Fatty acid profile, oxidative stability and toxicological safety of bayberry kernel oil

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Abbreviations: BKO, bayberry kernel oil; SC-CO_2, supercritical carbon dioxide; SFE, supercritical fluid extraction; NIH, National Institutes of Health; ICR, Institute of Cancer Research; NADP, nicotinamide adenine dinucleotide phosphate; FAMEs, fatty acid methyl esters; FID, flame ionization detector; POV, peroxide value; AV, acid value; PCB, polychlorinated biphenyl; SD, standard deviation.

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Abstract: The fatty acid profile, oxidative stability and toxicological safety of bayberry (*Myrica rubra* Sieb. et Zucc.) kernel oil (BKO) extracted by supercritical carbon dioxide (SC-CO_2) and solvent of diethyl ether were assessed. Fatty acid profile was determined by gas chromatography, oxidative stability by placing the sample of 25 g in a blast oven at 50 ± 1 °C to accelerate oxidation and toxicological safety by bacterial reverse mutation (Ames test) and acute oral toxicity in mice. The results demonstrated that in comparison to lard and rapeseed oil, the peroxide values of BKO were higher but the acid values were similar during the incubation test. The Ames test demonstrated no mutagenicity and no obvious acute toxicity were observed, suggesting that the BKO has potential as a novel edible oil.

Key words: Bayberry kernel oil; Supercritical carbon dioxide extraction; Fatty acid profile; Oxidative stability; Toxicological safety.

1. Introduction

Bayberry (*Myrica rubra* Sieb. et Zucc.), belonging to the family of *Myricaceae*, has a cultivation history of more than 2000 years in China (Chen, Xu, & Zhang, 2004). Bayberries are mainly cultivated in the southern side of the Yangtze River, where Zhejiang province is the major production area, with an annual yield of 350,000 tons (2010 data provided by Zhejiang Provincial Department of Agriculture). Bayberry fruit is very popular to the local people because of its enticing sweet/sour taste, exquisite flavor and attractive colour. It is high in carbohydrates, organic acids, proteins, minerals, and vitamins (Chen et al., 2004). However, because bayberry is harvested ripe in the hot and wet seasons of mid-June to early July, it can only be kept fresh for 3 days at 20-22 °C or 9-12 days at 0-2 °C (Xi, Zheng, Ying, & Chen, 1994), and the taste and flavor deteriorate quickly. To reach a wider market, shelf-life is extended by processing the fruits into juice and wine. During processing, bayberry seeds, which account for > 10% of the total fruit weight, are discarded as waste (Cheng, Ye, Chen, Liu, & Zhou, 2008b). Each bayberry seed has one kernel, which is a potential source of edible oil since the oil content is very high at 62%-68% of the kernel weight (Chen, Xu, & Xia, 2005). Nine types of fatty acids have been previously reported in bayberry kernel oil (BKO), which consists of ~85% unsaturated fatty acids (Chen et al., 2005). Intake of unsaturated fatty acids in human diet has the potential to reduce the risk of cardiovascular diseases, therefore, given its fatty acid profile bayberry kernel may have potential as a healthy edible oil source.

Conventional methods of extracting oil from fruit seeds include physical

extraction by pressing, as well as chemical extraction using solvents, the efficiency of which can be increased by continuous solvent recycling as in Soxhlet method or by using microwave assisted extraction or superheated hexane extraction (Abbasi, Rezaei, & Rashidi, 2008; Eikania, Golmohammada, & Homamib, 2012). More recently, the applications of supercritical fluid extraction (SFE) for oils have increased. Supercritical CO_2 (SC-CO_2) is widely used for extracting heat-sensitive and high-value components from biomaterials. The advantages of using CO_2 are its lack of toxicity, nonflammability, high availability, and low cost (Abbasi et al., 2008; Nodar, Gomez, & Martinez, 2002). It has been reported that oils such as *Hibiscus cannabinus* L. seed oil (Chan, & Ismail, 2009), *Opuntia dillenii* Haw. seed oil (Liu et al., 2009) and pomegranate seed oil (Liu, Xu, Gong, He, & Gao, 2012) extracted by SC-CO_2 maintain high antioxidant activity. SC-CO_2 has previously been used to extract oil from bayberry kernel (Zhang, Hu, Li, Ouyang, & Ma, 2007; Xia, Lu, Yang, Chen, Zheng, & Xing, 2009), however, the effect of this technology on BKO quality, including fatty acid profile and storage stability has not been reported. The toxicological safety of bayberry kernel has been tentatively confirmed using an Institute of Cancer Research (ICR) rat model, in which the medium lethal dose was >20.0 g/kg body weight (Cheng et al., 2008a). There is however no report on the mutagenicity and toxicity of the BKO. The acute oral toxicity test applied in the present work was also used for evaluation of other new oil sources, such as cashew seed oil (Konan, Bacchi, Lincopan, Varela, & Varanda, 2007) and pomegranate seed oil (Meerts, Verspeek-Rip, Buskens, Keizer, Bas saganya-Riera, & Jouni, 2009).

