Tropical Journal of Pharmaceutical Research January 2014; 13 (1): 41-46
ISSN: 1596-5996 (print); 1596-9827 (electronic)
© Pharmacotherapy Group, Faculty of Pharmacy, University of Benin, Benin City, 300001 Nigeria.
All rights reserved.

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v13i1.6

Original Research Article

A Green and Solvent-Free Process for Preparation of High-Purity (–)-Borneol from Leaves of *Blumea balsamifera* (L) DC

Yuan-Hui Wang[1], Hong-Yun Tian[1], Ai-Mei Wang[1], Hong-Xin Wang[1*] and Chun-Li Zou[2]

[1]State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Wuxi 214122, [2]Gui Zhou Ai Yuan Eco-Pharmaceutical Development Co Ltd, Luodian 550100, China

For correspondence: Email: xblack1983@eyou.com; **Tel: +86 510 85917795; **Fax:** +86 510 85876799*

Received: 13 May 2013 *Revised accepted: 1 December 2013*

Abstract

Purpose: *To develop a simple and low cost process for the preparation of high-purity (–)-borneol from leaves of Blumea balsamifera (L.) DC..*
Methods: *An improved hydrodistillation (IHD) equipped with a vertical condenser tube was designed for extracting the volatiles (crude (–)-borneol) without solvent, and comparing with hydrodistillation-solvent extraction (HDSE) and simultaneous distillation and extraction (SDE). The sublimation was used to separated high-purity (–)-borneol. The purities of (–)-borneol products were quantitatively analyzed by gas chromatography (GC), and the (–)-borneol product was analyzed by optical activity and nuclear magnetic resonance (NMR), and the antimicrobial activity was evaluated.*
Results: *The (–)-borneol content of the volatiles was 82 % in IHD, and much higher than that of HDSE (45 %) and SDE (44 %). After sublimation, the purity of separated (–)-borneol was 92 %, and the recovery was 96 %. The NMR spectral data confirmed that the product was (–)-borneol, and the specific rotation was –36.4° (20 °C, ethanol). Meanwhile, the performances of the (–)-borneol product and standard (–)-borneol were the same in antimicrobial activity.*
Conclusion: *The work provides a green, efficient and solvent-free process for preparation of high-purity natural (–)-borneol from B. balsamifera leaves.*

Keywords: *Blumea balsamifera (L.) DC., (–)-Borneol, Green method, Solvent-free distillation.*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

(–)-Borneol is a bicyclic monoterpenoid alcohol and possesses the property of sublimation. It is also one of two optical isomers of borneol that are the composition of traditional Chinese and Japanese medicine and a fragrance ingredient [1]. (–)-Borneol has shown various activities to be applied in the pharmaceutical, food and cosmetic industries, such as antimicrobial activities, enhancing drug permeability, increasing cell viability, [2-4], and it can be used to synthesize chiral substance and chiral ionic liquid, which were widely used in synthetic chemical industry [5-8]. Although (–)-borneol can be synthesized, the synthetic product is a mixture and belongs to the racemate, and contains (+)-borneol, (–)-borneol and isoborneol [9]. These chiral compounds are difficult to separate from the mixture. Furthermore, the synthetic product gradually deteriorates thus rendering it unsafe to use [10]. Because of this, more and more

researchers now pay attention to preparation of (–)-borneol from natural raw materials.

Natural (–)-borneol source is scarce in nature. *Blumea balsamifera* (L.) DC. is an important plant source for the extraction of natural (–)-borneol [11]. Chinese Pharmacopoeia names natural (–)-borneol as "Aipian", and requires that the content is > 85 %. *B. balsamifera* (L.) DC. is a half-woody, evergreen shrub that grows widely in tropical and subtropical areas, such as East Asia and Southeast Asia [12], and belongs to the family, Asteraceae [13]. The plant has been reported to promote digestion, reduce phlegm, and has antispasmodic, and sudorific properties [14,15]. It has been harvested as a medicinal plant for decades in Luodian County, China [16]. Based on its physicochemical properties, (–)-borneol can be extracted using steam distillation and organic solvent of low boiling-point extract. At present, we know from production enterprise that natural (–)-borneol is extracted from leaves of *B. balsamifera* (L.) DC. by steam distillation using the general distillation apparatus, but the method has some disadvantages, such as low yield, low recovery, unstable product quality, To the best of our knowledge, there has been no report on organic solvent extraction of (–)-borneol since the method requires the use of a large quantity of organic solvent and this would not be suitable for industrial scale-up.

