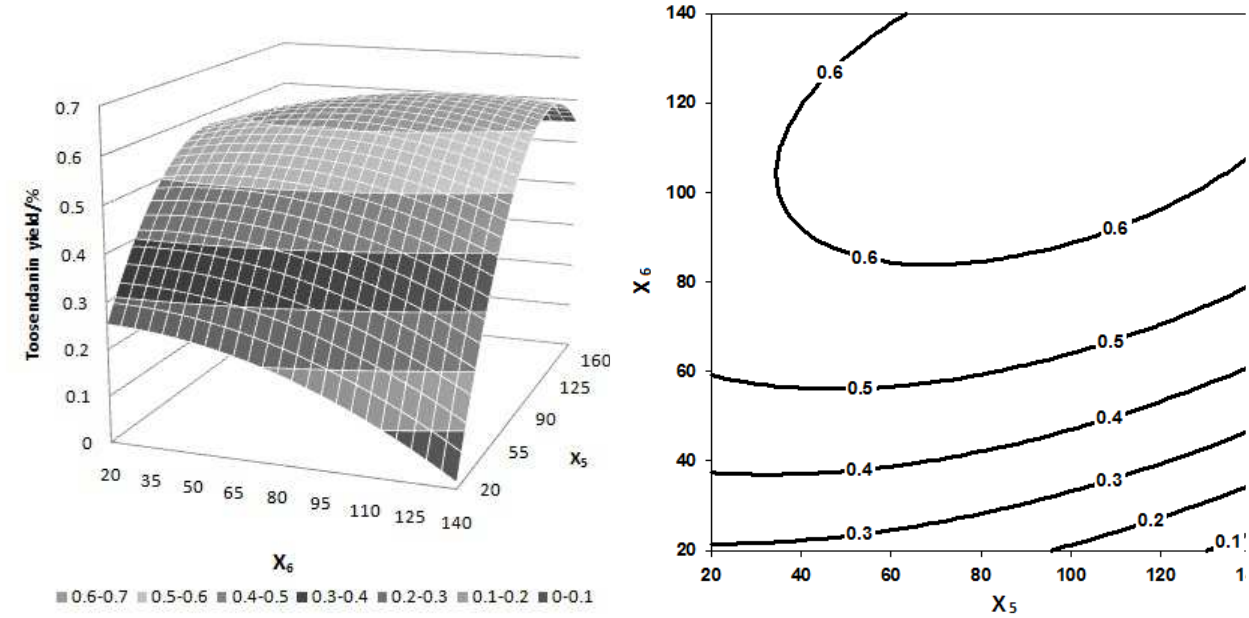

Figure 4. Three-dimensional graphic surface (left) and contour plots (right) for the effects of the volume (X_5, ml) and extraction time (X_6, min) of ethyl acetate on the toosendanin yield

Fig. 4 was drafted by the same way of Fig. 3. It showed the interaction between extraction volume (X_5) and extraction time (X_6) with ethyl acetate. The tortuose surfaces showed the dramatically complex interaction effects between X_5 and X_6. The elliptical contour plot demonstrated that the mutual interactions between extraction volume and extraction time were significant, which correspond to the result in Table 1. For less ethyl acetate volume, a higher yield of toosendanin was obtained with increasing extraction time. It was indicated that a higher yield could be obtain when the lower extraction volume and the higher extraction time were selected.

Seasonal variation of toosendanin content in different part of chinaberry

As shown in Fig. 5, the highest content of toosendanin in leaves from chinaberry was 4.31, 2.29, 4.11 times than the lowest value of leaves collected in 2011, 2012 and 2013, respectively. The fluctuations of the toosendanin contents were significant among different seasons. However, it gave a same trend in the three years. That is, the samples collected in May and June consistently showed a superiority of toosendanin content in comparison with leaves harvested in autumn. In north of China, the flower period of chinaberry is the duration of April to May. In May and June, the flowers mostly wither away, at the same time, the fruits just start to appear. The leaves are rapid growing without other nutrition competition at this time. In autumn, the trees step into full fruits stage and the leaves stop growing. These may be the reasons for the seasonal variation of toosendanin contents in the leaves. It could be imaged that the time would be changed if the flower period changed with different climate. Although leaves contain less toosendanin than that in other parts of the trees, its larger amounts still supplied more resources for the production of toosendanin. However, the collection of leaves would eventually have to face the practical difficulties involving their low mass density.