Therefore, the objective of this work was to investigate the effects of SC-CO_2 extraction on the fatty acid compositions of BKO, and evaluate the oxidative stability and toxicological safety of the oil in order to determine the commercialization potential of BKO as a novel edible oil.

2. Materials and methods

2.1 Materials and reagents

Bayberry fruits (cultivar Dongkui) were harvested on June 30, 2011 in an orchard in Taizhou city, Zhejiang province, transported to laboratory and used to separate the seeds on the same day. The seeds were squeezed out by a mini-juicer (Midea, Shunde, China) and dried at 50 °C overnight for further use. Five hundred grams of the kernels were taken out by cracking using a hammer from 1500 g of the seeds separated from 15 kg of fresh fruits. The kernels were dried at 50 °C for 6 h, sealed in a plastic jar, and stored at -18 °C as samples. The samples were ground into powder using a laboratory blender (Yili Instrument, Jinhua, China) before analyzed. CO_2 (99.995%) was purchased from Jingong Special Gas Co. Ltd (Hangzhou, China). Rapeseed oil and lard were purchased in the local food market. Diethyl ether and toluene (chromatography grade), fatty acid methyl ester standards (99.0%), acetyl chloride, sodium carbonate and TWEEN-80 (analytical grade) were purchased from Jiecheng Biotechnology Co. Ltd. (Hangzhou, China), Glucose-6-phosphate sodium, sodium azide, fenaminosulf and 2-aminofluorine were purchased from Sigma-Aldrich (Shanghai, China); nicotinamide adenine dinucleotide phosphate (NADP), and D-biotin were purchased from Boao Biotechnology Co., Ltd (Shanghai, China).

2.2 Extraction of BKO by SC-CO_2

Based on our previous work on SC-CO_2 extraction of BKO (Xia et al., 2009), the optimized extraction conditions with a high oil yield were selected. The details of the apparatus and extraction process have been described in detail previously (Xia et al., 2009). Briefly, 5.0 g of bayberry kernel powder was extracted using the 100 mL extraction vessel of the Spe-ed supercritical fluid extraction apparatus (Applied Separation, Pennsylvania, USA). The extraction process included two stages: during the static extraction stage the extraction conditions were maintained at 45 °C and 35 MPa for 60 min, with the oil outlet valve switch "off"; the dynamic extraction stage commenced when the oil outlet valve was switched "on", during which time the CO_2 flow rate was 4 $L.min^{-1}$. The dynamic extraction time was 50 min. The oil was collected in an automatic mode and transferred to a container, then stored at -18 °C until further analysis. Triplicate extractions were conducted.

2.3 Extraction of BKO by the Soxhlet method

The BKO was extracted from the kernels by the Soxhlet method following the procedure of Yazan, Fooa, Ghafara, Chana, Tahirc, & Ismaila (2011). Briefly, 5.0 g of bayberry kernel powder was transferred into a Soxhlet extractor and 300 ml of diethyl ether was added into the round bottom flask. During distillation/extraction the solvent flow rate was manually adjusted to 7 $min.cycle^{-1}$ and the extraction was terminated after 100 cycles. The solvent was removed from the oil by vacuum rotary evaporation under the temperature of 45 °C.