The aim of this work was to design a green and organic solvent-free process for preparing high-purity natural (–)-borneol from *B. balsamifera* leaves using an improved hydrodistillation (IHD) facility equipped with a vertical condenser tube. The developed method was compared with hydrodistillation-solvent extraction (HDSE) and simultaneous distillation and extraction (SDE) methods.

EXPERIMENTAL

Plant material

B. balsamifera (L.) DC. leaves were randomly collected in November 2011 from plants growing wild in Luodian County (Southwest China), localized at 800 m altitude, 25° 04′ N and 106° 28′ E. The plant was identified by Professor YN He (Institute of Biotechnology, Guizhou Academy of Agricultural Sciences, China), and a voucher specimen (CGA-Dafengai-Guizhou-2009-11) was deposited in the herbarium of the Institute of Biotechnology, Guizhou Academy of Agricultural Sciences. Before being distilled, the leaves were washed and air-dried in dark conditions.

Chemicals

(–)-Borneol (97 %) was purchased from Sigma-Aldrich (Shanghai, China); naphthalene (99.5 %), Aipian (–)-borneol with a purity of 87 %, and all analytically pure solvents used were supplied by Sinopharm Chemical Reagent Co Ltd (Shanghai, China).

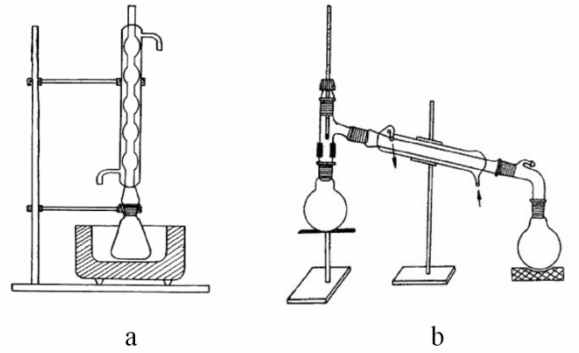

Figure 1: Improved hydrodistillation (a) and general hydrodistillation (b) apparatuses

Extraction of (–)-borneol by improved hydrodistillation

Powdered leaves (20 g, 40 mesh) were carefully weighed and put into a 2 l distillation flask, 1 l of distillated water was added, and the distillation flask linked with a vertical condenser tube (Fig 1a). The mixture was heated at boiling temperature, and there were white volatiles on the surface of condenser inner wall. At the end of distillation, the white volatiles were scraped off using a scraper and air-dried in the dark; its yield was measured (n = 6). The ratio of material/water (1:10, 1:20, 1:30, 1:40, 1:50, 1:60, w/v) and distillation times (10, 20, 30, 40, 50 and 60 min) were tested. The (–)-borneol content of white volatiles was quantitatively determined by gas chromatography (GC).

Separation of (–)-borneol by sublimation

Volatiles (2 g) were carefully weighed and placed in an evaporating dish, and a funnel was upended on the dish. The dish was heated to sublime (–)-borneol, with the sublimate ((–)-borneol) on the surface of the funnel's inner wall. The effect of temperature (100, 120, 140, 160, 180 °C) and time (10, 20, 30, 40 min) were investigated. The sublimates were collected and weighed, and their yield was determined (n = 6). The (–)-borneol content of sublimates was quantitatively determined by GC.