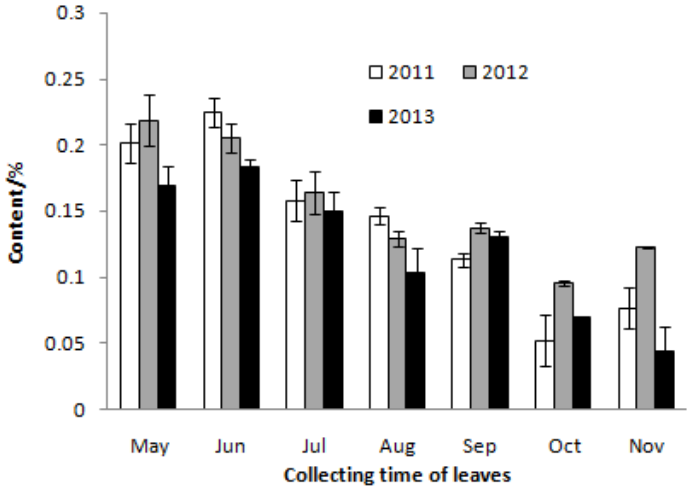

Figure 5. Contents of toosendanin in the leaves of chinaberry

Moverover, the fruits of chinaberry contained much more toosendanin than that in leaves. The results from Fig. 6 showed that the highest content of toosendanin in fruits from chinaberry was 6.06, 2.43, 3.19 times than the lowest value of fruits collected in 2011, 2012 and 2013, respectively. The fruits collected in November and December showed a higher toosendanin content among all of the samples. In generally, fruit of chinaberry is ripe between October and December. From November, skin of most fruits turns to be dry, that means the fruits get matured and the toosendanin content reaches a high level at the same time. Hence, toosendanin is accumulated in the fruit during its growing period and comes to a peak at the end of its maturation period. Due to the easier collection and higher content of toosendanin in the fruits in comparison with those of leaves, the fruit of chinaberry presents a potential source as industrial raw material.

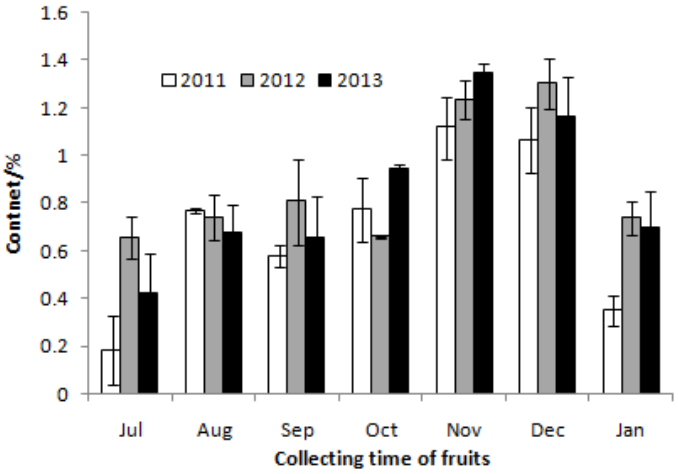

Figure 6. Contents of toosendanin in the fruits of chinaberry

Furthermore, the bark of chinaberry is a common part for the extraction of toosendanin due to the high content of toosendanin. As shown in Fig. 7, the highest toosendanin content in the bark was 1.82, 2.47, 1.63 times than the lowest value of barks collected in 2011, 2012 and 2013, respectively. The data exhibited the smaller inter-monthly variations than those of the leaves and the fruits. However, the samples collected in June, November, and December still showed the higher toosendanin contents than those in other seasons by the research of data during the three year, it implied that two peaks of toosendanin content were exhibited in summer beginning and winter. These two peaks should be related with the bark growth. The growth of trees bark shows a notable seasonal variation especially for those in temperate zones. For the industrial application, it is important to investigate a suitable technology to peel off the bark but protecting the tree from dead.

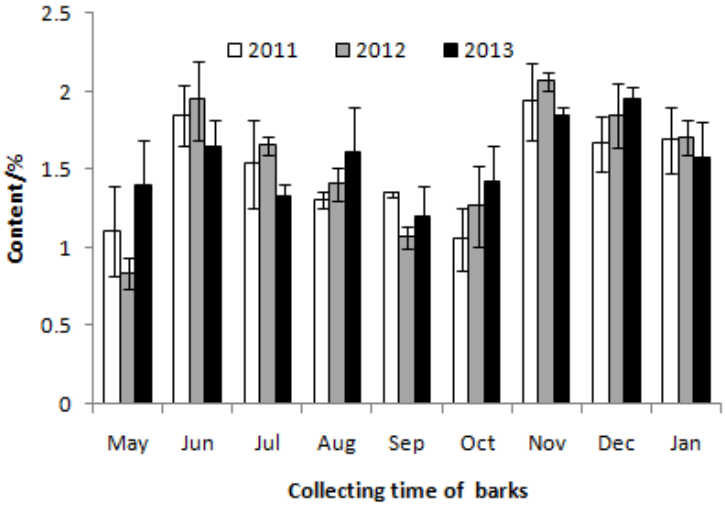

Figure 7. Contents of toosendanin in the barks of chinaberry

The seasonal variations for the toosendanin content were exhibited with the data for three years. Although inter-annual differences were observed, fortunately, the toosendanin content peaks were found. One year data were commonly used in the researches [23-24]. However, the conditions of plant are affected by climate, soil, water, et al. and these factors changes every year. Therefore, the data from three years were supplied for more reliable results.

CONCLUSION

Uniform design and regression analysis were employed to optimize the organic solvent extraction process of toosendanin, which would supply a reference for the industrial production of toosendanin from chinaberry fruits. The results showed a higher yield of toosendanin could be obtained when the shorter extraction time and the higher extraction volume of ethanol and longer extraction time and lower extraction time of ethyl acetate were selected. Moreover, by the determinations of toosendanin contents during different seasons in three years, it was found that extraction of toosendanin from the leaves, fruits, and barks of chinaberry occurred in May and June, November and December, and June, November, and December, respectively. Finally, compared with the barks and leaves, the mature fruits of chinaberry had great potential to be used as raw materials for the production of toosendanin from chinaberry on an industrial scale in future.

Acknowledgments
The authors thank for the financial support from “the Fundamental Research Funds for the Central Universities” (QN2011159) and the “Research Funds for Doctors” from Northwest A&F University.

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