2.4 Analysis of fatty acid profile

The fatty acids of BKO were transesterified into fatty acid methyl esters (FAMEs), according to the procedure of Arens, Schulte, & Weber (1994). The analysis of FAMEs was performed using a 7890A gas chromatograph (Agilent Technologies, Palo Alto, USA), equipped with a flame ionization detector (FID). The flow rate of the nitrogen carrier gas was 1.0 $ml.min^{-1}$ and the split ratio was 10:1. A 1μl sample was injected onto a 100 m×0.25 mm×0.20 μm film thickness HP-88 capillary column (Agilent Technologies, Palo Alto, USA). The injector and FID temperatures were set at 260 °C. The initial column temperature was 150 °C for 1 min, increasing by 3 °C /min to 240 °C and maintained at 240 °C for 10 min. The FAME peaks were identified by retention time compared to FAME standards. Each sample was analyzed in triplicate. The individual fatty acids were quantified from their peak areas and expressed as percent of total fatty acid weight.

2.5 Oxidative stability test

The modified oven method (Jiang, Ma, & Wu, 2004) was used to test the oxidative stability of the BKO along with lard and rapeseed oil controls which are two most commonly consumed edible oil used by the local community in Zhejiang, China. This procedure was conducted by placing the sample of 25 g in a blast oven at 50 ± 1 °C to accelerate oxidation. The oil samples were taken out every 12 h for 72 h duration to determine the peroxide value (POV) and acid value (AV) to assess their relative oxidative deterioration. The POV and AV were determined using titrating methods according to the China national standard method of GB/T 5009.37-2003 (Method for analysis of hygienic standard of edible oils, Standardization

Administration of the People's Republic of China).

2.6 Bacterial reverse mutation study (Ames test)

Four strains of *Salmonella typhimurium* (TA97, TA98, TA100, TA102) were obtained from Molecular Toxicology Inc. (NC, USA), and polychlorinated biphenyl (PCB) induced rat liver S9 were obtained from Zhejiang Academy of Medicinal Sciences, Hangzhou, China. The S9/cofactor mix was used as a metabolic activation system and was prepared immediately prior to use as described previously (Gomes-Carneiro, Viana, Felzenszwalb, & Paumgartten, 2005). Standard Ames test procedures were conducted using the plate incorporation method (Maron & Ames, 1983) which in brief was as follows: the BKO was mixed with TWEEN-80 and emulsified in distilled water by a mixer using oil concentrations determined in a preliminary experiment. The emulsion was then sterilized at 121 °C for 15 min. The experiment was performed both with and without the S9 activation system, and the dosage of BKO evaluated were 40, 200, 1000 and 5000 μg.plate^{-1} respectively. Standard mutagens used as positive controls in each experiment were 2-aminofluorene (10 μg.plate^{-1}), fenaminosulf (50 μg.plate^{-1}) respectively for TA97 and TA98 with and without S9; 2-aminofluorene (10 μg.plate^{-1}), sodium azide (1.5 μg.plate^{-1}) for TA100 with and without S9 respectively; and fenaminosulf (50 μg.plate^{-1}) for TA102 without S9. The treatments were performed in triplicate. All strains were tested by colony count（Xunshu Microbiology Company, Hangzhou, China）for spontaneous revertant colonies using distilled water as a negative control.

If the number of revertant colonies is more than triple the background average

number on a plate, or if there is a dose-related increase in revertant colonies, the test is considered as positive and the test material can be concluded as mutagenic. On the contrary, if the results do not reach to this criterion, the test material can be considered as non-mutagenic (Yan, Li, Lin, Song, & Jiang, 2010).

2.7 Acute oral toxicity study in mice

This experiment was carried out in accordance with the guidelines of the "Principles of Laboratory Animal Care" (NIH publication No. 85-23, revised 1996). Male and female ICR mice at four weeks old were obtained from Zhejiang Academy of Medicinal Sciences (Hangzhou, China) with their body weights ranging from 18-22 g. Mice were housed in solid-bottom polycarbonate cages in a controlled environment (temperature 23 ± 3 °C, relative humidity 50 ± 10%, and artificial lighting was sequenced at 12-h light/dark cycles). Mice were quarantined for 3 days before the experiment and were fasted overnight prior to being randomly divided into two groups, each group containing 11 males and 11 females. To one group (test), BKO was feed at a single dose of 9.446 $g.kg^{-1}$ by intra-gastric gavage. To the other group (control) an equal volume of distilled water was given. Mice were observed for clinical signs of intoxication or mortality at 10 and 30 min, and 1, 2, 3, 4, 5 and 6 h after dosing. Mice were sacrificed on day 15 and necropsy examinations were conducted to inspect acute intoxication on all external surfaces, organs and orifices.