Identification of the separated (–)-borneol

The separated (–)-borneol was qualitatively identified by optical activity and nuclear magnetic

resonance (NMR). The optical activity of sample was determined by WZZ-2A automatic polarimeter (INESA, Shanghai, China). The sample was dissolved in ethanol at 20 °C for optical activity test. NMR was performed on a Bruker Avance III 400MHz Digital NMR Spectrometer (Bruker, Swiss) with a 9.4-T Ultrashield™ Plus magnet at 25 °C. The test sample was prepared in 99.8 % chloroform-d ($CDCl_3$). The final concentration was 10 mmol/l. NMR spectra were recorded using 5 mm Z-Gradient S1 probe. All NMR data were processed using Bruker Topspin 2.1 software.

Comparison of methods for extracting (–)-borneol

Process productivity is defined as the mass of (–)-borneol processed per time unit. Solvent consumption is expressed as the volume of organic solvent produced per mass unit of (–)-borneol in processing. The process time is the time for a complete purification cycle, that is, the sum of the time for extraction and separation. In order to compare with the improved hydrodistillation, other methods, hydrodistillation-solvent extraction (HDSE) and simultaneous distillation and extraction (SDE), were used for the extraction of (–)-borneol.

In HDSE, leaves and water were used in similar amounts as that for the improved hydrodistillation; the general distillation apparatus was used is shown in Fig 1b. The mixture was heated to boiling temperature and distilled for 30 min. The distillated aqueous solution was collected and extracted with diethyl ether anhydrous, and then the diethyl ether anhydrous was evaporated at room temperature, under vacuum, in a rotary evaporator and the yield of volatiles determined (n = 6). In SDE, leaves and water were weighed and added to a distillation flask; 100 ml diethyl ether anhydrous was placed in another 500 ml distillation flask, and the two flasks linked with modified Likens-Nickerson apparatus for simultaneous distillation and extraction. After 30 min of distillation, and diethyl ether anhydrous was evaporated in a rotary evaporator to obtain the volatiles, whose yield was determined (n = 6). The (–)-borneol content of volatiles in each case was determined quantitatively by GC.

Determination of (–)-borneol by GC

The content of (–)-borneol was quantitatively determined according to Chinese Pharmacopoeia [10]. Naphthalene was used as the internal standard (IS). The samples and IS were prepared in ethyl acetate. The final concentration was 0.20 mg/ml. GC analysis was performed using a gas chromatograph (Shimadzu-2010Plus, Tokyo, Japan) with AOC20i autosamplers and a flame ionization detection system (FID). Data were collected with GCSolution software (Shimadzu). The gas chromatograph was equipped with a CP-WAX capillary column (30 m × 0.32 mm × 0.22 µm) (Varian, USA). The oven temperature was initiated at 80 °C (held for 5 min), then raised to 220 °C at 10 °C/min, held for 5 min. The injection port was held at 250 °C and used in split mode with a split ratio of 1:5. The detector temperature was 250 °C. Pure nitrogen (99.999%) was used as the carrier gas at constant flow rate of 3 ml/min; H_2: 47.0 ml/min; air: 400 ml/min. The analyses were executed in triplicate.

The linear range was 0.6-1875 µg/ml for (–)-borneol. The calibration curve for quantifying (–)-borneol was: $Y = 0.863\ X - 0.0094$, with a correlation coefficient of 0.9992 (Y: Peak area ratio of (–)-borneol to IS, X: concentration ratio of (–)-borneol to IS). The precision of the method was measured by RSD and recovery. Eight replicate analyses of standard solutions were performed. By their peak areas, the calculated RSD values for (–)-borneol was 0.16 %, and recovery 97 %.

Microbial strains

The samples were tested against six bacteria: two Gram-positive bacteria, *Staphylococcus aureus* (ATCC 6538), and *Listeria monocytogenes* (ATCC 19111), and four Gram-negative bacteria, *Escherichia coli* (ATCC 25922), *Shigella flexneri* (ATCC 12022), *Salmonella enterica* (ATCC 14028) and *Pseudomonas aeruginosa* (ATCC 27853). They were also tested against four fungi: *Candida albicans* (ATCC 90029), *Aspergillus flavus* (ATCC 28539), *Aspergillus brasiliensis* (ATCC 16404), *Trichophyton rubrum* (ATCC 28188). All of the strains were grown on Luria-Bertani agar (LBA) for the bacteria and Sabouraud dextrose agar (SDA) for fungi. Vancomycin, kanamycin sulfate, and amphothericin B were used as the positive antimicrobial agents against Gram-positive bacteria, Gram-negative bacteria, and fungi.

Determination of minimum inhibitory concentration of (–)-borneol

Minimum inhibitory concentration (MIC) was determined using the dilution agar method. Serial dilutions of the tested samples were carried out in Luria-Bertani agar medium (bacteria) or Saboureaud dextrose agar medium (fungi).

Appropriate volumes of every dilution were added to this medium to obtain the required concentration range and a final concentration of acetone of 10 % (v/v). Two controls were included in this test. Each dish contained a sterile solution of acetone and the culture medium, respectively. After incubation at 37 °C for 24 h for bacteria and at 30 °C for 48 h for fungi, MIC was taken as the lowest concentration of the test samples at which the microorganism did not demonstrate visible growth. Antibiotics (vancomycin, kanamycin sulfate and amphothericin B) used were also tested as described above. The test samples and the controls were tested in duplicate, and the experiments were performed in triplicate.

Statistical analysis

Software package Origin 8.0 (OriginLab, Northampton, MA, USA) was used to compute mean, standard deviations and to perform analysis of variance (ANOVA). The significance level applied was $p < 0.05$.

RESULTS

Efficiency of (–)-borneol extraction by improved hydrodistillation

As seen from Fig 2a, when the ratio of material/water was 1:50, the extraction rate of (–)-borneol was significantly higher than other ratios ($p < 0.05$). Therefore, 1:50 was chosen as the optimal ratio of material/water. The extraction rate of (–)-borneol did not increase significantly ($p > 0.05$) with distillation time from 30 min to 60 min (Fig 2b), while energy consumption significantly increased ($p < 0.05$); thus 30 min was chosen as the best extraction time. In the optimized process, the extraction rate of the volatiles was 10.5 mg/g (extract/leaves) and (–)-borneol content was 82 %, and the extraction rate of (–)-borneol was 8.6 mg/g ((–)-borneol/leaves, Fig. 2c).

Purity of (–)-borneol

After hydrodistillation, the volatiles were treated to obtain high-purity (–)-borneol according to the above sublimation, and the results are shown in Fig 3. When the sublimation time was 10 min, the purity of (–)-borneol (sublimates) decreased with increase of temperature, whereas recovery of (–)-borneol increased (Fig 3a and b). As seen from Fig 3c, the purity of (–)-borneol decreased with elapsed time from 94 to 84 %. Recovery of (–)-borneol did not remarkably increase ($p > 0.05$) after 30 min (Fig 3d). Therefore, 120 °C for 30 min were considered to be the best temperature and sublimation time, and this produced a purity of 92 % and recovery of 96 %.

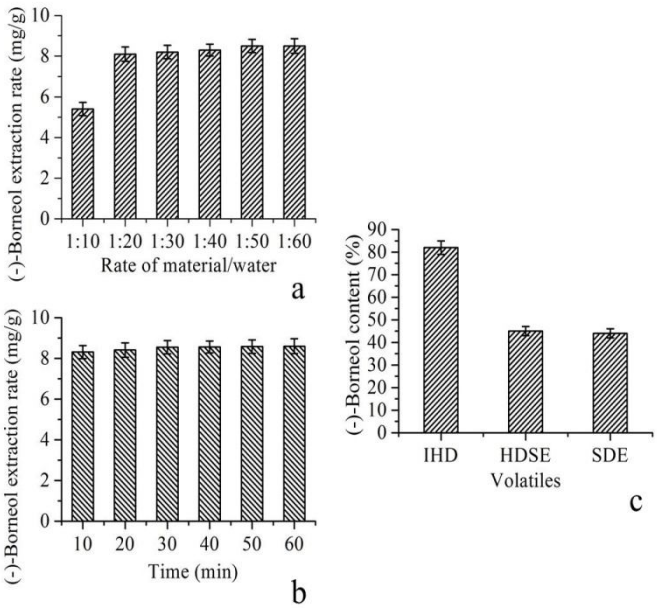

Figure 2: Optimal conditions of hydrodistillation; (a) optimization of ratio of material/water; (b) optimization of distillation time; (c) comparison of (–)-borneol content of volatiles for three methods