2.8 Statistical analyses

Data were expressed as mean ± standard deviation (mean ± SD). ANOVA and Duncan's post-hoc test were performed using SPSS software package version 17.0

(SPSS Inc., Chicago, IL) to identify significant differences between sample means. $P < 0.05$ was considered significant in all analyses.

3. Results and discussion

3.1 Effect of SC-CO_2 extraction on the fatty acid profile of BKO

The gas chromatography of fatty acid profile of BKO was given in Figure 1. The chromatogram (Figure 1a) identified seven fatty acids namely: palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2）, punicic acid (C18:3) and arachidic acid (C20:0）. Unlike the report of Chen et al. (2005), no myristic (C14:0) nor arachidic acid (C20:2）was found in our BKO. This may be attributed to different bayberry cultivars, since there are discrepancy in fatty acid profile among different cultivars (Cheng, 2008a). Chen et al. (2005) used the Biqi cultivar while the Dongkui cultivar was used in the present study.

Our results showed that oleic (~40% of total fatty acids) and linoleic (~45%) acids are the major fatty acids in BKO extracted by SC-CO_2, followed by palmitic acid (~10%), stearic acid (~3%) and palmitoleic acid (~1%). The content of the polyunsaturated fatty acid of punicic acid was < 1% (Table 1). These results agreed with the reports of Chen et al. (2005) in that oleic and linoleic acids are the major fatty acids in BKO, irrespective of cultivars.

The fatty acids profile of BKO from Soxhlet extraction is similar to that from SC-CO_2 extraction. However, arachidic acid was not detected in the Soxhlet extracted oil and the content of punicic acid was significantly lower ($p<0.05$) than that from SC-CO_2 extracted sample (Figure 1b and Table 1). This finding suggests that the

higher temperature applied in the Soxhlet method may have destroyed some of the highly unsaturated fatty acid punicic acid. The arachidic acid in bayberry kernel oil was very low (~ 0.1%) when SC-CO_2 extraction was used (Table 1), thus it may not have been extracted by the Soxhlet method under the present conditions.

3.2 Oxidative stability of BKO

The POV of BKO extracted by SC-CO_2 and Soxhlet as well as the lard and rapeseed controls all got higher with incubation time, but the POV values of the BKO from either extraction method were higher than those of lard and rapeseed oil (Figure 2). These finding suggest that for effective long term storage of BKO, it may be necessary to store at low temperature and/or stabilize by the addition of food grade antioxidants such as TBHQ to delay oxidation during storage.

The increase in POV observed in the present study is likely to have arisen through auto-oxidation rather than through photo-induced oxidation as the samples were tested under dark conditions. The free radical chain reaction mechanism of auto-oxidation of lipids, once initiated, will continue to occur even in the absence of exogenous free radicals and will lead to the increased POV values (Muik, Lendl, Molina-Diaz, & Ayora-Canada, 2005). In general the higher the content of unsaturated fatty acids in an oil, the more prone it will be to be oxidation. The content of unsaturated fatty acids (oleic and linoleic acids) in BKO (~85%) (Table 1) was significantly higher than that of lard (~58.8%) (Wu, Ji, & Go, 2009) and slightly higher than that of rapeseed oil (~81.2%) (Liu, Zhu, Huang, Duan, & Wang, 1997). These differences in unsaturated fatty acid levels could account for the higher POV of

BKO than those of lard and rapeseed oil. In addition, the POV values of BKO extracted by Soxhlet method were significantly higher than those by SC-CO_2 method at the same time-point (Figure 2). The higher temperatures required to evaporate the diethyl ether solvent from the extracted oil after the Soxhlet extraction compared to the exposure temperature in SC-CO_2 extraction might account for the higher POV of the Soxhlet extracted BKO.