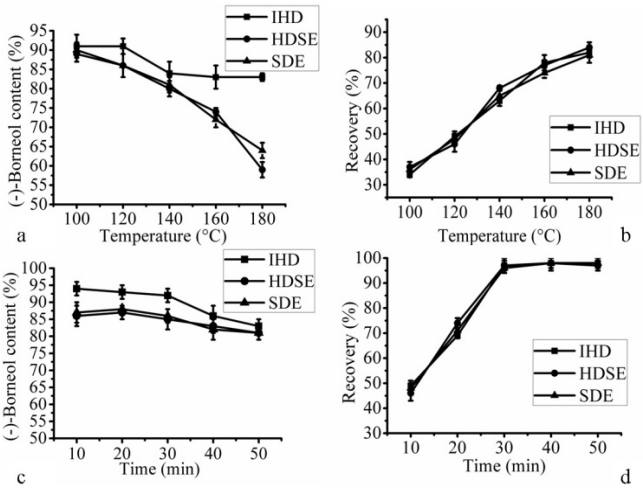

Figure 3: Optimization of sublimation; (a) (–)-borneol content of three sublimates at different temperatures; (b) recovery of three sublimates at different temperatures; (c) (–)-borneol content of three sublimates at different times; (d) recovery of three sublimates at different times

Effect of method on (–)-borneol extraction

Extraction rates of (–)-borneol by HDSE and SDE were 8.8 and 9.2 mg/g, and while (–)-borneol contents of the volatiles were 45 and 44 %, respectively (Fig 2c). As shown in Fig 3c, the purities of (–)-borneol separated from the

volatiles (HDSE and SDE) were 85 and 86 % while that of the new method was 92 %

Table 1: Comparison of efficiency of three distillation methods

Extraction method	Time (min)	Solvent consumption (ml)	(–)-Borneol extraction rate (mg/g)
IHD[a]	30	0	8.6
HDSE[b]	30	300	8.8
SDE[c]	30	100	9.2

[a] *Improved hydrodistillation;* [b] *Hydrodistillation-solvent extraction;* [c] *Simultaneous distillation and extraction*

Identification of the isolated (–)-borneol

Both the isolated and standard (–)-borneol were determined by polarimeter, and their specific rotations were –36.4° and –35.1° (20 °C, ethanol), respectively, which show that the isolated (–)-borneol was levoisomer. The NMR spectral data are as follows: ^{1}HNMR ($CDCl_3$, δ): 0.85 (3H, s, $-CH_3$), 0.86 (3H, s, $-CH_3$), 0.87 (3H, s, $-CH_3$), 0.95 (1H, dd, H-6b), 1.25 (2H, m, H-5b, H-6a), 1.62 (1H, t, -OH), 1.70 (2H, m, H-3b, H-5a), 1.90 (1H, m, H-3a), 2.28 (1H, m, H-4), 4.04 (1H, m, H-2). ^{13}CNMR ($CDCl_3$, δ): 13.4 (C-10), 18.9 (C-8), 20.5 (C-9), 26.3 (C-6), 28.5 (C-5), 39.2 (C-3), 45.2 (C-4), 48.3 (C-7), 49.9 (C-1), 77.7 (C-2). The specific rotation and NMR spectral data confirmed that the isolated (–)-borneol was truly (–)-borneol.

Antimicrobial activity of isolated (–)-borneol

The antimicrobial activity of the standard (–)-borneol, isolated (–)-borneol and commercial (–)-borneol (Aipian), expressed as minimum inhibitory concentration (MIC), is presented in Table 2.