Changes in AV of BKO (extracted by either the SC-CO_2 or the Soxhlet method), lard and rapeseed oil were all similar (Figure 3). The AV is a measure of free fatty acids and is considered to reflect oil quality, degree of refining, and quality changes during storage (Rao, Xiang, Zhou, Wang, Xie, & Xu, 2009). In all samples the AV peaked quickly (within ~12 h), maintained this high value for 12 h and then declined within the next 12 h. After this period it remained relatively constant until the end of the incubation (~72 h). Generally, reports have indicated that the AV of an oil increases with increasing levels of free fatty acids due to triglyceride hydrolysis; typically after long storage of oil or oil-rich products (Fuse, Kusu, & Takamura, 1997; Yuan, Liu, Ma, Zhu, Liu, & Na, 2006). However, it has also been reported that AV can decrease in the later periods of storage after initially rising in earlier storage period (Guo, Bai, Jiang, Shao, Wang, & He, 2009; Wang, Huang, & Wang, 2010; Ding, & Fei, 2011). This may be as a result of the hydrolysis of free fatty acids into even smaller molecules such as alcohols, ketones and lactones, and the rate of which would be related to the conditions such as the temperature and pH of the food (Guo, et al. 2009; Ma & Liu, 2005). The results of the present study suggest that the BKO

extraction technique has little effect on its AV and that the AV stability during storage of BKO is similar to that of lard and rapeseed oil.

3.3 Toxicological safety of BKO

The results of the Ames test (Table 2) indicated that regardless of the presence of S9 fraction, the numbers of revertant colonies in the four bacterial strains tested at any concentrations of BKO did not exceed the level indicative of mutagenicity; namely twice that of negative control. In addition, no obvious dose-response (between the dose of BKO and Revertant colonies) relationship was observed. However as expected, the numbers of revertant colonies did increase highly in all positive control compared with those in negative control. These results were confirmed in an independently repeated experiment (Table 3). Therefore, according to the criteria described in section 2.6, the BKO would not be classified as a mutagenic material. To our knowledge, this is the first report regarding the potential mutagenicity of BKO, which would be an initial supporting data to promote the commercialization of this new oil resource.

3.4 Acute oral toxicity study

During the acute oral toxicity experiment at the BKO feeding dose level of 9.446 g/kg body weight, no clinical signs were observed and no animal died. In addition, physical observations did not reveal any obvious treatment-related health changes for the mice. Body weight increased in all groups, and no significant difference was detected between the control group and test group (Figure 4), which suggested that the animals fed with BKO grew normally comparing with the control animals. Therefore,

no obvious acute toxicology of BKO was observed.

4. Conclusions

Bayberry kernel oil (BKO) was extracted by SC-CO_2 and Soxhlet methods. Oleic acid and linoleic acid were the major fatty acids in BKO either extracted by SC-CO_2 or Soxhlet method. During the incubation test, the POV of the BKO extracted by Soxhlet were higher than those from SC-CO_2 extraction, but both were all higher than those of lard and rapeseed oil, indicating BKO was more prone to oxidation. The acid value of the BKO either extracted by SC-CO_2 or Soxhlet method was similar to those of other two commonly consumed edible oils investigated. The Ames test indicated a lack of mutagenicity of BKO and no obvious acute toxicology was observed in the acute toxicological study. Overall the findings of this study indicated potential of BKO as a novel edible oil.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Table 1 Fatty acid profile of bayberry kernel oils obtained from different extraction methods (%)*

Extraction method	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Punicic acid (C18:3)	Arachidic acid (C20:0)
SC-CO_2	11. 4 ± 0.14[a]	1.0 ± 0.10 [a]	3.3 ± 0.20 [a]	39.2 ± 0.20[a]	44.3 ± 0.30[a]	0.5 ± 0.10[a]	0.1 ± 0.02
Soxhlet	11.5 ± 0.20[a]	1.1 ±0.10 [a]	3.3 ± 0.10 [a]	38.7 ± 0.20[a]	45.0 ± 0.20[a]	0.3 ± 0.10[b]	ND **

* Values are given as mean ± SD from triplicate determinations. Values in the same column having different superscript letters are significantly different ($p < 0.05$).

** ND, Not detected.

Table 2 Results of Ames test of bayberry kernel oil (BKO) (first round)

Sample	Dose	Revertant colonies							
	(μg/plate)	TA_{97}		TA_{98}		TA_{100}		TA_{102}	
		$-S_9$	$+S_9$	$-S_9$	$+S_9$	$-S_9$	$+S_9$	$-S_9$	$+S_9$
Solvent control	0	96.3±10.1	145.0±7.0	35.7±3.0	42.7±3.8	153.0±12.3	181.0±12.1	264.3±26.0	292.0±11.5
BKO	40	90.7±0.6	158.3±0.6	33.0±3.6	44.0±2.0	167.3±15.3	171.7±14.0	261.7±1.5	288.0±3.6
	200	107.3±14.6	142.7±3.5	35.7±1.5	44.3±4.0	191.7±10.7	216.0±12.1	280.0±10.4	292.3±3.0
	1000	129.0±18.7	150.7±6.1	38.7±1.5	45.3±0.6	184.0±10.1	172.7±15.5	267.3±13.0	293.3±3.0
	5000	102.0±14.2	164.0±2.6	31.7±1.1	45.3±4.0	173.7±11.6	198.3±8.0	264.3±4.0	299.3±5.0
sodium azide	1.5	-	-	-	-	>1000	-	-	-
fenaminosulf	50	>1000	-	>1000	-	-	-	890.0±3.6	-
2-aminofluorene	10	-	>1000	-	>1000	-	>1000	-	-

Table 3 Results of Ames test of bayberry kernel oils (second round)

Sample	Dose	Revertant colonies							
	(μg/plate)	TA_{97}		TA_{98}		TA_{100}		TA_{102}	
		$-S_9$	$+S_9$	$-S_9$	$+S_9$	$-S_9$	$+S_9$	$-S_9$	$+S_9$
Solvent control	0	93.7±	135.0±	39.7±	45.7±	157.7±	180.3±	272.0±	295.7±
		2.5	6.0	1.5	4.2	12.3	4.0	12.1	7.6
	40	91.7±	147.7±	34.0±	46.7±	174.7±	173.3±	270.7±	293.7±
BKO		1.5	3.0	2.0	1.1	8.1	9.6	2.1	5.8
	200	106.3±	138.3±	40.3±	47.3±	192.3±	198.7±	282.7±	295.7±
		4.5	2.1	1.5	2.1	7.5	6.5	9.6	4.6
	1000	128.0±	148.7±	39.0±	46.3±	187.3±	173.0±	276.7±	299.0±
		10.5	1.5	2.0	3.5	10.0	9.5	10.7	6.2
	5000	105.0±	156.3±	37.7±	46.7±	175.0±	201.3±	271.0±	305.3±
		6.0	4.0	3.0	3.2	4.6	6.0	12.8	8.5
sodium azide	1.5	-	-	-	-	>1000	-	-	-
fenaminosulf	50	>1000	-	>1000	-	-	-	888.7±6.6	-
2-aminofluorene	10	-	>1000	-	>1000	-	>1000	-	-