Table 2: Antimicrobial activities of various (–)-borneol products

Microorganism	MIC[a] (µg/ml)			
	Standard (–)-borneol[b]	*Isolated (–)-borneol*[c]	*Aipian*[d]	*ATB*[e]
Gram-positive bacteria				
Staphylococcus aureus	1000	1000	1000	0.59
Listeria monocytogenes	1000	1000	1000	1.2
Gram-negative bacteria				
Escherichia coli	1000	1000	1000	0.29
Shigella flexneri	1000	1000	1000	1.2
Salmonella enterica	1000	1000	1000	0.58
Pseudomonas aeruginosa	1000	1000	1000	0.29
Fungi				
Aspergillus flavus	500	500	1000	3.7
Aspergillus brasiliensis	500	500	1000	0.92
Trichophyton rubrum	125	125	250	3.7
Candida albicans	250	250	250	0.92

[a] *Minimum inhibitory concentration;* [b] *Standard (–)-borneol (97 %) (250 µg);* [c] *Isolated (–)-borneol (92 %) (250 µg);* [d] *Aipian (87 %) purchased from market (250 µg);* [e] *Antibiotic, vancomycin (25 µg) for Gram-positive bacteria, kanamycin sulfate (25 µg) for Gram-negative bacteria, amphotericin B (25 µg) for fungi*

DISCUSSION

In IHD process (Fig 1a), the water was heated to vaporize, was and the vapour cooled to liquid in the vertical condenser tube and refluxed into the flask. Meanwhile, (–)-borneol cannot reflux into flask because the temperature of boiling water in the flask is high. Therefore, (–)-borneol would absorb on the inner surface of the condenser tube. At the end of distillation, the volatiles which contained (–)-borneol could be scraped off from the inner surface, and the process did not require the use of organic solvent.

In order to examine the efficiency of IHD, two other methods, namely, HDSE and SDE, were used for the extraction of (–)-borneol. Solvent consumption and (–)-borneol extraction rate were used as reference for comparison. No organic solvent was used in IHD, but HDSE and SDE required the use of organic solvent. (–)-Borneol extraction rate for the three methods was in the following rank order: SDE > HDSE > IHD, and there was no significant difference among them ($p > 0.05$). In addition, the (–)-borneol content of volatiles by IHD was much higher than those of HDSE and SDE. High (–)-borneol content of the volatiles benefits the follow-up sublimation.

At present, the sublimation process in factory is an extensive operating mode. The flame (200-300 °C) was used during heating, so that the temperature was too high and cannot be easily controlled. A variety of sublimated components were achieved under higher temperature [17].

Thus, the purity and yield of (–)-borneol collected from sublimation is low. In the study, the sublimation temperature and time were investigated, and the easily operated sublimation method (120 °C, 30 min) was established to separate (–)-borneol from the volatiles. The purity of the isolated (–)-borneol was superior to the standard of "Aipian" recorded in Chinese Pharmacopoeia and was nearly the same as that of standard (–)-borneol. The advantage was also originated from high (–)-borneol content of the volatiles from IHD.

Furthermore, the specific rotations, NMR spectrals, and antimicrobial activities of the isolated (–)-borneol and standard (–)-borneol are similar, which show that the quality of isolated (–)-borneol prepared in the proposed process is high. In a word, the improved hydrodistillation and sublimation method is superior to the current technological situation in factory, and the whole process is simple and suitable for industrial scale-up.

CONCLUSION

A green, efficient and organic solvent-free process has been successfully developed for the preparation of high-purity (–)-borneol from *B. balsamifera* leaves. The (–)-borneol extraction rate is close to that of HDSE and SDE, but with a higher yield than these other two methods. The purity of the isolated (–)-borneol is 92 %, thus exceeding the "Aipian" pharmacopoeial standard; recovery of (–)-borneol is 96 %. Moreover, the antimicrobial activities of the separated (–)-borneol and standard (–)-borneol were consistent. For industrial scale-up, the equipment would be suitable but needs to be further developed for automation and reliability.