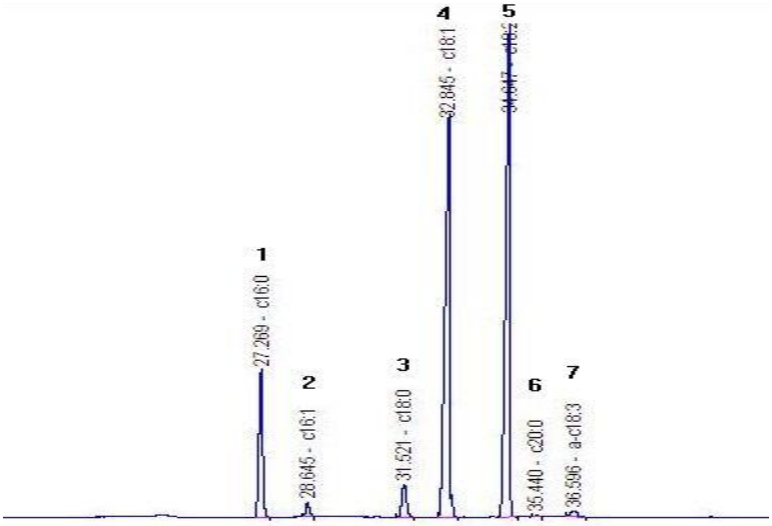

Fig.1 The gas chromatography of bayberry kernel oil extracted by SC-CO_2. Peak identity: 1, Palmitic acid (R_t = 27.269 min); 2, Palmitoleic acid (R_t = 28.645 min); 3, Stearic acid (R_t = 31.521 min); 4, Oleic acid (R_t = 32.845 min); 5, Linoleic acid (R_t = 34.647 min); 6, Arachidic acid (R_t = 35.44 min); 7, Punicic acid (R_t = 36.596 min).

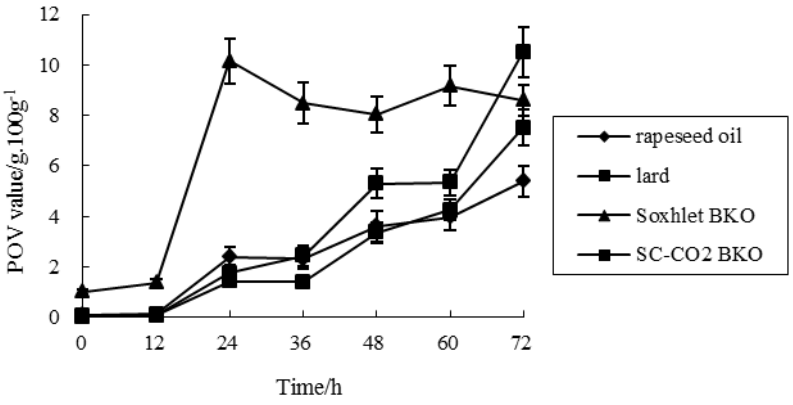

Fig.2 The change of peroxide value

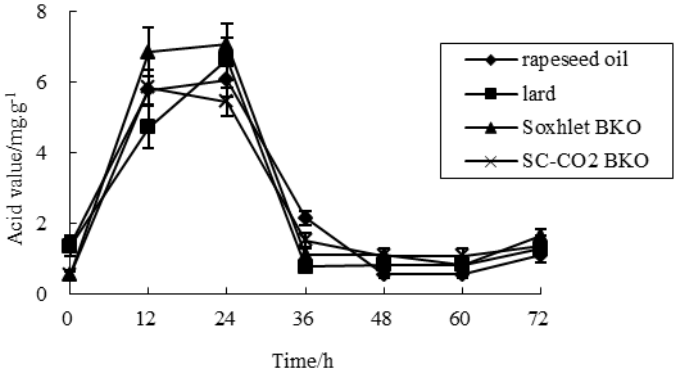

Fig.3 The change of acid value

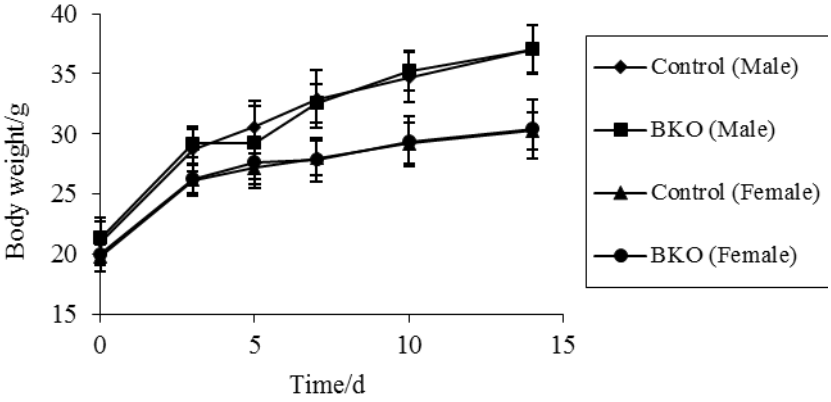

Fig.4 Body weight of mice treated orally with BKO for acute toxicity test