ACKNOWLEDGMENT

This research was financially supported by Chinese Innovation Fund for Technology-Based Firms (no. 11C26215205838) and Guizhou's Innovation Fund for Technology-Based Firms (Qian Ke He Zi {2011} 5002).

REFERENCES

1. *Bhatia SP, McGinty D, Letizia CS, Api AM. Fragrance material review on l-borneol. Food Chem Toxic. 2008; 46: S81-S84.*
2. *Al-Farhan KA, Warad I, Al-Resayes SI, Fouda MMG, Ghazzali M. Synthesis, structural chemistry and antimicrobial activity of (–)-borneol derivative. Cent Euro J Chem. 2010; 8: 1125-1131.*
3. *Cherneva E, Pavlovic V, Smelcerovic A, Yancheva D. The effect of camphor and borneol on rat thymocyte viability and oxidative stress. Molecules. 2012; 17: 10258-10266.*
4. *Yang H, Xun Y, Li Z, Hang T, Zhang X, Cui H.) Influence of borneol on in vitro corneal permeability and on in vivo and in vitro corneal toxicity. J Int Med Res. 2009; 37: 791-802.*
5. *Farber C, Wolmershauser G, Sitzmann H. Formation of quaternary stereogenic centers by wagner-meerwein rearrangement-synthesis of optically active cyclopentadienyl complexes from borneol and fenchol. Z Naturforsch. 2009; 64b: 25-40.*
6. *Matos RAF, Andrade CKZ. Synthesis of new chiral ionic liquids based on (–)-menthol and (–)-borneol. Tetra Lett. 2008; 49: 1652-1655.*
7. *Nizamov IS, Gabdullina GT, Al'metkina LA, Shamilov RR, Batyeva ES, Cherkasov RA. (1S)-endo-(–)-borneol in the synthesis of optically active phosphorus dithioacids. Rus J Gene Chem. 2012; 82: 1751-1752.*
8. *Sitzmann H, Faerber C, Wolmershaeuser G. Optically active metallocenes from borneol and fenchol. Abs Papers Ame Chem Soc. 2005; 229: U964-U965.*
9. *Chen L, Su J, Li L, Li B, Li W. A new source of natural D-borneol and its characteristic. J Med Plants Res. 2011; 5(15): 3440-3447.*
10. *Xu XF, Wang Y, Zhang YJ, Liu CX, He X. Pharmacokinetic Study of Borneol and Menthol in Rats after Oral Administration of Qingyan Drop Pills. Planta Medica. 2011; 77: 1600-1604.*
11. *Chinese Pharmacopoeia Commission. Pharmacopoeia of The People's Republic of China volume 1. Beijing: China Medical Science Press; 2010.*
12. *Ruangrungsi N, Wongpanich V, Tantivatana P, Cowe HJ, Cox PJ, Funayama S, Cordell GA. Traditional Medicinal Plants of Thailand, V. Ancistrotectorine, a New Naphthalene-Isoquinoline Alkaloid from Ancistrocladus tectorius. J Nat Prod. 1985; 48: 529-535.*
13. *Lin R. Flora Republicae Popularis Sinicae volume 75. Beijing: Science Press; 1979. 19 p.*
14. *Zhu TC, Wen YX, Wang HS, Huang YL. Chemical constituents in Blumea balsamifera (l). Guihaia. 2008; 28: 139-141.*
15. *Bhuiyan MNI, Chowdhury JU, Begum J. Chemical components in volatile oil from Blumea balsamifera (L.) DC.. Bangladesh J Bot. 2009; 38: 107-109.*
16. *Shirota O, Oribello JM, Sekita S, Satake M. Sesquiterpenes from Blumea balsamifera. J Nat Prod. 2011; 74: 470-476.*
17. *Riu-Aumatell M, Vargas L, Vichi S, Guadayol JM, Lopez-Tamames E, Buxaderas S. Characterisation of volatile composition of white salsify (Tragopogon porrifolius L.) by headspace solid-phase microextraction (HS-SPME) and simultaneous distillation-extraction (SDE) coupled to GC-MS. Food Chem. 2011; 129: 557-564.*